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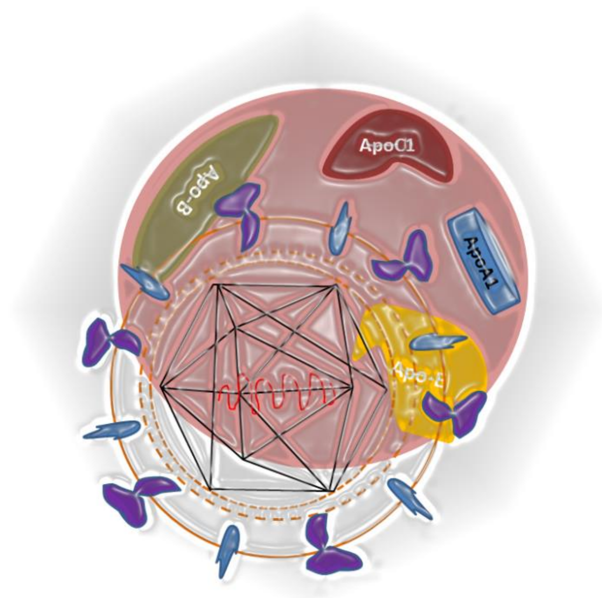
Protection from HCV infection – Identification of mechanisms of resistance to HCV infection in exposed uninfected injection drug users.



By

Isaac Thom Shawa

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Author Declaration.

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Sub-Committee. Work submitted for this research degree at the Plymouth University has not formed part of any other degree either at Plymouth University or at another establishment.

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Relevant scientific seminars and conferences were regularly attended at which work was often presented; external institutions were visited for consultation purposes and several papers were submitted for publication.

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Signed……………………….

Date………………………….

ABSTRACT

Protection from HCV infection – Identification of mechanisms of resistance to HCV infection in exposed uninfected injection drug users.

By Isaac Thom Shawa

Hepatitis C virus (HCV) is a leading cause of chronic liver disease. In the developed world, injection drug use (IDU) through sharing of infected needles and other paraphernalia remains the principal risk factor for HCV transmission. Effective but expensive treatment is now possible but there remains a pressing need for a vaccine.

A proportion of people who inject drugs (PWIDs) remain uninfected despite HCV exposure from a long history of sharing needles and other paraphernalia. These cases are termed exposed but uninfected (EU) and test negative for both HCV antibodies and RNA and exhibit a phenotype of resistance to HCV infection. Improved understanding of the mechanisms that confer resistance in the EUs has the potential to aid development of an effective vaccine and novel therapeutic strategies.

This thesis reports on the findings from 3 different strategies to identify characteristics of HCV resistance. I used urinary metabolomics, serum lipidomics and the study of adaptive and innate immune responses. Each of these methods has demonstrated clear differences between EU cases and healthy controls and/or spontaneous resolvers of HCV infection. Urinary metabolomics suggest a potential role of the gut microbiome, the serum lipidomics showed marked differences in lipid profiles in EU cases pointing towards a perturbed lipid/virus interaction, and the immune studies confirmed previous work identifying low level T cell responses in many EU cases but has also identified a marked upregulation of interferon alpha production to low dose viral RNA in EU cases utilising ELISA assay.

In conclusion, this thesis reports data that identifies a number of new findings that provide insight into mechanisms of resistance to HCV infection. My findings suggest that the complex interplay between the virus and lipids together with an upregulated innate immune response may together help determine the outcome following HCV exposure.

In summary, studies performed in this thesis have demonstrated that there are different pathways that define the EU phenotype. Despite being a heterogenous subgroup of PWIDs, the EUs are clearly distinct from a healthy control population.

Publications related to this thesis.

1. **Shawa IT**, Felmlee DJ, Hegazy D, Sheridan DA, Cramp ME. Exploration of potential mechanisms of HCV resistance in exposed uninfected intravenous drug users. *Journal of Viral Hepatitis*. 2017; 00: pp.1 – 7.
2. **Shawa IT**, Sheridan DA, Felmlee DJ, Cramp ME. Lipid interactions influence Hepatitis C Virus susceptibility and resistance to infection. *Clinical Liver Disease*. 2017; 10(1), pp.17 – 20.

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1. **Shawa IT**, Gomez-Romero M, Pechlivanis A, Felmlee DJ, Crossey M, Holmes E, et al. Serum lipid profiling using ultra-performance liquid chromatography mass spectrometry (UPLC-MS) discriminates HCV exposed uninfected injection drug users from those susceptible to infection. In: British Association for the study of the Liver; Basic Science Retreat. 2017.
2. **Shawa IT**, Gomez-Romero M, Pechlivanis A, Felmlee DJ, Crossey M, Holmes E, et al. Serum lipid profiling using ultra-performance liquid chromatography mass spectrometry (UPLC-MS) discriminates HCV exposed uninfected injection drug users from those susceptible to infection. In: British Association for the study of the Liver; Annual meeting: Manchester. 2016.
3. **Shawa IT**, Cox IJ, Riva A, Fullerton JN, Sheridan DA, Felmlee DJ, et al. Urine metabolic profiling distinguishes HCV exposed uninfected injection drug users from those with chronic or resolved HCV infection. In: British Association for the study of the liver; Annual meeting: Manchester. 2016.
4. Sheridan DA, Gomez-Romero M, Bridge S, Crossey M, **Shawa IT**, Neely D, et al. P725: Lipidomics analysis of fasting serum identifies novel lipid biomarkers specific for HCV genotype 3 and genotype 1 chronic hepatitis C virus infection. *Journal of Hepatology*. 2015; 62, p.S596.

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List of Abbreviations.

^{1}H NMR …………… Proton nuclear magnetic resonance

ABCA1…………….. ATP-binding cassette transporter A1

ACAT……………….Acyl-CoA-cholesterol acyltransferase

AHCV……………… Acute hepatitis C virus

ANOVA………….... One-way analysis of variance

APC…………………Antigen presenting cell

Apo………………… Apolipoprotein

ATP…………………Adenosine triphosphate

BCR………………... B cells' membrane-bound receptor

bnAb……………….. Broadly neutralizing antibody

BSA…………………Bovine serum albumin

C_2H_3N……………… Acetonitrile

C_3H_8O……………… Isopropanol

CARD……………… Caspase recruitment domain

CCR5………………. C-C chemokine receptor-5

CD…………………..Cluster of differentiation

cDNA……………….Complementary deoxyribonucleic acid

CE………………….. Capillary electrophoresis

CEFT………………. Cytomegalovirus, Epstein Barr virus, Influenza virus, & Tetanus toxin

CH_2O_2………………. Formic acid

CHCV………………. Chronic HCV infection

CLDN1…………….. Claudin-1

CLR…………………C-type lectin receptor

CM………………….Chylomicron

CTD………………... C-terminal domain

DAAs……………….Direct acting antivirals

DC…………………. Dendritic cell

DC-SIGN………….. Dendritic-cell-specific intercellular adhesion molecule-3-grabbing non- integrin

DDA……………….. Data-dependent acquisition/analysis

DMA………............Dimethylamine

DMSO………………Dimethyl sulfoxide

DNA……………….. Deoxyribonucleic acid

ds……………………Double-stranded

EDTA……………… Ethylenediaminetetraacetic acid

EGFR……………….Epidermal growth factor receptor

EIA……………….…Enzyme immunoassay

ELISA ……………...Enzyme immunosorbent assay

ELISpot……………..Enzyme-linked immunospot

EMCV………………Encephalomyocarditis virus

EphA2……………... Ephrin receptor A2

ER………………….. Endoplasmic reticulum

ESI…………………. Electrospray ionisation

EU………………….. Exposed uninfected

FBS……………….... Foetal Bovine serum

FFA……………….... Free fatty acids

GS-MS…………….. Gas chromatography mass spectrometry

GVB-B……….......... GB virus B

GWAS……………... Genome-wide association studies

HAV……………….. Hepatitis A virus

HBV………………... Hepatitis B virus

HC............................ Healthy control

HCC......................... Hepatocellular carcinoma

HCV.......................... Hepatitis C virus

HCVpp..................... HCV producing Pseudo particle

HCVRUK................. Hepatitis C virus Research UK

HDL.......................... High density lipoprotein

HIV........................... Human immunodeficiency virus

HLA C-1……………Human leucocyte antigen class 1

HMDB...................... Human Metabolome Data Base

HRA……………….. Health research authority

HRP…………………Horseradish peroxidase

HS-GAG……......... Highly sulphated glycosaminoglycan

Huh 7………………. Human hepatoma cell line

HV……………........ Healthy volunteer

HVR……………….. Hypervariable region

IDU……………….... Injection drug user

IFN…………………. Interferon

IL…………………... Interleukin

IPS-1……………….. Interferon-β promoter stimulator 1

IRES……………….. Internal ribosomal entry site

IRF3………………... Interferon regulatory factors 3

ISG…………………IFN-stimulated gene

IU…………………... International units

IVDU………………. Intravenous drug use

JEV……………….... Japanese encephalitis virus

JFH-1………………. Japanese fulminant hepatitis isolate 1 (Full length replicon for HCV genotype 2a that was derived from a Japanese patient).

KIR………………… Killer-cell immunoglobulin-like receptor

LCAT ……………... Lecithin cholesterol acyltransferase

LC-MS……………... Liquid chromatography mass spectrometry

LD………………….. Lipid droplet

LDL…………………Low density lipoprotein

LDLR……………….Low density lipoprotein receptor

LGP2………………. Laboratory of genetics and physiology 2

LPCAT…………….. Lysophosphatidylcholine acyltransferases

LPL………………… Lipoprotein lipase

L-SIGNL……………Liver/lymph-node-specific intercellular adhesion molecule-3 grabbing integrin

LVP…………………Lipoviral particle

MAVS …………..... Mitochondrial antiviral-signalling *protein*

MDA-5…………….. Melanoma differentiation-associated protein 5

MHC……………….. Major Histocompatibility Complex

miRNA…………….. MicroRNA

MS…………………. Mass spectrometry

MTP………………... Microsomal triglyceride transfer protein

NANB……………… Non-A, non-B

NANBH……………. Non-A, non-B hepatitis

NAT………………... Nucleic acid testing

NCR.......................... Natural cytotoxicity receptor

NH_4HCO_2.................Ammonium formate

NK- κB…………….. Nuclear factor kappa B

NK…………………..Natural killer

NKG2……………… Natural killer group 2

NKR………………... Natural killer receptor

NKT………………... Natural killer T cell

NLR………………... Nucleotide-binding oligomerisation domain -like receptor

NMR……………….. Nuclear Magnetic Resonance

NOD……………….. Nucleotide-binding oligomerisation domain

NPC1L1……………. Niemann-Pick C1-like 1

NRES………………. National Research Ethics Service

NS………………….. Non-structural

nt…………………... Nucleotide

NTR……………….. Non-translated regions

OCLN……………… Occludin

OPLS-DA………….. Orthogonal projections to latent structures discriminant analysis

ORF………………... Open reading frame

PAMP……………… Pathogen-associated molecular pattern

PBMC ……………... Peripheral blood mononuclear cell

PBS…………………. Phosphate Buffered Saline

PC………………….. Phosphatidylcholine

PCA…………………Principal component analysis

PCR…………………Polymerase chain reaction

PD-1……………….. programmed death-1

pDC……………….. Plasmacytoid dendritic cell

PDME…………….. Phosphatidyldimethylethanolamine

PE………………….. Phosphatidylethanolamine

PEG-IFN………….. Pegylated interferon

PEMT…………….... Phosphatidylethanolamine *N*-methyltransferase

PFU………………… Plaque forming units

PHA………………... Phytohaemagglutinin

PI4KIII……………... Phosphatidyl-inositol-4-kinase-III

PI4P………………... Phosphatidylinositol-4-phosphate

PKR……………….. Protein kinase RNA-activated

PMME……………... Phosphatidylmonomethylethanolamine

PolyIC…………….. Polyinosinic-polycytidylic acid

ppm………………… Parts per million

PRR…………………Pattern recognition receptor

PVDF…………….... Polyvinylidene difluoride

PWID………………. People who inject drugs

QC…………………..Quality control

QD…………………..Quantum dot

Q-ToF……………… Quadrupole time-of-flight

R848……………….. Resiquimod

RBV………………... Ribavirin

RF………………….. Radiofrequency

RIBA………………. Recombinant immunoblot assay

RIG-I…………….... Retinoic acid-inducible gene 1

RLR………………... Retinoic acid-inducible gene 1-like receptor

RNA……………….. Ribonucleic acid

rpm………………… Revolutions per minute

RPMI………………..Roswell Park Memorial Institute medium

rt …………………… Retention time

SD………………….. Standard deviation

SDC…………………Syndecans

SEM……………….. Standard of error of mean

SFU………………… Spot forming units

SNP…………………Single nucleotide polymorphism

SOP…………………Standard operating procedure

SP………………….. Streptavidin-peroxidase

SR-BI………………. Scavenger receptor class B type I

ss……………………single-stranded

STAT1……………... Signal transducers and activators of transcription 1

SVR…………………Sustained viral response

SW…………………. South West

T_c……………………Cytotoxic T-cells

TCR…………………T cell receptor

TfR1……………….. Transferrin receptor 1

TG………………….. Triglyceride

Th……………………Helper T-cells

TLR…………………Toll-like receptor

TMA………………...Trimethylamine

TMAO………………Trimethylamine- N-oxide

TMB……………….. Tetramethylbenzidine

TRL…………………Triglyceride rich lipoproteins

TSP………………….Trimethylsilyl propanoic acid

UPLC………………. Ultra-performance liquid chromatography

UTR……………….. Untranslated region

VIP…………………. Variable Importance for the Projection

VLDL………………Very low density lipoprotein

WHO………………. World Health Organisation

1 CHAPTER ONE

1.1 Introduction.

1.2 Background.

This introduction will outline the HCV life cycle highlighting the involvement of HCV-lipid interactions during the viral lifecycle, and also unravel the innate immunological factors that describe the potential putative mechanisms of resistance for HCV infection in exposed but uninfected intravenous drug users (IVDU).

Hepatitis C virus (HCV) is a major cause of liver disease, and is a global public health problem that requires worldwide active interventions for effective prevention and control of the infection (Gower *et al.*, 2014). Humans are the only known natural host (Pybus *et al.*, 2009) but HCV experimental transmission to chimpanzees can occur. The World Health Organisation (WHO) estimates that 71 million people are persistently infected with HCV worldwide. An estimated 399,000 people die annually from HCV associated liver diseases (WHO, 2017). An HCV global epidemiology report for people who inject drugs (PWIDs) in 77 countries indicated a midpoint prevalence estimate of 60 – 80% of PWIDs had HCV detecting antibodies, with over 80% prevalence in 12 countries (Nelson *et al.*, 2011). Approximately 10 million PWIDs have HCV detecting antibodies worldwide with an estimated 1.6 million in China, 1.5 million in the United States of America , and 1.3 million in Russia (Nelson *et al.*, 2011).

54 – 86% of the acutely infected individuals develop chronic hepatitis annually (Hoofnagle, 2002); while 20% are able to clear the virus spontaneously in the first 6 months after exposure (Thomas *et al.*, 2009). However, some studies also reported spontaneous resolution of HCV infection after one year (Scott *et al.*, 2006; Mosley *et al.*,

2008). Liver cirrhosis develops in approximately 20 – 30% of chronic patients within 20 years (Lauer and Walker, 2001); 1 – 4% may progress to liver cancer such as hepatocellular carcinoma (HCC) (Fattovich *et al.*, 2004). Approximately 25% of primary HCC cases are due to chronic HCV(CHCV) infection worldwide (Tanaka *et al.*, 2006).

In the developed world, injection drug use through sharing of needles and other paraphernalia, remains the highest risk factor for HCV transmission, with HCV prevalence rates of greater than 90% reported among injection drug users (IDUs) (Tseng *et al.*, 2007).

The outcome of HCV exposure is affected by a complex set of interactions between the host and the virus. Exposure to HCV may be considered to result in one of three outcomes; remain antibody seronegative and aviraemic by sensitive RNA PCR (uninfected) (Thurairajah *et al.*, 2008), spontaneous clearance (detectable HCV-antibodies but HCV-RNA negative), or chronic infection (both HCV antibody and RNA positive) (Knapp *et al.*, 2010). Only chronic HCV infection leads to cirrhosis and HCC, although the risk of HCC persists in those with cirrhosis even after successful antiviral treatment of HCV (European Association for Study of Liver, 2014).

Several mechanisms have previously been described in relation to the immune failure resulting in persistent HCV infection. Other researchers described the peripheral and intrahepatic virus-specific T cell responses targeting different HCV epitopes in HCV clearance (Rehermann, 2009). Therefore occurrence of mutations in epitopes targeted by virus-specific CD8+ T cells in HCV acute infections (Tester *et al.*, 2005) contributes to development of HCV infection. HCV employs strategies in order to escape the host immune responses. The host adaptive immune responses are mediated by both cellular and humoral immunity. Therefore, CD4+ and CD8+ T cells play an essential role in

the outcome of HCV infection. The CD8+ T cells prevent establishment of HCV infection by inhibiting viral replication through activation of cellular cytolytic mechanisms (Thimme, Binder and Bartenschlager, 2012). The function of CD8+ T cell cytolytic activities is dependent on CD4+ T cells, therefore failure of CD4 T helper cells function compromises the function of CD8+ T cells (Penna *et al.*, 2007; Sun, Rajsbaum and Yi, 2015).

There is no effective vaccine developed yet to prevent HCV infection due to a high degree of strain variation (Forns, Bukh and Purcell, 2002; Torresi, Johnson and Wedemeyer, 2011). Researchers have studied different targets for design and development of a vaccine in animals; but currently HCV vaccine testing has reached phase II clinical trial in humans (Halliday, Klenerman and Barnes, 2011; Young *et al.*, 2015) but extreme diversity of HCV is one of the major challenges for vaccine design.

Numerous research efforts have been made to understand HCV infectivity and factors that could potentially confer resistance in exposed individuals. In the last decade, HCV standard of care has been a combination of pegylated interferon (PEG-IFN) and ribavirin (RBV), but these have many side effects as a result of poor tolerability, suboptimal sustained viral response rates in difficult-to-treat cases, and reports of resistance have emerged (Pawlotsky, 2011). The licencing of multiple direct acting antivirals (DAAs) e.g. Sofosbuvir that could potentially reduce the treatment duration and adverse effects has enabled efforts to improve efficacy and tolerability of HCV therapy.

Over the years, our research group and others (reviewed in (Mina *et al.*, 2015)) characterized a cohort of IDUs called 'exposed uninfected' (EU) who are at risk of HCV infection but still remain negative for both HCV antibody and HCV RNA (Thurairajah *et al.*, 2008). The phenotype of repeated exposure to HCV without the

development of infection is of considerable interest and suggests these individuals are in some way resistant to HCV infection. In this chapter, I have considered the phenotypic definitions of the EU cohort, and the distinct immunological, and genetic features that characterise the EU group for insights into possible mechanisms of HCV resistance (Shawa, Felmlee, *et al.*, 2017).

1.3 Hepatitis C virus discovery.

Before the discovery of HCV in 1989, the common hepatitis viruses were hepatitis A virus (HAV) discovered in 1973 (Feinstone *et al.*, 1975) and hepatitis B virus (HBV). Viral hepatitis that was caused by neither hepatitis A nor hepatitis B viruses, was previously termed 'Non-A, non-B' (NANB) hepatitis. In 1975, Feinstone et al, reported that transfusion-associated hepatitis was not due to type A or B hepatitis viruses due to absence of serological markers of NANB viruses. They further reported that nearly 10% of transfusions resulted in NANB hepatitis, a condition characterised by persistent liver damage in majority of cases (Feinstone *et al.*, 1975), and increased serum transaminases (transaminasemia) (Alter and Houghton, 2000). The existence of the NANB hepatitis (NANBH) aetiological agent was demonstrated following intensive investigations and development of numerous immunological and serological assays to identify reliable and reproducible biomarkers specific for NANB hepatitis. An experimental chimpanzee model was developed using NANBH patient blood which successfully demonstrated the presence of an NANBH transmissible agent (Alter *et al.*, 1978; Hollinger *et al.*, 1978; Tabor *et al.*, 1978). Immunoscreening of bacterial complementary deoxyribonucleic acid (cDNA) derived from samples from chimpanzees infected with NANBH, led to the isolation of a single cDNA clone (5-1-1) which enabled the sequencing and identification of the whole viral genome. Antibodies derived from an NANBH patient were used for an immunoscreening for identification of a cDNA clone that encodes non-

structural (NS) protein 4 epitope. Further analysis of a larger overlapping 'clone 81' showed that the clone was bound to a single-stranded ribonucleic acid (ssRNA) molecule derived from NANBH infected blood samples (Houghton, 2009). Following years of vigorous experimental and clinical studies, on 21st April 1989, Michael Houghton and his colleagues in collaboration with Daniel Bradley using molecular approaches, discovered the aetiological agent of NANB hepatitis termed '*Hepatitis C virus*' (Choo *et al.*, 1989).

1.4 Virology.

1.4.1 HCV genome organisation.

The HCV has a positive-sense single-stranded RNA (+ssRNA) genome that is approximately 9.6kb that belongs to *Flaviviridae* family (Houghton, 2009). The HCV genome contains one long open reading frame (ORF) coding for a polyprotein precursor of approximately 3000 amino acids (aa) flanked by non-translated regions (NTRs) at both ends (Choo *et al.*, 1989). HCV belongs to the genus *Flavivirus*. Other viruses that belong to *Flavivirus* include: *yellow fever, west Nile virus, Dengue virus*. Previous studies have grouped HCV together with GB virus B (GVB-B) in *Hepacivirus* genus (Simons *et al.*, 1995). GVB-B was used as a surrogate model for HCV; is phylogenetically related to HCV, and they both have common 5' structural protein-3' non-structural protein organization (Muerhoff *et al.*, 1995) that is required for replication and initiation of translation. The 5' NTR which is ~341 nucleotide (nt) in length, contains an internal ribosomal entry site (IRES) that is essential in facilitating the translation of viral RNA (Buratti *et al.*, 1998). HCV encodes 10 different structural (Core, envelope 1 (E1), envelope 2 (E2)) and non-structural (NS) viral porin (p7), (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins (Welbourn and Pause, 2007) (Figure

1.1). The HCV genome has high genetic variability, with high mutation rates in different regions (Martell *et al.*, 1992).

Figure 1.1: Hepatitis C virus genome organisation.

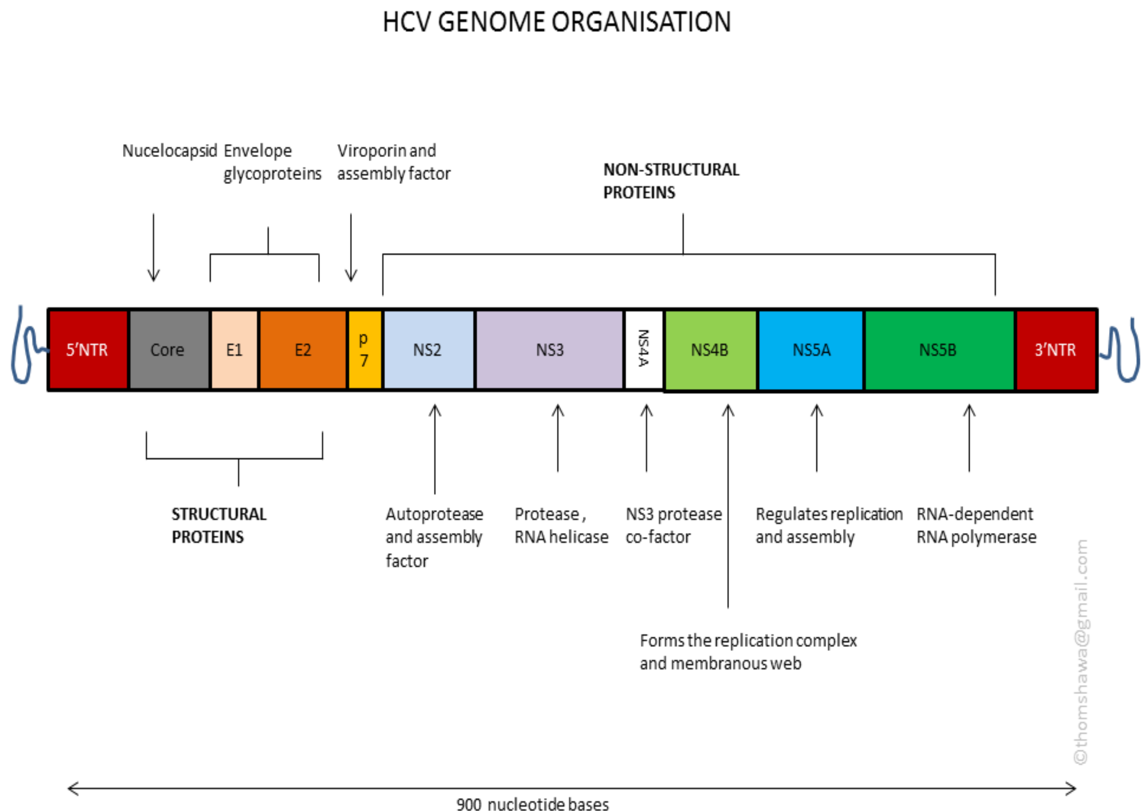


Figure 1.1: HCV genome organization. HCV genome contains a single open reading frame (ORF) flanked by 5′ and 3′ non-translated regions (NTRs). The 5′ and 3′ NTR consists of four highly structured domains that contain the internal ribosome entry site (IRES); and stable stem-loop structures respectively. HCV ORF translation is directed via 5′ NTR that function as an IRES. It permits binding of ribosomes to the ORF start codon. The viral polypeptide is cleaved co- and post-transcriptionally by protease enzymes encoded by both the virus and the host for production of structural and non-structural (NS) proteins.

1.4.2 Features of structural and non-structural viral proteins.

1.4.2.1 Structural proteins.

1.4.2.1.1 Core.

The HCV core protein released in a 191 aa precursor is an RNA-binding protein that forms the viral capsid. The HCV core contains three distinct hydrophilic domains in the first 120 aa: an N-terminal hydrophilic domain (domain D1) that contains immunodominant antigenic sites; a C-terminal hydrophobic domain (domain D2) (Penin *et al.*, 2004), and the last 20 aa that serve as signal peptides for E1 (Majeau *et al.*, 2004). Domain D1 is involved in binding RNA, whereas D2 facilitates the association of the core protein with the endoplasmic reticulum (ER) and lipid droplets (LDs) (Ren *et al.*, 2004; Suzuki *et al.*, 2005). The HCV core protein was reported to facilitate the accumulation of LDs in vitro (Harris *et al.*, 2011) whereby core protein is loaded onto LDs. The junction between core-loaded LDs and the viral replication complex-rich ER membranes acts as a site for assembly of progeny virions. Mechanisms of the HCV core protein assembly still remains unclear (Penin *et al.*, 2004). The structural peculiarity of the HCV core describing its domains provides some useful explanations for the physiopathological differences between HCV and the other flaviviruses. The structural and functional role for D2 was thoroughly characterized. Thus, functional studies of the relevance of D2 and the interaction of the core protein and lipid droplets was understood following development of HCV cell culture (HCVcc) system in a cell culture to enable the description of the HCV life cycle. Further analyses also revealed that the D2 domain was responsible for HCV production efficiency (Shavinskaya *et al.*, 2007).

1.4.2.1.2 E1 and E2 glycoproteins and p7.

The two HCV envelope glycoproteins E1 and E2 assemble as noncovalent heterodimers (Op De Beeck, Cocquerel and Dubuisson, 2001) and play important roles in the HCV life cycle. The E1 and E2 transmembrane glycoproteins, with N-terminal ectodomains of 160 and 334 aa respectively, are essential in viral attachment to host receptors together with entry and fusion with host cell membranes (Deleersnyder *et al.*, 1997; Bartosch *et al.*, 2003; Nielsen *et al.*, 2004). The HCV E2 glycoprotein sequence has hypervariable regions (HVR) (Weiner *et al.*, 1991) whose aa sequence differs by 80% among HCV genotypes. The first segment of E2 is HVR1 which forms HCV neutralization epitope (Farci *et al.*, 1996). The second hypervariable region HVR2 was described in HCV genotype 1, and together with HVR1 plays a role in host cell recognition and attachment (Roccasecca *et al.*, 2003). The HCV structural and non-structural proteins are separated by p7 (63aa), a small intrinsic membrane protein that belongs to the viroporin family, and is located between the E2 and NS2 region. p7 was reported to mediate membrane ion permeability and also plays a role in the formation of progeny viruses, maturation and release (Harada, Tautz and Thiel, 2000; Pavlovic *et al.*, 2003).

1.4.2.2 Non-structural proteins.

1.4.2.2.1 NS2.

The non-structural proteins are encoded by HCV and form a group of viral enzymes (viral replicase) that are involved in host-viral interactions. The NS2 (250aa) is a nonglycosylated integral membrane protein whose function is unclear. Some studies suggest that N2 participates in proteolytic cleavage at the NS2-NS3 junction of the polyprotein (Yamaga and Ou, 2002) and interacts with both structural and non-structural proteins in HCV particle assembly (Popescu *et al.*, 2011).

1.4.2.2.2 NS3 – 4A complex.

The NS3 protease (500aa) is a serine protease located within the ER, and forms a complex with NS4A (54aa) (Love *et al.*, 1996). The NS3 is a multifunctional enzyme that interacts with other NS proteins NS4B, NS5A and NS5B within the replication complex (Ishido, Fujita and Hotta, 1998). The NS4A is a cofactor of NS3 protease activity that provides its stability, localization at ER membrane and cleavage at other NS proteins complex junctions (Bartenschlager *et al.*, 1995). The NS3 – NS4A complex is essential in the life cycle and pathogenesis of HCV infection. The NS3 – NS4A protease is an important viral target for the development of antiviral therapeutic agents. The NS3 – NS4A complex plays a significant role in its interaction with host cell pathways and proteins in HCV life cycle. The NS3 – NS4A protease was reported to catalyse HCV polyprotein cleavage at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions (Yan *et al.*, 1998).

1.4.2.2.3 NS4B.

The NS4B is a small integral hydrophobic membrane protein located within the ER and is essential in recruiting other viral proteins (Hügle *et al.*, 2001). It plays a role in inducing ER morphological changes during the formation of the membranous web that harbours the HCV genome, structural and NS proteins (Egger *et al.*, 2002; Gosert *et al.*, 2003). Therefore, it is believed that NS4B induces alteration of specific membranes that serve as a scaffold for the formation of the viral replication complex (reviewed in (Penin *et al.*, 2004)).

1.4.2.2.4 NS5A.

The NS5A is a membrane associated phosphoprotein whose function in the HCV replication cycle is still unclear. The NS5A was reported to be involved in the formation of the functional viral replication complex (Bartenschlager, 2002). The NS5A enzymatic activity is observed through interaction with other viral and cellular proteins. The NS5A an enzyme processed by NS3 proteases, exerts a wide range of activities on cellular pathways such as induction of innate immune response, host cell growth and proliferation (reviewed in (Reed and Rice, 2000)). The interaction of NS5A with multiple host cell and viral proteins suggests its significant role as part of the replication complex in mediating viral replication, viral-host interactions, and viral pathogenesis.

1.4.2.2.5 NS5B.

The NS5B RNA-dependent RNA polymerase belongs to a class of membrane proteins commonly known as tail-anchored proteins (Ivashkina *et al.*, 2002). Non-nucleoside inhibitors and the new licenced direct acting antiviral drugs target NS5B to prevent viral replication (Biswal *et al.*, 2006). The NS5B forms an integral part of the membrane bound replication complex, and is essential for HCV replication through transcription of the viral positive-sense RNA strand. The resultant negative-sense RNA strand serves as a template for the synthesis of viral RNA genome (Lohmann, 2013). The NS5B nucleoside analogues interfere with HCV genome replication by inducing chain termination that results in interruption of transcription and translation of viral polypeptides (Sofia *et al.*, 2010). The NS5B catalytic site is highly conserved in different HCV genotypes; therefore NS5B RNA-dependent RNA polymerase has emerged as a major target for antiviral intervention.

1.4.3 HCV life cycle.

The HCV must attach to and infect hepatocytes in order to carry out its life cycle. Key steps in HCV life cycle occur outside the host's nucleus, and include attachment of the virus to the host cellular receptors, entry, uncoating of viral capsid, translation of viral proteins, replication, assembly, maturation and egress of virions via the lipoprotein pathways.

1.4.3.1 Formation of lipoviral particles (LVPs).

Figure 1.2: Lipoviral particle.

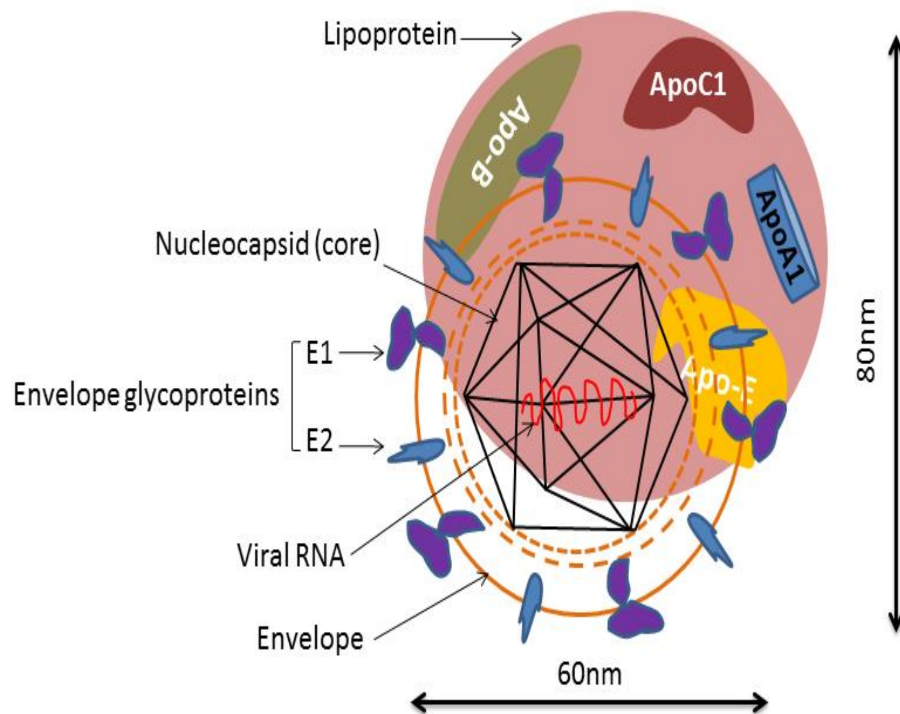


Figure **1.2** shows a schematic cartoon of LVP. The HCV particles circulate in the blood in association with lipoproteins in a complex called 'lipoviral particles' that are rich in cholesterol, triglycerides, and apolipoproteins (apo) B, E and C1. The LVPs help to attach the virus to the host cell via cellular lipoprotein receptors (Shawa, Sheridan, *et al.*, 2017).

An essential stage in the HCV lifecycle is the formation of lipoviral particles (LVPs) (Figure **1.2**). These circulating HCV particles are associated with different classes of lipoproteins (Dao Thi, Dreux and Cosset, 2011). HCV binds directly to low density lipoprotein (LDL), very low density lipoprotein (VLDL), and chylomicrons (CMs) (Diaz *et al.*, 2006) which leads to the viral particle's heterogeneous buoyant density (Felmlee *et al.*, 2010). LVPs are composed of triglyceride (TG) and cholesterol-rich lipoproteins that contain apoA1, apoB, apoE (Diaz *et al.*, 2006; Felmlee *et al.*, 2010), apoC1 and viral envelope proteins E1and E2; and nucleocapsids. LVPs are characterised by low buoyant density and larger size inherent from the lipoprotein interaction, and LVPs have higher infectivity than non-lipoprotein bound HCV virions (Miyanari *et al.*, 2007).

Evidence suggests that LVPs are formed from viral assembly within the hepatocyte, where the machinery for viral replication and assembly is dependent on very low density lipoprotein (VLDL) synthesis and export (Bassendine *et al.*, 2013). A subpopulation of LVP may also be formed within the vascular compartment by transfer of HCV particles onto native triglyceride rich lipoproteins (TRL) derived from both the liver (VLDL) and intestine (chylomicrons), and thus LVPs have been noted to increase after a high fat meal (Felmlee *et al.*, 2010).

The HCV particles that redistribute to VLDL and chylomicrons in the vascular compartment after a high fat meal have a very short half-life (<180 mins), implying that very-low density LVP that appear post-prandially are rapidly taken up by the liver (Felmlee *et al.*, 2010). *In vitro* studies revealed a pan-genotypic capacity for extracellular transfer of HCV onto TRL 'acceptors', and this transfer enhances infectivity of HCVcc (Felmlee *et al.*, 2010).

LVPs have inherent increased infectivity in cell culture and in animal models compared to non-lipoprotein bound HCV particles (Miyanari *et al.*, 2007). Silencing of apoE disrupts the formation of LVP and inhibits infectivity in vitro (Benga *et al.*, 2010). Furthermore, apoE-poor HCV particles are more sensitive to neutralizing antibodies, showing that apoE interaction is a viral adaptation to escape immune surveillance (Fauvelle *et al.*, 2016). Evidence from human studies also supports the model that LVPs are important in determining the natural history of early acute HCV infection. A study of patients with early acute HCV infection from the Australian Trial In Acute Hepatitis (ATAHC) and Hepatitis C Incidence And Transmission in Prisons Study (HITs P cohorts) reported low LVP levels were associated with spontaneous resolution of early acute HCV (Sheridan *et al.*, 2014). Previous studies of LVP in chronic HCV infection found an association between increased LVP concentrations and insensitivity to interferon based antiviral therapy (Bridge *et al.*, 2011; Sheridan *et al.*, 2012). In chronic HCV G1, LVP levels correlated negatively with markers of interferon sensitivity, and higher non-LVPs were associated with the interferon lambda 3 (IFNL3) CC genotype (Sheridan *et al.*, 2012).

The close association of virus and lipoprotein in the LVP masks HCV epitopes from antibody mediated neutralisation (Grove *et al.*, 2007). It is thought that there is an inverse relationship between density and infectivity, such that as HCV particles bind to immunoglobulin, so the density increases and infectivity diminishes. Formation of LVP could therefore be a mechanism that enables evasion of antibody mediated neutralisation (reviewed in (Felmlee *et al.*, 2013)). Recent evidence indicates that apoE association in itself may be sufficient for escape from neutralization (Fauvelle *et al.*, 2016). Thus any mechanism that would disrupt the formation of LVP could potentially expose viral epitopes that may increase the likelihood of antibody mediated clearance and decreased infectivity of HCV.

1.4.3.2 HCV attachment and entry.

The HCV involves lipid metabolism at each step of its life cycle. The viral entry steps involve complex processes involving viral attachment, clathrin-mediated endocytosis, and membrane fusion. Several studies have demonstrated that HCV utilises virally encoded envelope glycoproteins, and cellular protein apoE (Jiang *et al.*, 2012) for attachment which is the first step of virus–host cell interactions, and thus represents a good target for antiviral therapeutic agents. Figure 1.3 below outlines an HCV life cycle model.

Figure 1.3: HCV life cycle.

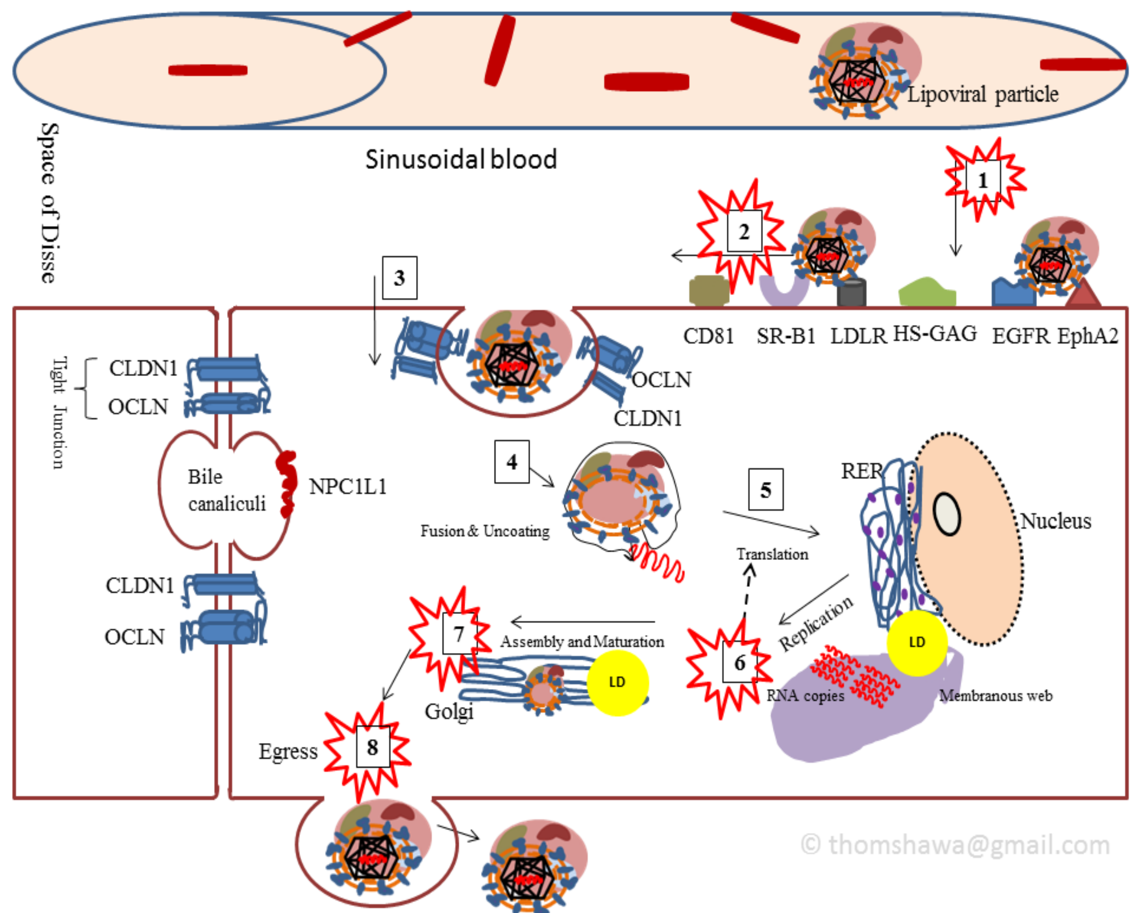


Figure 1.3 HCV circulates in the blood as lipoviral particles and enters the liver cells via the space of disse through fenestrated endothelia. L-SIGN (CD209L) and DC-SIGN (CD209) capture viral particles and transmit them to target cells to interact with receptor molecules such as HS-GAG and LDLR with high affinity for apolipoproteins. **2.** The viral particles then bind to SR-B1, CD81, OCLN and CLDN1 receptors/entry factors. **3.** The internalization (endocytosis) process is facilitated by the assembly of clathrin (ubiquitous route of receptor invagination into cells) and associated proteins on intracellular plasma membranes which mature into early endosomes. **4.** Low pH in endosomes enables fusion of viral particles with endosome. Uncoating of viral capsid delivers viral genomic material to cytoplasmic replication site. EGFR, EphA2, TfR1 and NPC1L1 are some of the putative entry factors. **5.** The successful viral entry through lipoprotein channels leads to translation of polypeptides in the endoplasmic reticulum. The expression of the viral NS4B protein induces alteration of cellular membranes leading to formation of a membranous web. **6.** The replication complex forms the +ssRNA strands via the –ssRNA intermediates in membranous web which shows characteristics of lipid rafts. **7.** Assembly and maturation of LVP. **8.** Secretion of mature virions via VLDL pathway. Note: The red stars (numbers 1,2,6,7 and 8) highlight stages of HCV-lipid interactions. Any defect in such interaction may provide a mechanism of resistance.

Several putative HCV receptor candidate molecules have been suggested to be involved in viral attachment and entry. The HCV entry is believed to be a highly orchestrated system that involves multiple viral and host cell factors, via receptor-mediated endocytosis and subsequent fusion of viral and host cellular membranes (Blanchard *et al.*, 2006; Meertens, Bertaux and Dragic, 2006). The circulating HCV particles gain access to hepatocytes through liver sinusoidal blood. The sinusoidal blood percolates to hepatocytes through the space of disse in fenestrated endothelium which lacks a basement membrane. The viral particles get trapped by sinusoidal cells mediated by specific molecules such as liver/lymph-node-specific intercellular adhesion molecule-3 grabbing integrin (L-SIGN) commonly known as CD209L, which are expressed by liver sinusoidal endothelial cells (LSECs), and dendritic-cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) also known as CD209 which are both expressed by liver kupffer cells. The HCV envelope glycoprotein E2 binds to L-SIGN; and DC-SIGN with high affinity; a process that captures and delivers viral particles to hepatocytes (Pöhlmann *et al.*, 2003). Several host receptors have been suggested to be involved in viral attachment and entry into hepatocytes, but the exact hierarchy still remains elusive. The HCV entry mechanism is a highly orchestrated multistep process that involves viral envelope glycoproteins E1 and E2, and apoA-1, apoB, apoC-1 and apoE (Jiang *et al.*, 2012) that attach to host receptors and co-receptors to facilitate viral internalization into endosomal compartment.

The initial attachment of the virus to hepatocytes occurs via low affinity interaction with highly sulphated glycosaminoglycans (HS-GAGs) (Barth *et al.*, 2003) via apoE and the low density lipoprotein receptor (LDLR) (Agnello *et al.*, 1999; Molina *et al.*, 2007). The docking of viral particles to HS-GAGS helps to concentrate them on the target cell surface for further horizontal interaction with other host entry factors (Barth *et al.*, 2003; Zeisel *et al.*, 2011) such as Scavenger receptor class B type I (SR-BI) which is highly

expressed at the sinusoidal surface of hepatocytes in the liver and other tissues. SR-BI binds to HDL, LDL, oxidized LDL, and viral envelope protein E2 (Scarselli *et al.*, 2002). SR-BI physiologically maintains lipid homeostasis by transporting cholesterol from HDL to hepatocytes (Rhainds and Brissette, 2004). In HCV entry, SR-BI binds directly to virus-associated lipoproteins (Dreux *et al.*, 2009) such as ApoE, ApoB, ApoC1 and ApoA-1 and the HCV envelope glycoprotein E2 (reviewed in Dao (Dao Thi, Dreux and Cosset, 2011)). Furthermore, serum HDL accelerates viral entry into hepatocytes (post-binding stage) by transferring apoC-1 from HDL to HCV during SR-BI mediated lipid transfer. SR-BI plays a dual role in attachment and post-attachment entry process and enhances viral infectivity (reviewed in (Zeisel *et al.*, 2011)).

The role of apoE in mediating viral attachment through heparan sulphate is well described (Jiang *et al.*, 2012). The formation of HSPG involves covalent interaction of HS and the cell membrane core proteins. The currently identified HSPG core proteins are syndecans (SDC), glypicans, perlecans, and agrin. SDC1 and SDC4 have recently been reported as LVP attachment co-factors, and important mediators that facilitate VLDL remnant clearance (Lefèvre *et al.*, 2014) (Shi, Jiang and Luo, 2013).

The LDLR was proposed as another HCV receptor (Agnello *et al.*, 1999; Molina *et al.*, 2007) but its role still remains controversial. It is yet to be established whether LDLR is a true host cellular receptor or a mediator of LVP attachment to hepatocytes. Other researchers described LDLR as an HCV co-receptor that interacts with apoE; further proposing that availability of apoE-containing lipoproteins such as VLDL but not LDL facilitates infection through interaction with LDLR (Owen *et al.*, 2009).

The tetraspanin CD81 is another HCV co-receptor that interacts with HCV E2 (Nencioni *et al.*, 1990) as a post-binding entry molecule. The interaction of CD81 with HCV E2, induces conformational changes in the HCV envelope glycoproteins which

primes HCV to respond to low pH of the endocytic compartment post entry. E2 contains a putative fusion domain which binds to CD81, and SR-BI. The hypervariable region 1 (HVR1) of E2 is essential in preserving the viral ability for replication through its interaction with SR-BI (Bartosch *et al.*, 2003). The crystal structure for highly conserved E2 antigenic site 412 to 423 was characterized in complex with the broadly neutralizing antibody AP33. The antibodies to CD81-E2 binding complex neutralize HCV infectivity by binding to E2 epitopes (Kong *et al.*, 2012). The CD81 – E2 complex facilitates the lateral movement of the virus to bind to other entry molecules such as Claudin-1 (CLDN1) (Evans *et al.*, 2007) and Occludin (OCLN) (Ploss *et al.*, 2009).

The CLDN1 is principle component of tight junction proteins required for late step viral entry into hepatocytes, but there is no clear evidence suggesting a direct interaction between HCV and CLDN1 (Evans *et al.*, 2007). However, CLDN1 was reported to form a complex with CD81 that promotes HCV entry (Harris *et al.*, 2008). Another critical component of tight junctions is OCLN which is involved in post-binding viral entry that confers HCV permissivity in mouse cell line (Ploss *et al.*, 2009). The OCLN interacts with HCV glycoprotein E2, but it is still unclear how OCLN/E2 interaction takes place. Whether OCLN binds directly to E2, or binds to CD81/CLDN1 complex is still elusive (Zhu *et al.*, 2014). What is known is the fact that OCLN acts as an anchor to the tight junction complex by providing cell-cell adhesion (Peng, Lee and Campbell, 2003).

The HCV internalization into the cell cytosol (endocytosis) is mediated by clathrin (Blanchard *et al.*, 2006) promoted by CD81 in association with CLDN1 (CD81/CLDN1 co-receptor complex) (Farquhar *et al.*, 2012). A recently identified entry factor transferrin receptor 1 (TfR1) was reported to assist in HCV internalisation after binding

CD81 (Martin and Uprichard, 2013). It was reported by Coller et al. (Coller *et al.*, 2009) that HCV endocytosis does not preferentially take place at cell-cell contact junctions as observed by imaging studies.

The final step of HCV cell entry which involves fusion of viral and host cell membranes (a process triggered in a pH-dependent fashion), depends on viral particles lipoprotein density and envelope protein integrity (Haid, Pietschmann and Pécheur, 2009). The endosome acidic pH, and virus – receptor interactions triggers viral penetration through fusion of cell receptors and envelope glycoproteins that contain fusion peptides (Smith, 2004). In vitro fusion assays showed that HCVpp/liposome fusion does not only depend on acidic pH and temperature but cholesterol as well (Lavillette *et al.*, 2006). Apart from the above explained viral entry route, a direct cell-cell viral infection has been described which potentially avoids the effects of neutralizing antibodies (Grupp *et al.*, 2007).

Other entry factors include epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2) (Lupberger *et al.*, 2011) and Niemann-Pick C1-like 1 (NPC1L1) proteins (Sainz *et al.*, 2012). No single entry factor permits HCV entry into a susceptible cell. Viral attachment and entry is a complex multistep process, with no clear hierarchical order fully elucidated. However, combined expression of four entry factors (CD81, SR-BI, OCLN, and CLDN1) is essential for HCV to productively infect hepatocytes (Da Costa *et al.*, 2012).

1.4.3.3 HCV RNA translation and replication.

The successful viral entry mediated by cathrin leads to translation of polypeptides in the endoplasmic reticulum. The RNA translation is initiated by the involvement of host cellular factors (Niepmann, 2013). The HCV genome contains a single open reading frame flanked by 5′ and 3′ non-translated regions (NTRs). The NTRs contain RNA elements that are important for RNA translation and replication (Lohmann, 2013). The 5′ NTR contains IRES which initiates HCV RNA translation into polypeptides which are later processed into structural and NS proteins by viral and host encoded proteases (Welbourn and Pause, 2007).

After translation, there is formation of a membrane-associated replication complex composed of HCV proteins associated with altered host cell membranes derived from the ER. The replication machinery constitutes viral proteins (NS3/4A, NS4B, NS5A, and NS5B) and the replicating RNA, which replicate the +RNA genome through a –RNA intermediate (Lohmann, 2013). The single +RNA genome template and the newly synthesized –RNA strand are base-paired which results in the formation of double-stranded (ds) RNA (Quinkert, Bartenschlager and Lohmann, 2005) (Targett-Adams, Boulant and McLauchlan, 2008).

The HCV replication takes place on double membrane vesicles known as lipid droplets (LD) with co-localization of structural core protein and non-structural NS5A (Masaki *et al.*, 2008). Viral replication induces formation of micro-environment in the host cell cytoplasm called 'membranous web.' Compartmentalisation of the replication complex protects the viral genome from double-stranded RNA (dsRNA) – mediated host defences, and exogenously administered nucleases and proteases (Moradpour *et al.*, 2002; Miyanari *et al.*, 2003). The intermediate dsRNA is copied multiple times for generation of +RNA progeny virions. The dsRNA serves as a pathogen-associated

molecular pattern (PAMP) and induces host innate immune system as a result of its recognition by toll-like receptors (TLR). One of the important replication factors identified is Phosphatidyl-inositol-4-kinase-III (PI4KIII) which interacts with NS5A to induce production of phosphatidylinositol-4-phosphate (PI4P) within the membranous web. Disruption in or absence of PI4KIII and NS5A interaction confers conformational changes in the membranous web (Reiss *et al.*, 2013). MicroRNAs (miRNA) are small RNAs involved in inhibition of RNA molecules (Hobert, 2008). The HCV is believed to utilise liver-specific miRNA-122, the most abundant miRNA in hepatocytes, for replication (Jopling, 2005) through the binding of miRNA-122 to two HCV binding sites in the 5' NTR of HCV genome (Machlin, Sarnow and Sagan, 2011). The HCV infection induces expression of lipogenic genes in hepatocytes that facilitate replication and assembly of infectious viral particles.

ApoA-I is also thought to play a role in the HCV replication as evidenced by the siRNA-mediated silencing of apoA-I that resulted in reduced concentration of viral RNA (Mancone *et al.*, 2011). However, the exact role of apoA-I in HCV replication is poorly understood.

1.4.3.4 HCV assembly and exit.

The later stages of the HCV life cycle involve assembly of viral proteins for production of progeny virions, and exit. The core protein forms the viral nucleocapsid of assembled virions but also binds to intra-cellular lipid droplets for production of infectious virions. It was observed that the HCV core causes steatosis in HCV genotype 3 patients by inducing lipid accumulation within hepatocytes; it also inhibits microsomal triglyceride transfer protein (MTP) activity (Jhaveri *et al.*, 2008). Thus, steatosis was reported to be more frequent in HCV genotype 3 infected patients than those infected with HCV genotype 1. The mechanisms underlying the significant

association between HCV genotype 3 and steatosis are unclear (Asselah *et al.*, 2006). The MTP plays a crucial role in VLDL synthesis; therefore its inhibition could potentially affect VLDL production and HCV assembly and exit.

The NS5A mediates the assembly of viral RNA genome in progeny virus by binding to apoE (Benga *et al.*, 2010). The NS5A also binds to HCV RNA and contributes to formation of the membranous web. The emergence of NS5A resistance associated variants in relapsing patients treated with DAAs on the interaction with apoE is unknown. Effective HCV particle formation involves packaging of nucleocapsid and envelope proteins into progeny virions that bud through the endoplasmic reticulum lumen via the VLDL secretory pathway (reviewed in (Felmlee *et al.*, 2013)). The assembly of infectious virions is dependent on an intact VLDL pathway. Co-dependency of HCV assembly on VLDL secretion is demonstrated by silencing of apoB, apoE and MTP, all of which inhibit HCV production in Huh 7 cells (an hepatocyte derived cellular carcinoma cell line) (Benga *et al.*, 2010). MTP plays a crucial role in VLDL synthesis; therefore its inhibition could potentially affect VLDL production and HCV assembly and exit. Expression of HCV core protein in transgenic mice was reported to inhibit MTP activity which also decreases VLDL secretion (Perlemuter *et al.*, 2002).

During HCV assembly, the HCV core protein is located on the cytosolic side of the ER, it is therefore believed that assembly occurs in the cytosol, whereas maturation and egress occur on the luminal side of ER, to enable mature virions to exit the cell via the low density lipid secretory pathways (reviewed in (Jones and McLauchlan, 2010)). Endogenous VLDL secretion occurs daily with an estimated 10 particles produced every 24 hours; whereas approximately 10^{12} HCV virions per day are produced from a complete HCV life cycle (Neumann *et al.*, 1998; Bassendine *et al.*, 2013).

Defects at any stage of the HCV replication and assembly pathway including induction of lipid droplets by core to facilitate viral replication, or defects in MTP or apoE or other proteins involved in HCV assembly through the VLDL secretory pathway may therefore provide resistance to establishing chronic infection in EUs.

1.5 HCV transmission.

HCV is primarily transmitted via parenteral routes. Risk for HCV transmission include factors with potential percutaneous exposure to unsafe blood or its products such as: unprotected sexual intercourse (Terrault *et al.*, 2013), solid organ transplant from an infected donor, blood transfusion before 1992 (Schreiber *et al.*, 1996), healthcare associated exposures (Grebely, Prins and Hellard, 2012), intravenous drug use (IVDU), acupuncture, tattooing (Pérez *et al.*, 2005), intranasal cocaine use and other unknown risk factors (Bunchorntavakul et al. 2014). Multiperson use of contaminated injecting needles, syringes and other injection paraphernalia is the common mode of transmission among IDUs (Hagan *et al.*, 1995). 5% of children with HCV infection, were infected through vertical transmission (Gibb *et al.*, 2000). Heterosexual intercourse poses a low risk to HCV infection than men who have sex with other men (Terrault *et al.*, 2013). Cell-to-cell HCV transmission has been reported in some studies, this poses a potential evasion of the virus from anti-HCV neutralizing antibodies as well as host immunity (Brimacombe *et al.*, 2011).

1.6 HCV epidemiology, genotype and geographical distribution.

Global HCV epidemiology has been reported based on seroprevalence. HCV is distributed worldwide among all races, age groups, gender and regions. HCV prevalence is high in economically less developed countries; with a total anti-HCV global prevalence ranging between 1.3 – 2.1 % (Gower *et al.*, 2014). In the developed world, HCV infection is also a problem with a prevalence of (0.9%) in Western Europe (United Kingdom 0.6%) (Gower *et al.*, 2014). Egypt has the highest reported HCV prevalence in the world owing to use of non-sterile injecting needles during the widespread use of a tartar emetic to treat schistosomiasis (Lehman and Wilson, 2009). Since variabilities in HCV geographical distribution have been reported, determination of specific HCV genotypes has become a useful method to predict the disease outcome, and treatment options. However, the impact of genotypes on outcome of HCV infection seem to be minimal in long term (reviewed in (Bukh, 2016)). Six major HCV genotypes have been reported, each comprising multiple subtypes and subspecies. The recently additional genotypes 7 through 11 have been suggested as variants of genotype 6 (Gower *et al.*, 2014). Genotypes 1, 2, and 3 have a worldwide distribution despite predominantly prevalent in western countries where in Europe alone, 90% of HCV infection are genotypes 1, 2 and 3 (Esteban, Sauleda and Quer, 2008; Messina *et al.*, 2015). The HCV genotype 4 seems to be confined in Egypt and the middle-east (Gower *et al.*, 2014). In Egypt genotype 4a is prevalent due to national anti-schistosomiasis mass treatment/injection campaigns from 1960s through to the 1980s (Ray *et al.*, 2000; Pybus *et al.*, 2003); whereas genotype 5 and 6 are highly prevalent in South Africa and Hong Kong respectively (Tokita *et al.*, 1998).

1.6.1 HCV epidemiology in injecting drug users.

In developed countries, injection drug use is the dominant mode of HCV transmission (Alter, 2002). As injection drug use still remains the highest risk factor for HCV infection, a long duration of injection usage is associated with high anti-HCV prevalence, and decreased HCV incidence rates (Lorvick *et al.*, 2001). HCV seroprevalence among long term IDUs is greater than 90% (Lorvick *et al.*, 2001; Alter, 2002; Tseng *et al.*, 2007) among IDUs who had an injection history of more than 6 years (Thomas *et al.*, 1995; Diaz *et al.*, 2001). Studies have reported a seroprevalence of less than 50% in IDUs who had an injection history of less than 5 years; but annual HCV incidence rates remain very high (10 – 40%) among uninfected IDUs (Diaz *et al.*, 2001; Hahn *et al.*, 2001; Miller *et al.*, 2002; Des Jarlais *et al.*, 2003). The HCV transmission is sustained by injecting drug practices such as sharing injecting needles and other paraphernalia (Thorpe *et al.*, 2002). There is a low risk of developing persistent HCV viraemia in IDUs who successfully cleared the infection even with continued exposure to the virus through injecting drug use (Grebely *et al.*, 2006). There is little data about the prevalence of HCV among IDUs in the developing world.

1.7 HCV natural history.

Exposure to HCV may be considered to result in one of three outcomes; remain antibody seronegative and aviraemic by sensitive RNA PCR (exposed uninfected) (Thurairajah *et al.*, 2008), spontaneous clearance (detectable HCV-antibodies but HCV-RNA negative), or establishment of chronic infection (both HCV antibody and RNA positive) (Knapp *et al.*, 2010) (Figure 1.4).

Figure 1.4: HCV Natural History

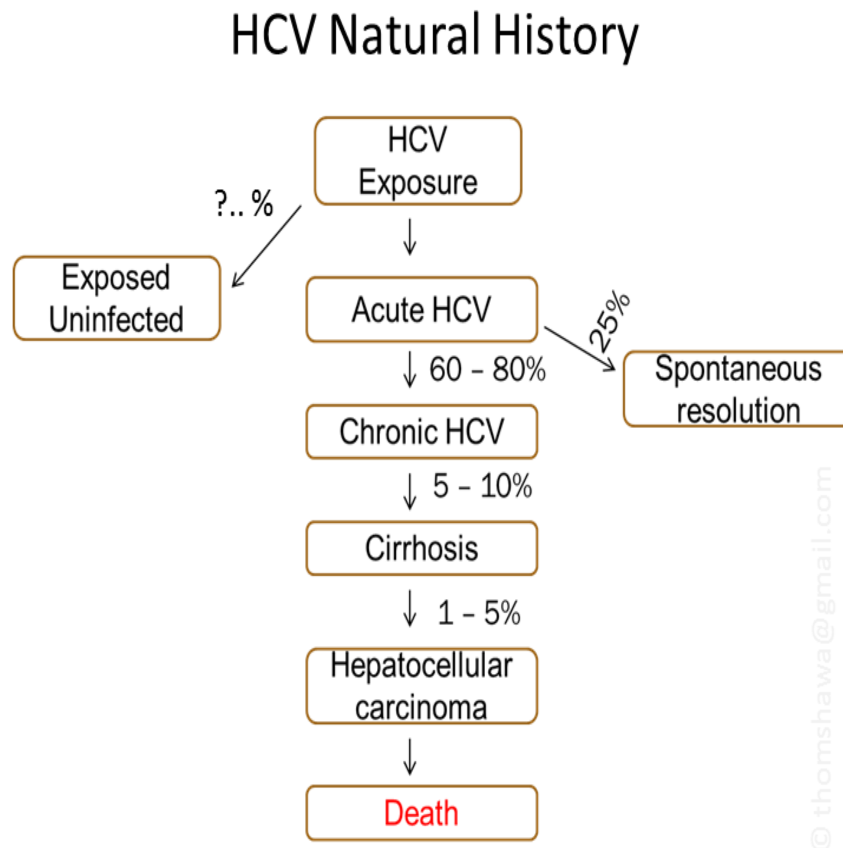


Figure 1.4: HCV natural history in untreated cases. Untreated HCV infection can lead to chronic infection, and liver cirrhosis. The liver-related mortality rates are due to decompensated liver cirrhosis or hepatocellular carcinoma. World health organisation (WHO) estimates that between 350,000 – 700,000 HCV infected individuals die every year. The estimated percentage for the exposed uninfected population among IDUs is yet to be reported globally. Plymouth city has more than 200,000 recorded cases of IDUs.

The outcome of acute HCV (AHCV) infection is dependent on host and viral factors that include age, sex, clinical presentations, viral co-infections, immune antiviral response and immunogenetic polymorphism. HCV infection is self-limiting within 12 – 16 weeks after onset of acute infection. During the first 12 weeks of disease onset, spontaneous viral clearance is achieved in a minority of self-limiting patients but beyond 24 weeks may suggest viral chronicity (Maasoumy, 2012). Approximately 80% of acutely infected individuals may progress to chronic hepatitis (Shepard, Finelli and Alter, 2005; Te and Jensen, 2010) because they fail to eliminate the virus (Mas Marques et al. 2009). It still remains elusive why some individuals clear the virus while others do not. However, Ball et al. (Ball, Tarr and McKeating, 2014), in their review indicated that immune T cell response and rapid induction of cross-reactive neutralizing antibody responses was an important factor for viral spontaneous clearance in acute infection. Women are twice likely to clear the virus than men. The mechanism behind such a phenomenon is unclear, though some findings have suggested that high oestrogen levels could be associated with sustained viral clearance (Baden et al. 2014).

1.7.1 HCV exposed uninfected.

There are subsets of individuals who remain uninfected with HCV despite long term repeated exposure through injection drug use; the global figures are yet to be reported. This naturally resistant unique cohort of IDUs has been referred to as ‘exposed uninfected’ (EU) (Thurairajah *et al.*, 2008). There is clear evidence that there is an absence of demonstrable infection in the EU cohort, who might represent a phenotype that is resistant to HCV infection. These individuals are serially negative for anti-HCV antibody and HCV RNA on at least 2 occasions more than 6 months apart with extremely high risk of HCV exposure, typically through injection drug use and sharing

of injection paraphernalia (Mizukoshi *et al.*, 2008; Knapp *et al.*, 2010). The probability of true HCV exposure has been determined on the basis of risk questionnaires and/or by the presence of HCV specific immune responses by interferon gamma (IFN-γ) enzyme-linked immunospot (ELISpot) assay.

IDUs who share needles with individuals who are known to have HCV infection are at high risk of HCV exposure. High risk of exposure is associated with high incidence of HCV infection during the first year of sharing drug injection equipment. Likewise, HCV sero-prevalence increases with a long duration of injection history (Law *et al.*, 2003; Roy *et al.*, 2007) as probability of actual exposure increases. The EU group needs to be carefully distinguished from unexposed by close scrutiny of the probability of actual HCV exposure.

An example utilising exceptional high-risk behaviours was described by Sugden et al, using a composite risk index for HCV exposure based on a time dependent cox-regression analysis of 14 separate weighted risk factors of risk behaviours and demographics (Sugden *et al.*, 2014). Significantly higher composite risk scores were found in those that subsequently became infected rather than uninfected. However, within the uninfected group there were individuals that remained uninfected despite high risk behavioural profiles. Separation of the uninfected group's risk into tertiles (two points that divide data into three equal parts) indicated that those in the highest tertile had a pattern of risk actually higher than that observed in subjects who subsequently become infected (Sugden *et al.*, 2014). Thus based on statistical models it is highly probable that these subjects had been exposed to HCV but were uninfected. Some people were infected with HCV through receiving of blood products before 1992 (Williams *et al.*, 2005). The HCV lookback programme described the outcome of HCV exposure and number of HCV infections following blood transfusions (The English

National Blood Service HCV Lookback Collation Collaborators, 2002). The residual risk of HCV transmission was reduced by implementation of an HCV RNA testing programme for individual blood donations (Legler *et al.*, 2000).

An alternative definition of HCV exposure in EUs is the presence of HCV specific immunological responses (Sugden *et al.*, 2014) in the absence of anti-HCV antibodies and HCV RNA. T cell responses can be measured to an array of HCV peptides from viral structural and non-structural proteins, and may thus be used as a marker of immunological exposure to actual HCV particles. Up to 60% of high risk individuals have detectable HCV specific T cells responses by ELISpot (Knapp *et al.*, 2010), but this definition of EU may exclude others who may have alternative pathways of resistance other than immunological. The ELISpot is a technique employed for detection and analysis of individual cells that secrete specific proteins such as cytokines in vitro (Czerkinsky *et al.*, 1983). ELISpot is one of the sensitive cellular assays that allows for detection of one cell in 100,000; and is between 20 and 200 times more sensitive than the conventional enzyme immunosorbent assay (ELISA) (MABTECH, 2016). Cellular IFN-γ production is used as readout of single cell activation (T helper 1 cells) in the specific immune response. Generation of an individual spot (cellular footprint) represents an individual cytokine secreting cell (reactive cell) (MABTECH, 2016).

The detection of an HCV-specific T cell immune response may not conclusively suggest the presence of HCV infection, as adaptive immune response can also be detected in healthy individuals who show cross-reactivity between HCV NS3 proteins and influenza virus neuramidase proteins (Wedemeyer *et al.*, 2001). Therefore, testing against an array of other HCV NS proteins could potentially increase the specificity of ELISpot assays as a marker of HCV exposure. Cross-reactivity to an array of HCV

structural and non-structural antigens may be common among IDUs due to exposure to different antigens from contaminated injection equipment (Zeremski *et al.*, 2009) and may therefore be a more robust marker of HCV exposure.

HCV resistance may represent a spectrum, with some individuals having high level resistance even after exposure to high concentrations of HCV, such as a few cases that received known HCV contaminated blood products. Other EU cases may have a lower degree of resistance, potentially on the same spectrum as those ~20% that spontaneously clear HCV by immune mediated pathways (Elliot *et al.*, 2006). Our research group characterised the EU cohort, defined on the basis of high risk behaviours but without demonstrable HCV infection (Thurairajah *et al.*, 2008) supports the notion of a spectrum of levels of HCV resistance, because HCV specific T-cell immune responses are demonstrated in more than 50% , but not all EU cases (Thurairajah *et al.*, 2008).

1.7.2 HCV spontaneous resolution.

A number of research groups have investigated the ~ 20% that are able to clear HCV spontaneously (spontaneous resolvers) (Thomas *et al.*, 2009). HCV spontaneous resolution has been associated with multi-faceted cellular immune response with neutralising antibodies (nAb) playing a vital role. There is growing evidence suggesting that protective natural immune response allows viral clearance without seroconversion. In-vitro studies have also demonstrated that genetic polymorphism can confer partial resistance to establishment of HCV infection (Liu *et al.*, 2009). The discovery that genetic resistance for *Human immunodeficiency virus* (HIV) infection was conferred via the homozygosity for the truncation mutation of C-C chemokine receptor-5 (CCR5)

gene (HIV entry co-receptor also known as CD195) (Olivieri *et al.*, 2007), underpins the belief that HCV clearance could be conferred in a similar fashion. Establishment of HCV primary infection is deterred by a characterised combination of genetic and environmental factors. There was a major breakthrough in 2009 regarding identification of some host factors that are associated with viral clearance.

Single nucleotide polymorphisms (SNP) in the interferon lambda (IFN-λ) 3 gene locus (IFNL3) linked to interleukin 28B (IL-28B) are the dominant host genetic factors associated with HCV spontaneous resolution (Ge *et al.*, 2009; Thomas *et al.*, 2009; Rauch *et al.*, 2010) and PEG-IFN treatment induced viral clearance (Sugiyama *et al.*, 2011). The interferon lambda genes encode 3 distinct but related proteins denoted IFN-λ 1, -λ2 and -λ3 also known as interleukin (IL)-29 (IL-29), IL-28A and IL-28B respectively which form a group of cytokines called 'type III IFNs' (Kelly, Klenerman and Barnes, 2011). These cytokines share a common signalling pathway with type I IFNs but exert their actions via a receptor complex that is distinct from type I IFNs (Ank *et al.*, 2008). Therefore IL-28B is a significant marker that could be used to distinguish the healthy population, spontaneous resolvers, chronic infection and EUs.

Spontaneous resolution of acute HCV infection is associated with a sustained HCV-specific T cell response. However, such a T cell response is weak, and transient in CHCV patients (Mizukoshi *et al.*, 2008). The crucial change in gene expression that occurs during HCV infection is the activation of the type 1 interferon response. The complex cell signalling process inducing the cytokine production during initial steps of viral infection allows recruitment of coordinated and effective innate and adaptive immune responses. However, HCV interferes with cytokines at various levels including escaping the surveillance of the immune responses by inducing a Th2 cytokine profile.

1.7.3 Acute HCV infection.

Acute hepatitis C virus (AHCV) is usually misdiagnosed because the majority of infected individuals are asymptomatic (Deterding *et al.*, 2009). Only 20 – 30% of the clinically asymptomatic infected adults develop clinical symptoms. Even in symptomatic acute hepatitis, the symptoms are very unspecific which last for few weeks. The HCV incubation period ranges from 3 to 12 weeks and varies depending on the transmission route. The longer incubation period is experienced when one is infected with low viral load (Mosley *et al.*, 2005). Therefore, >1 log viraemia fluctuation and low titre HCV-RNA are enough to support the AHCV laboratory diagnosis. Notable symptoms include; fever, nausea, anorexia, abdominal discomforts, mild jaundice and malaise. Jaundice is the hallmark for liver disease and is apparent in 50 – 84% of infected overt patients. Seroconversion takes 8 – 12 weeks after viraemia. HCV RNA can be detected within 1 to 3 weeks after exposure, whereas anti-HCV antibodies are negative at this stage, but they could be detected at the onset of clinical symptoms in some patients. Therefore antibody diagnostic assays are unreliable in acute infection (Maasoumy, 2012; Bunchorntavakul *et al.*, 2014). AHCV infection is reported to be declining but increased risk factors such as IVDU, sexual activities and occupational exposures are some of the predisposing factors that increase the incidence of AHCV infection (Maasoumy, 2012; Baden, Rockstroh and Buti, 2014; Bunchorntavakul *et al.*, 2014).

1.7.4 Chronic HCV infection.

The chronic hepatitis could be defined as the presence of HCV RNA in the blood for at least 24 weeks after infection (Seeff and Hoofnagle, 2002). The spontaneous resolution is rare once chronic hepatitis is established (Maasoumy, 2012). It is clear that HCV employ multiple strategies to persist within the infected host. The AHCV is often

followed by chronic infection in approximately 85% of infected individuals (Seeff, 2002). There is strong evidence associating chronic HCV infection (CHCV) to development of liver fibrosis, cirrhosis and HCC. Approximately 500,000 new cases of liver cancer are reported annually whereby 22% of such new cases are due to chronic HCV infection (Lozano *et al.*, 2012). Once infection is established, the viral – host interactions underpin HCV pathogenesis. The CHCV is typically acquired during adulthood, and disease progression is advanced by greater age, alcoholism, obesity, HIV co-infection. Interferon based therapy is less effective in chronic infection but DAAs have improved the sustained viral response (SVR) rate (Dabbouseh and Jensen, 2013). Other researchers defined SVR as undetectable serum HCV RNA based on a transcription-mediated amplification assay, maintained for 24 weeks after treatment (Barnes *et al.*, 2009).

1.8 HCV laboratory diagnosis.

HCV diagnostic assays include serological assays that detect HCV antibodies, and molecular techniques that detect, quantify and characterize the HCV RNA genome. Enzyme immunoassay (EIA) assays are screening methods for detection of anti-HCV antibodies in CHCV patients' serum; whereas recombinant immunoblot assay (RIBA) is a supplementary method designed to resolve false positive results generated by screening methods. HCV recombinant proteins and synthetic peptides from core, NS3, and NS5 proteins (immunodominant epitopes) were used as antigens that lead to successful development of RIBA anti-HCV IgG immunoglobulins detection assays (Colin *et al.*, 2001). Detection of anti-HCV by RIBA is based on immobilisation of HCV recombinant antigens into a test strip membrane that appear as individual bands. Positive results are detected by reactivity with ≥ 2 proteins, whereas reactivity to 1 protein indicates indeterminate results (Gerlach *et al.*, 2003). Anti-HCV antibodies are

undetectable in early AHCV infections and severely immunocompromised patients (Alter *et al.*, 2003). Anti-HCV antibody concentrations persist in the circulation in the absence of HCV RNA following spontaneous or treatment-induced resolution (Chevaliez and Pawlotsky, 2008; Kamili *et al.*, 2012).

Diagnosis of AHCV or CHCV is dependent on detection of HCV RNA in body fluids by sensitive molecular detection techniques (lower detection limit <9.3 HCV RNA international units (IU)/ml in plasma) such as polymerase chain reaction (PCR). Confirmation of active HCV infection is established by detection of HCV RNA by PCR in patients' samples, as well as monitoring treatment antiviral response to therapy. HCV viral load is determined by a sensitive quantitative PCR. Nucleic acid testing (NAT) remains the gold standard for active HCV confirmatory testing, and HCV RNA is detectable in plasma as early as 1 week post-exposure (Pawlotsky, 2003). NAT testing in clinical laboratories requires skilled technical personnel, the reagents and consumables are expensive, as well as dedicated procedure areas (Hosseini-Moghaddam *et al.*, 2012). The current NAT testing is based on PCR (relies on in-vitro amplification of the target sequence), branched DNA signal amplification (a signal amplification technology that does not require amplification of a target sequence), and transcription mediated amplification. NAT testing exhibits approximately 99% specificity across all 6 major HCV genotypes (Kamili *et al.*, 2012). Advances in molecular techniques have improved that led to the development of prototype nanoparticle-based diagnostic assays that detect HCV biomarkers. Examples of the developed nanoparticles are quantum dots (QDs) and gold nanoparticles (Azzazy, Mansour and Kazmierczak, 2006). HCV blood metabolites profiling have recently been successfully quantified by Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) techniques (Gowda *et al.*, 2008).

Identification of AHCV infection is seldom possible because the majority of patients test positive for anti-HCV antibodies or HCV RNA at diagnosis. Therefore no anti-HCV IgM immunoglobulins serve as early markers for HCV infection because HCV IgM are variably detected in both AHCV and CHCV infections (Quinti *et al.*, 1995). AHCV and CHCV infection are distinguished by monitoring fluctuations of HCV viral load (McGovern *et al.*, 2009), variations in anti-HCV titres (Coppola *et al.*, 2009), and determination of IgG avidity to HCV (Kanno and Kazuyama, 2002). The rapid HCV serologic assays for detection of anti-HCV antibodies have been introduced for rapid screening of HCV infections, but discrimination between active HCV infection and resolution requires molecular techniques.

1.9 HCV treatment and drug resistance.

The CHCV treatment has significantly been revolutionized recently with the licencing of DAAs. Interferon based standard therapy in combination with RBV has been the cornerstone for HCV treatment for the past two decades but has many adverse effects. The common adverse effects associated with PEG-IFN/RBV treatment include cough, haemolytic anaemia, neuropsychiatry, muscle aches, teratogenicity, poor tolerability and significant toxicity which result in premature discontinuation of therapy in 10 – 20% of patients (Russo and Fried, 2003). The HCV treatment with PEG-IFN and RBV is relatively ineffective with 50% SVR rate (Niederau *et al.*, 2012). Because of reduced SVR for interferon-based therapy, there has been a motivation for development of alternative therapeutic agents with minimal adverse effects, and a convenient drug administration route as opposed to the subcutaneous administration of PEG-IFN/RBV (Abraham and Spooner, 2014). The DAAs target HCV specific NS proteins to disrupt viral replication and subsequent infection. The following four classes of DAAs have been defined depending on their therapeutic targets and mechanisms of action: NS3/4A

protease inhibitors, NS5A inhibitors, NS5B nucleoside polymerase (NS5B RNA-dependent RNA polymerase) inhibitors, and NS5B non-nucleoside polymerase inhibitors (Table 1.1) (Poordad and Dieterich, 2012). In 2011, NS3/4A serine protease inhibitors (Boceprevir and Telaprevir) were the first DAAs to be approved for HCV treatment in conjunction with PEG-IFN and RBV; which improved the SVR to about 75% in CHCV genotype 1 treatment naïve patients (Jacobson *et al.*, 2011; Poordad *et al.*, 2011), but they have to be used in combination with the PEG-IFN and RBV standard of care regimen. SVR is defined by absence of detectable viremia (HCV RNA in blood) 12 or 24 weeks post HCV treatment (Swain *et al.*, 2010). The successful SVR is associated with reduced risk of HCC development but does not fully offer protection against development of HCC (Russo, 2010; Swain *et al.*, 2010). Sofosbuvir is an oral NS5B nucleotide polymerase inhibitor that was licenced in Europe in January 2014 and has been reported to have HCV pan- genotypic effect, either as a dual therapy with RBV or triple therapy with NS5A inhibitors, and protease inhibitors (reviewed in (Stedman, 2014)). Sofosbuvir is administered orally once a day even without food dependence. It achieves its highest plasma concentration within 0.5 to 3 hours (Abraham and Spooner, 2014; Mariño *et al.*, 2014). Sofosbuvir's virologic efficacy rate varies between 70 – 90%, has high genetic barrier to resistance, and can easily be eliminated renally (Jacobson *et al.*, 2013).

The USA biotechnology company, Gilead Sciences has developed an experimental HCV drug 'Ledipasvir' an NS5A inhibitor that has antiviral activities again genotype 1 subtypes only whereas Sofosbuvir works against genotypes 1 – 4 in combination with Ribavirin with or without PEG-IFN (Afdhal *et al.*, 2014). A 12 week or 24 week therapy combination of Ledipasvir and Sofosbuvir with or without Ribavirin has been reported to have an increased SVR in genotype 1 treatment naïve patients while the previously genotype 1 treated patients equally showed high SVR (between 94% to 99%).

Generally, a Ledipasvir and Sofosbuvir combination with or without Ribavirin has a >97% SVR regardless of treatment duration and patients' characteristics. Therefore, a single tablet regimen of Ledipasvir and Sofosbuvir without Ribavirin for 12 weeks is effective enough, but the addition of Ribavirin in the regimen has little benefit (Afdhal *et al.*, 2014).

HCV exists in quasispecies (a mixture of closely related but different genomes) in the host due to high degree of genetic variations as a result of high mutation rate (Forns, Purcell and Bukh, 1999). This renders the available treatment options less effective (Schweitzer and Liang, 2013; Zeng *et al.*, 2013). Other newly licenced DAAs include Simeprevir (an NS3/4A protease inhibitor) approved in May 2014, and Daclatasvir (an NS5A inhibitor) approved in September 2014 (The European Association for the Study of the Liver, 2014). Baseline predictors of treatment response include: viral factors (viral load, HCV genotype), ethnicity, IL-28B polymorphism, HIV co-infection, degree of liver fibrosis and previous HCV treatment (Delwaide *et al.*, 2005; Ramachandran *et al.*, 2012). In the current DAA era, HCV genotype 3 SVR is lower as opposed to genotype 2 (Jacobson *et al.*, 2013), making HCV genotype 3 as the more difficult –to-treat genotype (Goossens and Negro, 2014; Pawlotsky, 2014).

HCV becomes resistant to the commonly prescribed Interferon and Ribavirin due to selective pressure which results in adaptive mutations. The mutations in viral NS3 and NS5B regions are associated with protease and non-nucleotide inhibitors' resistance (Stedman, 2014). Nucleotide analogue inhibitors have a high barrier to resistance because they select viral resistant variants that hardly replicate in the presence of drugs, and rarely present at baseline. The other DAAs have low barrier to resistance because they select fit viral resistant variants in the presence or absence of the drugs (Pawlotsky, 2014). A 90% cure rate was achieved in phase III clinical trials (a single-pill

combination of Sofosbuvir plus Ledipasvir, Sofosbuvir plus Daclatasvir, and a two-pill combination of Sofosbuvir plus Simeprevir); however, a subset of CHCV patients (13 out of 316) had treatment failure (Zeuzem *et al.*, 2015). The CHCV patients who failed to clear the virus experienced post-treatment relapse to DAAs which have low barrier to resistance. There is a growing evidence confirming that patients who fail oral IFN-free treatment, contain highly resistant viral strains at the time of relapse (Dvory-Sobol *et al.*, 2015).

Table 1.1 Newly licenced HCV DAAs.

Classes of DAAs	Name of drugs	Mode of action	HCV regimen	Therapeutic target	Targetable HCV genotype
NS3/4A protease inhibitors	- Telaprevir - Boceprevir - Simeprevir - Paritaprevir - Asunaprevir - Faldaprevir - Elbasvir - Grazoprevir	Arrests protein synthesis	Paritaprevir + Ombitasvir + RBV	NS3/4A	Genotype 4
NS5A inhibitors	- Ledipasvir - Ombitasvir - Daclatasvir - Velpatasvir	Prevents replication	Lediprevir +Sofosbuvir + RBV	NS5A	Genotypes 1, 4, 5 & 6
NS5B nucleoside polymerase inhibitors	- Sofosbuvir	Prevents replication	Sofosbuvir + RBV	NS5B	Genotypes 2 & 3
NS5B non-nucleoside polymerase inhibitors	- Dasabuvir		Dasabuvir + Paritaprevir + Ombitasvir ± RBV	NS5B	Genotype 1

Table 1.1: Examples of IFN-free HCV regimens for treatment of CHCV in Europe (European Association for the Study of the Liver, 2017).

1.10 HCV reinfection following spontaneous resolution and/or treatment.

After clearance of HCV infection either spontaneously or following treatment, people remain at risk of reinfection with HCV. People who successfully resolve HCV infection do not possess protective immunity against reinfection, but available evidence suggests that reinfection does occur despite efficient immune responses that result in viral clearance (Grady *et al.*, 2012; Grebely *et al.*, 2012). Blackard defined HCV reinfection as a complete resolution of an initial infection prior to a subsequent infection either with a different genotype/subtype or the same type (Blackard and Sherman, 2007). On the contrary, recurrence of HCV viraemia within 24 weeks after completion of treatment is defined as a viral relapse. Viral coinfection is a simultaneous acquisition of more than one viral strain; whereas superinfection occurs in CHCV cases that are re-exposed to different HCV viral strain(s) (Grady *et al.*, 2013). Therefore detection of viraemia in individuals who previously cleared HCV infection either spontaneously or following cessation of therapy, are classified as reinfection cases.

Studies of HCV reinfection have provided insights into important aspects that offer against viral persistence. It was further reported that reinfection bouts are associated with improved control of HCV replication and an increased likelihood of resolving the infection as opposed to the primary infection (Bassett *et al.*, 2001; Prince *et al.*, 2005) possibly due to induction of HCV-specific T cell responses (Shoukry *et al.*, 2003; Abdel-Hakeem *et al.*, 2014). Cases of HCV reinfection were reported in PWIDs and men who have sex with other men (Grady *et al.*, 2012). The proportion of incidences of HCV reinfection after spontaneous resolution versus incidences of primary infection should be assessed. Grebely et al. explained that if most reinfections spontaneously cleared, there would be a strong logical argument for some level of protection. Determination of the size and length of HCV viraemia during reinfection as compared

to the primary infection aid to establish whether protection is generic or immunological. A simplification in the degree or duration of viraemia would suggest that acquired protective immunity resistance has a role, because fixed genetic factors would not adapt and become robust as does the immune response (Grebely *et al.*, 2013).

Notably, HCV reinfection does not always lead to viral persistence; data suggest that spontaneous clearance of reinfection also occurs even when infected with a different genotype from that of the primary infection (Page *et al.*, 2009). Therefore reinfection rates among IDUs are still low, but despite such low incidences, there is a need to provide awareness and sensitisation education to PWIDs regarding the possibility of HCV reinfection even after resolution of the initial infection.

1.10.1 Models for the study of HCV infection.

1.10.2 In vivo models.

The HCV clinical research and investigation is inherently hampered by the heterogeneity of human host, tissue tropism and its restricted access, and lack of a suitable small animal model. Numerous experimental tools have been developed to describe the HCV interaction with its human host. Efforts to identify a small animal model to study HCV life cycle are on-going. Chimpanzees (*Pan Troglodytes*) are well-studied, established wild animal species with a 98% genetic identity to humans that are susceptible to HCV infection. The chimpanzees have played a pivotal role in the discovery of HCV (reviewed in (Houghton, 2009)) and remains the gold standard for other small and large animal models. The HCV has a narrow host range (humans and chimpanzees only), therefore studying HCV in large animal models (great apes) is restricted by cost implications, ethical issues, and limited availability (Bukh *et al.*, 2001), which led to some countries banning the use of large apes as experimental models in research. The host immune response to HCV infection was described by

successful experimental infection of Chimpanzees which helped assessment of potential drug and vaccine candidates (reviewed in (Bukh, 2004; Houghton, 2011).

Various animal species have been challenged with HCV to identify alternative animal models. The simians and their orthologs of HCV attachment and entry factors were reported to share sequence similarities with humans and Chimpanzees (Flint *et al.*, 2006). The HCV replication in Simian hepatocytes is antagonised by different kinetics, and monkey cells' magnitudes of antiviral defences (Billerbeck *et al.*, 2013). Despite genetic similarities between the large primates and humans, establishment of infection in Chimpanzee models does not directly translate to human infection.

The tree shrew (*Tupaia belangeri*) – a wild small squirrel-like mammal related to primates, is a reported putative candidate for small animal models for HCV infection, which has shown to be susceptible to HCV infection. Establishment of HCV infection in tree shrew animal models is difficult due to transient viremia and need for immunosuppression. *Tupaia belangeri* orthologs of HCV entry factors have been reported to facilitate viral uptake (Tian *et al.*, 2009; Tong *et al.*, 2011). Intermittent and transient viremia was reported in *Tupaia belangeri* challenged with serum mixture of HCV genotypes (Xie *et al.*, 1998). Immunosuppression of *Tupaia* by X-ray irradiation increased infection frequency of 50%. However, *Tupaia* challenged with patient or cell culture derived HCV resulted in increased frequency of infection rates to more than 80% without the need for immunosuppression (Amako *et al.*, 2010). Despite the long-standing track of mice in biomedical research, they are not susceptible to HCV infection. Different transgenic mice strains have been studied to model HCV infection, but transgenic models have different pathways and strictures that limit their utility. Numerous approaches toward the development of small animal models for HCV infection such as xenotransplantation, non-primate hepacivirus, continue to be pursued.

1.10.3 Small animal model for the study of HCV.

Researchers previously observed that the CD81 and occludin were the human entry factors required to render mouse cells permissive to HCV entry in vitro (Ploss *et al.*, 2009). It was further reported that transgenic mice capable of expressing human CD81 and occludin factors support HCV entry into hepatocytes, but the establishment of HCV infection in vivo was restricted by the host innate and adaptive immune responses (Dorner *et al.*, 2013). The genetically humanized mouse models may offer new avenues for studying HCV infection in vivo. The transient expression of human CD81 and occludin entry factors by adenoviral delivery efficiently supported HCVcc entry into the mouse hepatocytes (Dorner *et al.*, 2011). The transgenic mice with four human entry factors but deficient in innate immune signaling pathways was reported to allow HCVcc entry into the hepatocytes, low level replication was also observed, and infectious viral particles were recovered in mouse serum (Dorner *et al.*, 2013).

1.10.4 The newly discovered hepaciviruses.

Until 2011, the only known HCV homolog was GB virus B (GBV-B) named after a surgeon (initials GB) suffering from an acute hepatitis in 1967 whose serum caused transmissible acute hepatitis in tamarins in 1995 (Stapleton *et al.*, 2011). Both the GBV-B and HCV belong to the hepacivirus that also include the recently accepted genus Pegivirus (Stapleton *et al.*, 2011), but GBV-B rarely establish persistent infection (Takikawa *et al.*, 2010). The HCV-related hepaci- and pegiviruses were discovered in animals such as horses, non-human primates, bats, dogs, and rodents (Scheel, Simmonds and Kapoor, 2015). Based on phylogenetic relationships and the genome organization, Pegivirus contains GBV-A, GBV-C, and GBV-D viruses (Stapleton *et al.*, 2011). The GBV-A was not shown to cause hepatitis in tamarins whereas GBV-B was

associated with the development of acute, self-limiting hepatitis infection in tamarins (Schlauder *et al.*, 1995).

A well-studied hepacivirus that belongs to the hepaciviridae family is non-primate hepacivirus (NPHV) also identified in dogs, bats, horses and wild rodents (El-Attar *et al.*, 2015). The NPHV are phylogenetically related to HCV (Burbelo *et al.*, 2012).

Another hepacivirus to be characterized and reported to infect a wild non-human primate (*Colobus guereza*) was *guereza* hepacivirus (GHV) that share some common features with the GBV-B. A novel equine Pegivirus (EPgV) has also been discovered recently that infects horses (Chandriani *et al.*, 2013). These new discoveries explain the host range and evolution of hepaciviruses.

1.10.5 In vitro models.

The lack of reliable cell culture systems in the past hampered the initiation of productive HCV infection in biomedical research. Molecular techniques enabled the development of efficient *in vitro* culture systems for study of HCV infection. Primary cells from humans and Chimpanzees were used in initial attempt to establish the *in vitro* HCV infection; but due to contamination problems observed in primary hepatocytes, researchers tried to develop immortalized human hepatoma cell lines (Castet *et al.*, 2002). Human liver cancer cell lines (Huh 7 cells and its derivatives), were proposed as an ideal *in vitro* human hepatic cell model for recapitulation of the HCV life cycle. The development of *in vitro* hepatocyte culture platforms has enables the elucidation of a detailed analysis of essential aspects of HCV pathogenesis. The *in vitro* models do not directly represent the clinical host responses and disease development *in vivo*. Hepatoma cell line 7721 (Song *et al.*, 2001) and human hepatocyte cell line PH5CH

(that is immortalised with simian virus 40 large antigen) were susceptible to HCV infection but with less efficiency (Kato *et al.*, 1996) suggesting that overexpression of viral oncogenes could be enough to support the *in vitro* growth of arrest of adult hepatocytes. However, human adult hepatocytes have limited proliferation capacity and do not undergo cell growth *in vitro*.

1.10.5.1 HCV sub-genomic replicon.

Woerz et al. defined a replicon as a nucleic acid (either DNA or RNA) that is capable of autonomous replication, but in this context, a replicon refers to RNA molecules capable only of intracellular self-replication i.e. unable to support the production of infectious viral particles (Woerz, Lohmann and Bartenschlager, 2009). The HCV replicon system facilitates the replication of a modified HCV genome in human hepatoma (Huh 7) cells. The HCV replicons contain either NS proteins for RNA replication (sub-genomic) only or the entire HCV genome (genomic) in length. Both replicon systems contain the neomycin phosphotransferase resistance gene for selection with G418 (geneticin). A bi-cistronic replicon was created with the inclusion of sub-genomic clones of HCV genotype 1b in combination with a heterogenous encephalomyocarditis virus (EMCV) IRES sequence for synthesis of HCV NS proteins (Lohmann, 1999). All genes were driven by the T7 promoter. Following transcription by T7 RNA polymerase, the sub-genomic replicon RNA was transfected into Huh-7 cell lines, to observe the intracellular replication of viral genome. RNA replication facilitates cell growth and colony formation in the presence of G418 antibiotic. Poor replication efficiency and reduced reproducibility of the replicon system were observed. However, to enhance replication levels, HCV genotype 1b clone adaptive mutations were developed in NS region. Some RNA replicons replaced the neomycin phosphotransferase gene with the luciferase gene in transient assays to identify adaptive mutations. The adaptive mutations observed in

the replicons were acquired through mechanisms that are unclear. Detection of adaptive mutations in HCV NS proteins NS3, NS5A and NS5B were observed (Blight, 2000) (Lohmann *et al.*, 2003). It was reported that IFN resistance was associated with NS5A adaptive mutations. Adaptive mutations increased viral genome replication (Bartenschlager, 2002). Characterization of cells harbouring replicons showed that they were able to sustain RNA autonomous replication for over one year, but effective clone replication decreased over time (Pietschmann *et al.*, 2001). The study of sub-genomic replicons systems has allowed elucidation of viral replication, and screening of chemicals for development of novel therapeutic agents with antiviral actions against HCV. Despite great strides made in studying *in vitro* HCV models, the production of intact viral particles for elucidation of a complete HCV life cycle is yet to be achieved.

1.10.5.2 HCV producing pseudo particle (HCVpp) and infectious HCV cell culture systems (HCVcc).

Infectious cell culture systems have been developed for in-vitro study of HCV replication. The development of full length replicon (Japanese fulminant hepatitis isolate 1 – JFH-1) for HCV genotype 2a that was derived from a Japanese patient with fulminant hepatitis represent a major breakthrough. This full length genomic replicon efficiently replicates in Huh-7 and other cell lines without requiring adaptive mutations (Kato *et al.*, 2003). Wakita and his colleagues developed a replicon system that efficiently propagates and secretes viral particles in Huh-7 cells using a full length JFH-1 sequence (genomic replicon). Transfection of JFH-1 genomic replicon supports the propagation of HCV in cell culture (HCVcc) that produces culture-derived HCV infectious particles (the HCVcc particles) as authentic virions (Wakita *et al.*, 2005; Zhong *et al.*, 2005; Lindenbach *et al.*, 2006).

Further improvements were made to the Huh-7 cell lines, to derive Huh-7.5.1 cell lines that are highly permissive to JFH-1 infection and increase viral titre to between 10^4 – 10^5 infectious units per ml of culture supernatant (Zhong *et al.*, 2005). The current models of HCV replication cycle are based on studies of JFH-1 infectious viral particles. Attempts for explanation of the early stages of HCV life cycle were provided by studying HCV pseudotype particles (HCVpp). Transfection of three vectors in human embryo kidney cells (293T) led to production of HCVpp (Da Costa *et al.*, 2012). The HCVcc system covers the complete viral replication cycle, but has its strong impact in dealing with late steps of the replication cycle (assembly and egress); whereas the HCVpp system is superior at providing an explanation for early stages of HCV life cycle (attachment, entry, and uncoating) (Baumert *et al.*, 1998; Blanchard *et al.*, 2002). The efficient production of viral particles in HCVcc is only restricted to JFH-1 isolates which is a major limitation. Other infectious viral particles were produced in H77 genotype 1a isolates, but the produced particles have low infectivity (Yi *et al.*, 2006). Generally, the HCV replicon systems attain a high genetic flexibility and cover a broader range of isolates and genotypes that offers avenues for the development of antiviral agents.

1.11 Lipid metabolism.

The host factors are important in viral infectivity, therefore this section will describe the host factors that play a role in HCV infection. HCV is associated with lipoproteins and their associated apolipoprotein components in the viral life cycle as already described in this Chapter from Section 1.4.3 above. Available evidence indicates the close connection between HCV and lipid metabolism through the formation of LVPs (Shawa, Sheridan, *et al.*, 2017).

1.11.1 Post-prandial lipid metabolism.

1.11.1.1 Exogenous lipid pathway.

This is the process that produces lipids from the diet in the intestines and are transported to the liver. The digestion of dietary fat starts in the stomach that involves mechanical emulsification, lipolytic action by lipase enzymes and bile salts action. Lipolysis describes the enzymatic breakdown of complex lipids that releases free fatty acids (FFA). The lipolysis of the emulsified fat is catalysed by pancreatic lipase (an enzyme that mainly acts on dietary triglycerides – TG). Dietary fats containing TGs as the principal lipid components in the diet, are emulsified in the small intestines, and absorbed in enterocytes (Ramasamy, 2014). The newly re-esterified TGs together with cholesterol esters associate with apolipoproteins and phospholipids and are packaged into CMs.

Nearly 90% of TGs in the intestine is absorbed that later forms the lipid content of CMs; whereas only 40% of dietary free cholesterol is absorbed (Griffin, 2013) through the NPC1L1 to be incorporated into the CMs. The FFAs generated through hydrolysis of TGs are taken up by enterocytes via fatty acid binding protein and are resynthesized into TG before being incorporated into CMs. The digested dietary lipids are efficiently assembled into CMs by the apoB-48, a truncated isoform of apoB exclusively found in the small intestines (Davidson and Shelness, 2000). The ApoB-48 is synthesized in the rough ER of the enterocytes and later transported to the smooth ER to combine with LDs. ApoB-48 is 48% of the size of apoB-100 (synthesized in the liver). ApoA-I, apoA-IV, and apoB-48 are the only apolipoproteins synthesized in the enterocytes. The combination of apoB-48, apoA-I and apoA-IV occurs in the Golgi apparatus within the enterocytes (Griffin, 2013).

Before their delivery into the lymphatic system, the TG-rich CMs acquire exchangeable apolipoproteins (apoA-I and apoA-IV) leave the enterocytes by exocytosis and travel via the thoracic duct to enter the vascular compartment. While in the circulation, the CMs mature through acquisition of apoC (I – III) and apoE from HDL (Ramasamy, 2014) that consequently activate lipoprotein lipase (LPL) to hydrolyse the TGs in order to release FFAs for storage in adipose tissue. TG hydrolysis results in shrinkage of the CMs, decrease in size and transfer of apoC (II & III) and cholesterol back to HDL. The ApoE remains/stays on the CM surface. The TG-depleted chylomicron remnants are cleared from the circulation via the liver (Ramasamy, 2014), mediated by interaction of apoE and cellular HSPG receptors since apoB-48 on the CMs lacks the binding domain for cellular receptors such as LDLR (Davidson and Shelness, 2000). Therefore apoA, apoC, and apoE are commonly known as exchangeable apolipoproteins that are able to dissociate from one lipoprotein and reassociate with another in the circulation (Sundaram and Yao, 2012).

1.11.1.2 Endogenous lipid pathway.

The liver is responsible for regulating the ingested lipids, non-esterified FFAs from adipocytes and de novo synthesis of lipids. As described in Section 1.11.1.1 above, CMs mediate the delivery of dietary lipids to the liver, whereas hepatocytes synthesize and secrete VLDL (another class of TG produced dependent on availability of lipid substrates for TG synthesis) that deliver endogenously synthesized TG to peripheral tissues (Ramasamy, 2014).

Similar to CM production, VLDL synthesis occurs in two main stages that eventually fuses nascent VLDL with LDs. Initial stages of VLDL synthesis involves lipidation of apoB-100 in the ER by MTP where they acquire TGs, cholesterol and other apolipoproteins (i.e. apoC and apoE) before being released into the systemic circulation,

while in the circulation there is sequential lipolysis of TGs by LPL to produce FFAs, VLDL remnants, and IDL. During the process of lipolysis, the IDL lose both apoC-II and apoE to eventually become LDL that are rich in apoB-100 and contain high cholesterol (Aizawa *et al.*, 2015).

The VLDL remnants, half of IDL (Aizawa *et al.*, 2015), and LDL are cleared from the circulation by binding to LDL receptor through recognition of apoE (exclusively in VLDL remnants, and IDL), and apoB-100 (present in LDL). The metabolic fate of VLDL is largely dependent on its size and lipid composition; but is eventually removed as VLDL remnants or by following the sequential VLDL-IDL-LDL pathway. The later pathway favours the removal of smaller VLDL (often referred to as pre-VLDL) (Griffin, 2013).

Table 1.2 Properties of lipoproteins in blood circulation.

Lipoprotein	**Size (nm)**	**Density**	**Apolipoprotein**
CM	Largest (80 – 1200) and most TG-rich lipoproteins	Lowest	B-48, A-1, C-II, E
VLDL	30 – 52	Very low	B-100, C-II, E
IDL	24 – 30	Intermediate	B-100, C-II, E
LDL	18 – 24	Low	B-100, C-II
HDL	8 – 15 (smallest)	Highest	A-I, A-II, E

Table **1.2** shows the properties of lipoproteins in blood circulation. Distinct families of lipoproteins have been described, each of which play different roles in lipid metabolism and transport. The lipoprotein classes contain characteristic apolipoproteins. Each lipoprotein class varies in size, density, and the lipid composition. The CMs are the VLDL are the largest lipoproteins but have lowest density, whereas the HDL are the smallest but have the highest density. CM, chylomicron; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; HDL, High density lipoprotein (Griffin, 2013).

1.11.1.3 Reverse cholesterol transport: HDL.

The bulk of free cholesterol that is absorbed from the stomach is re-esterified in the enterocytes through the action of acyl-CoA-cholesterol acyltransferase (ACAT). HDL transports cholesterol from the peripheral tissues to the liver (hence reverse transport) for excretion. This is the only route that eliminates cholesterol from the body. The cholesterol is an important constituent of cell membranes. The HDL is primarily synthesized in the gut and liver. The plasma HDL is highly heterogeneous (different size, density and composition) as a result of acquisition of cholesterol from peripheral tissues, and from VLDL and CM following LPL-mediated lipolysis of TGs (Kontush *et al.*, 2015). The initial steps for HDL biosynthesis begin with the interaction of apoA-I (forms 70% of HDL protein) and adenosine triphosphate binding cassette transporter A1 (ABCA1) proteins that facilitate cholesterol efflux to apoA-I to generate immature discoidal HDL (Wang and Smith, 2014). The re-esterification of free cholesterol by the lecithin cholesterol acyltransferase (LCAT) and the migration of cholesterol esters into immature discoidal HDL to generate mature spherical HDL_3. The acquired cholesterol esters are transported back to the liver directly as HDL or indirectly via a shuttle protein called cholesterol ester transfer protein. Thus, HDL_2 provides cholesterol influx into hepatocytes by transporting cellular cholesterol to the liver for excretion through SR-BI receptor (Ramasamy, 2014).

1.11.2 Lipid storage.

Lipids are mainly stored as TGs in adipose tissue but perform biological functions as phospholipids in cell membranes. TGs are synthesized exogenously in the intestines, and endogenously produced in the liver where they are transported in macromolecular complex with lipoproteins to tissues for oxidation or storage. Lipoprotein transport is described in relation to production and removal of cholesterol and TG from the vascular compartment. Forward transport refers to the influx of cholesterol from the gut and liver to the blood circulation and back to the liver again, whereas reverse transport describes the cholesterol efflux from peripheral tissues via HDL pathway to the liver (Sehayek and Hazen, 2008) that offer protection from development of atherosclerosis. Two types of adipose tissues exist in relation to the function and location namely: visceral and subcutaneous where visceral adipose tissue is located in close proximity with internal organs.

1.11.3 HCV modulates lipid metabolism.

The CHCV infection is characterised by abnormal accumulation of fat in the liver, a condition known as steatosis. The HCV genotype 3 is considered the most difficult to treat genotype in patients with advanced liver disease. The HCV genotype 3 is associated with hepatic steatosis, rapid progression to cirrhosis, reduced apoB-containing lipoproteins, and low LDL cholesterol as opposed to genotype 1 (Negro and Sanyal, 2009; Sheridan, Neely and Bassendine, 2013). This is due to the apparent viral inhibition of MTP transfer (Mirandola *et al.*, 2010). It is clear that different HCV genotypes utilise different pathways for HCV infectivity that consequently deregulate lipoprotein concentrations.

The HCV modulates lipid metabolism by reducing lipid oxidation and at the same time increasing lipid synthesis leading to increased accumulation of cellular fats. As described previously, apoE plays an essential role in HCV infectivity, and probably acts as a potential ligand for infectious LVPs through interaction with HSPG cellular receptor (Baumert *et al.*, 2014). Previous studies have reported the positive correlation of increased concentrations of apoB-containing lipoproteins (VLDL and LDL) and treatment outcome in CHCV patients receiving IFN-based therapy (Sheridan *et al.*, 2009).

Furthermore, other researchers suggested that apoE mediates the interaction between IFN-sensitivity, lipoprotein metabolism, and infectious viral particles in CHCV. Therefore apoE is associated with IFN-response (Sheridan *et al.*, 2012). The above information presents the important roles of apolipoproteins in modulating lipoprotein metabolism and as critical regulators of HCV life cycle involving lipid metabolism.

1.12 Innate and adaptive immunity.

Moving from host lipid interactions, this section describes the host immune responses that are crucial in determining the outcome of HCV exposure.

The innate immune system constitutes the non-specific defence mechanisms, and plays a crucial role in recognition and triggering pro-inflammatory response to pathogenic microorganisms (Medzhitov and Janeway Jr., 2000). The innate immune system recognises evolutionary highly conserved molecular structures of pathogens, termed pathogen-associated molecular patterns (PAMPs) (e.g. ssRNA). The PAMPs are invariant structures unique to microbes and distinguishable from host's 'self' (Schenten and Medzhitov, 2011). The recognition of PAMPs by innate immune system is

primarily mediated by the host's pattern recognition receptors (PRRs) that fall into several families such as Toll-like receptors (TLRs), cytoplasmic retinoic acid-Inducible Gene 1 (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) (Mogensen, 2009), and C-type lectin receptors (CLRs) that recognise fungal pathogens (Vautier, MacCallum and Brown, 2012). The RIG-I and melanoma differentiation-associated protein 5 (MDA-5) are important innate immune receptors that detect dsRNA in the cytosol (Kato *et al.*, 2006; Pichlmair *et al.*, 2006).

The classification of PRRs depends largely on specificity, function, and localisation of their ligands. Since PAMPs are perceived as molecular signatures of infection, their recognition by cellular transmembrane proteins such as TLRs leads to the induction of a cascade of downstream mechanisms that result in inactivation of invading pathogens, modification of innate immune response, and subsequent activation of adaptive immune response. These coordinated systems manage to either clear the pathogens or restrict their replication (Fejér *et al.*, 2005; Kumar, Kawai and Akira, 2011; Schenten and Medzhitov, 2011). PRRs are capable to distinguish self from non-self by binding PAMPs present in pathogens but typically absent on host molecules.

Likewise, the adaptive or acquired immune system comprises highly specialised cells that provide specific response to particular pathogens that induced the immune response. Adaptive immunity is characterised by elimination of invading pathogens, and generation of immunological memory (Bonilla and Oettgen, 2010). Unlike the innate immunity, adaptive immune system is highly specific and provides long-lasting protection through destruction of pathogens and their secreted toxins. Lymphocytes are the main type of leucocytes that are involved in adaptive immunity. The B cells and T

cells are the major lymphocytes involved in ‘humoral’ and ‘cell-mediated’ immune responses respectively (Schenten and Medzhitov, 2011).

1.12.1 Humoral immune response.

The B cells mature in the bone marrow and spleen and remain in peripheral tissues until activated by presence of a foreign antigen. The B cell activation requires two distinct signals, achieved by firstly, binding of antigen to B cells’ membrane-bound receptors (BCRs) whose binding moiety contain membrane-bound antibodies; and secondly, by T helper (Th) cell stimulation (Baumert *et al.*, 2014). Upon binding to B cell receptors, the receptor mediated endocytosis takes place that internalises the detected antigen, where it gets broken down and complexed to Major Histocompatibility Complex II (MHC-II) present on the surface of the B cells (immediately acting as antigen presenting cells – APCs). The B cell activation signal occurs via the interaction of B cells and Th cells; where antigen/MHC-II complex on the B cell surface is presented to Th cells through the T cell receptors (TCRs) present on Th cells. Binding of antigen/MHC-II complex to TCRs, results in T cell activation that consequently enables a second activation of the B cells achieved by presence of different peptides (Holgate, 2012). The activated B cells differentiate to produce memory cells, or plasma (effector) cells that secrete antibodies.

1.12.2 Cell-mediated immune response.

Contrary to the humoral immunity, cell-mediated response does not involve antibodies. T cells develop in the thymus, and enter the circulation. Upon reaching the peripheral lymphoid organs, they exit the blood stream and migrate through the lymphoid tissue and return again to the blood stream until they get activated by specific antigens. Naïve

T cells are the recirculating mature T cells before they encounter their specific antigens. The naïve T cells are induced by specific antigens in order to proliferate and differentiate into effector T cells (activated naïve T cells) that participate in adaptive immune response. The T cells are divided into Th cells that contain CD4 proteins on their cell surface; and killer T cells or cytotoxic T cells (Tc) that contain CD8 proteins on their cell surface (Bonilla and Oettgen, 2010).

The naïve T cells are activated by either exogenous or endogenous antigens that drive the activation of Th cells and Tc cells respectively. The T helper CD4 receptor binds to the MHC-II in order to regulate both innate and adaptive immune responses, whereas Tc CD8 receptors are attracted to the MHC-I in order to directly kill the infected cells (by releasing cytotoxins and granulysin (a protease) to infected cells to undergo apoptosis) (Bonilla and Oettgen, 2010). The activation of T cells is basically achieved by recognition of antigen/MHC-I or -II complex along with costimulatory signals from APCs.

Dendritic cells (DCs), macrophages, and B cells are specialised cells often known as professional APCs. The MHC-II carries peptide antigens from extracellular pathogens and activates Th CD4 cells that eventually differentiate into two types of effector T cells (i.e. Th1 and Th2 cells – CD4+), whereas MHC-I presents peptide antigens from intracellular pathogen that multiply in host cell cytoplasm to CD8 T cells that differentiate to Tc cells (CD8+) (Park and Rehermann, 2014). The Th1 CD4 T cells (inflammatory) are the main activators of cellular immunity that activate macrophages to kill cells, whereas Th2 CD4 T cells play a crucial role in humoral immunity that activate B cells for antibody production.

1.12.3 Innate immune receptors: Toll-like receptors.

Upon PAMPs recognition, the TLRs flag them (PAMPs) as biological markers of infection that activates intracellular signalling pathways, and trigger antimicrobial effector and pro-inflammatory responses (Janeway and Medzhitov, 2002). The TLRs first identified in 1997, are well studied examples of germ-line encoded PRRs; deriving their name from *Drosophila melanogaster* Toll protein (Mercurio *et al.*, 1997). Ten different TLRs were identified in humans and each recognises distinct PAMPs from different microorganisms either through direct interaction or via an intermediate PAMP-binding molecule (Figure 1.5). TLRs are expressed in APCs including sentinel cells such as macrophages and DCs (Akira, Uematsu and Takeuchi, 2006).

Figure 1.5: TLRs and the recognised PAMPs.

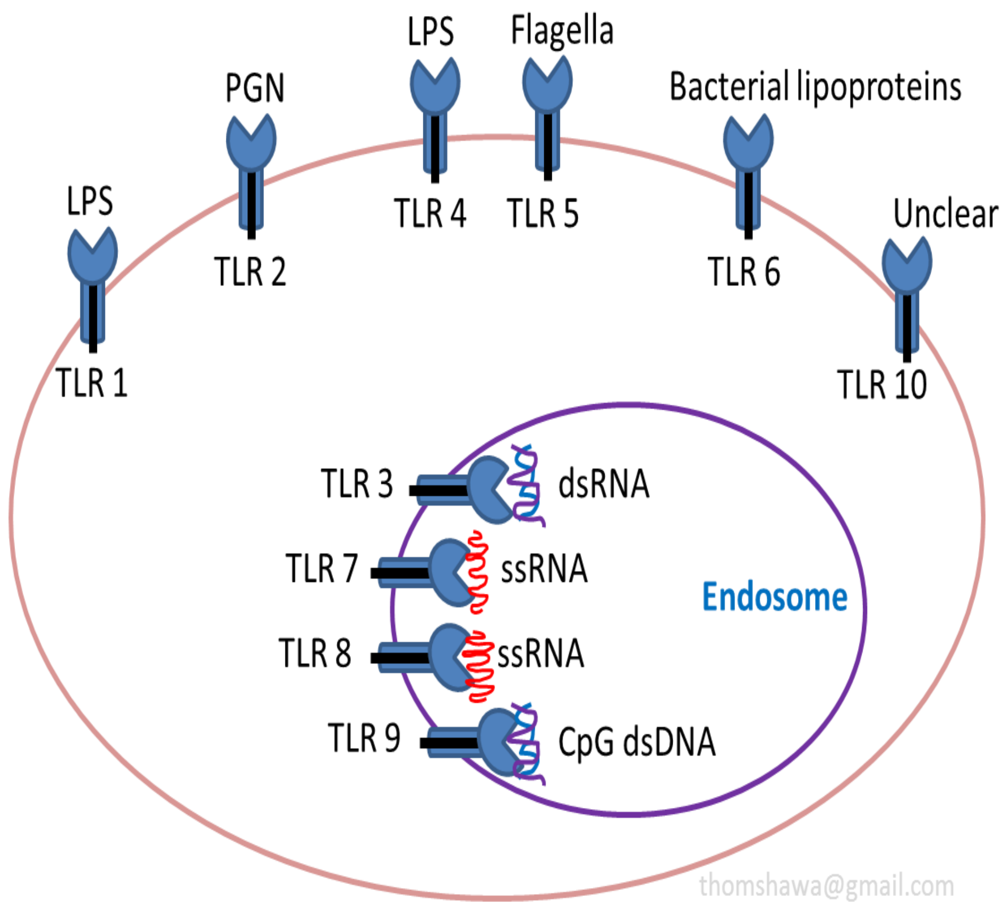


Figure 1.5**:** Toll pathway. Structurally, TLRs are cellular integral glycoproteins that have extracellular or luminal ligand-binding domains that recognise PAMPs. Human TLRs were divided into subfamilies TLR1, TLR2, TLR4, and TLR6 that recognise lipids; whereas TLR3, TLR7, TLR8, and TLR9 recognise microbial genomes such as DNA or RNA. Key: PGN = Peptidoglycans LPS = Lipopolysaccharide CpG = 'C..phosphate..G' (DNA regions with higher G+C content).

The recognition of PAMPs by innate immune system is primarily mediated by phagocytic cells and APCs such as dendritic cells, macrophages, and granulocytes. The activation of TLRs amplifies the initiation of adaptive immune responses (Janeway and Medzhitov, 2002). The formation of TLRs heterodimers between TLR2 and either TLR1 or TLR6 further distinguishes several bacterial PAMPs. The TLRs 1, -2, -4, -5, -6, and -10 are expressed at the surface of sentinel cells, whereas TLRs 3, -7, -8, and -9 are located in intracellular compartments such as endosomes and lysosomes (Ozinsky *et al.*, 2000; Iwasaki and Medzhitov, 2004). Triggering TLRs on these cells induces the activation of naïve T cells specific for antigenic peptides expressed on APCs in complex with either MHC-I or -II molecules.

1.12.4 Cytoplasmic pathogen recognition receptors.

The expression of TLRs in sentinel cell surface or endo-lysosomal membranes hinders their recognition of intracellular cytosolic microbes and their derivatives (e.g. nucleic acids). A group of cytosolic PRRs were identified that induced an immune response independent of TLR – PAMP recognition. There are three types of cytoplasmic PRRs in humans that sense viral replication; these are RIG-I, Melanoma differentiation-associated gene 5 (MDA-5), and laboratory of genetics and physiology 2 (LGP2). The cytosolic PRRs were subdivided into RIG-I-like receptors (RLRs) (Yoneyama *et al.*, 2004), and nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) (Kanneganti, Lamkanfi and N????ez, 2007). The MDA-5 is a member of RIG-I-like receptors family that are cytosolic PRRs that recognise intracellular viruses (especially dsRNA). Both the RIG-I and MDA-5 are IFN-inducible RNA helicases that recognise cytoplasmic RNA suggesting a similar mechanism of action for both PRRs (Yoneyama *et al.*, 2004). Studies have reported different roles played by both helicases; evidence suggested that RIG-I activates immune response to paramyxoviruses, HCV, and

influenza virus; whereas the MDA-5 induces an immune response to picornavirus, and norovirus (Kato *et al.*, 2006).

The RIG-I recognises 5′-phosphorylated short (<300bp) dsRNA ligands that have blunt ends; whereas MDA-5 internally recognises long kilobase-scale (>1000bp) genomic dsRNA with no end specificity (Kato *et al.*, 2006) (Pichlmair *et al.*, 2006). The RIG-I associates with interferon-β promoter stimulator 1 (IPS-1), upon sensing viral RNA nucleotides. IPS-1 overexpression induces interferon and interferon-inducible genes, via activation of transcriptional factors (Zeng *et al.*, 2010). The RIG-I and MDA-5 contain N-terminal caspase recruitment domains (CARDs) that induce a cellular response upon recognition of viral dsRNA containing 5′ triphosphate (5′-ppp-dsRNA) (Schlee *et al.*, 2009) via IPS-1 (Zeng *et al.*, 2010); whereas LGP2 lacks a CARD domain and does not induce a signalling response independently, but was reported to mediate positive regulation of RIG-I/MDA-5 antiviral response in a mechanism that is still unclear.

When the viral dsRNA nucleic acids are absent in the cytosol, the RIG-I stays in a closed inactive conformation but undergoes conformational rearrangement upon sensing the viral dsRNA ligands (Jiang *et al.*, 2011; Kowalinski *et al.*, 2011). RIG-I binding of the RNA through the helicase and C-terminal domain (CTD) (Jiang *et al.*, 2011) enables release of the CARDs and subsequently recruits and activates mitochondrial antiviral-signalling protein (MAVS) such as IPS-1that is highly associated with RIG-I (Zeng *et al.*, 2010).

Unlike RIG-I, the MDA-5 does not sequester CARDs in the absence of RNA ligands, but cooperatively forms dimers and ATP-sensitive filaments upon binding to the dsRNA. MDA-5 CTD is not used for RNA binding but rather for cooperative filament assembly (Berke and Modis, 2012).

1.13 Immune response to viral infections.

Virus-mediated PRR activation results in either successful clearance or establishment of infection. Viral immunostimulatory nucleotides produced during replication or possessed within the virus are detected by the host's nucleotide sensors such as TLRs. The TLR2 and TLR4 were also reported to sense viral glycoproteins apart from bacterial lipids (Mogensen, 2009). Formation of PRR–viral-PAMP complex induces signalling cascades that activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κB) and interferon regulatory factor 3 (IRF3) (both are transcription factors), that trigger activation of interferon-regulated genes and inflammatory responses through the MAVS (Dreux *et al.*, 2012).

Two subsets of mononuclear phagocytes namely plasmacytoid dendritic cells (pDCs) and $CD14^{dim}$ $CD16^{+}$ monocytes were identified to produce predominantly antiviral responses; such as secretion of type I interferons by pDCs, and pro-inflammatory cytokines by $CD14^{dim}$ $CD16^{+}$ monocytes (Barchet, Cella and Colonna, 2005; Cros *et al.*, 2010). In like manner, TLR9 induces type 1 IFN response when sensing DNA region with high G+C content (CpG DNA) in pDCs (Fejer *et al.*, 2008). Apart from pDCs, other cells also possess TLR-independent receptors that are capable of inducing an antiviral response during infection (Mogensen *et al.*, 2003). The activation of viral-infected cellular receptors releases signalling proteins such as type I and III IFNs that induce neighbouring cells to heighten the defence against viral infection. The inhibition of viral replication is done by the secreted cytokines (IFNs and tumour necrosis factor - TNF) that indirectly arrest the viral replication through activation of other antiviral immune cells and mechanisms (Kumar, Kawai and Akira, 2011). When mammalian cells are infected with viruses, the MHC-I display viral peptides on APCs to signal cytotoxic T cells to release toxic mediators that kill the infected cells. Cytotoxic T cells

possess T cell receptors (TCRs) on their surfaces that recognise virally-infected cells to initiate apoptosis. However, some viruses prevent MHC-I from displaying viral peptides, and consequently preventing apoptosis. NK cells have a special property of recognising and killing host cells with reduced number of MHC-I molecules. Cytotoxic cells also release IFN-γ and TNF-α, when augmented with the innate immune system, enhancing the killing of invading pathogens.

1.13.1 Specific immune response to HCV.

The HCV has evolved several immune evasion mechanisms to establish human infection. During acute viral infection, regulatory T (Treg) cells suppress the T cell immunity by inhibiting both $CD4^+$ and $CD8^+$ cell proliferation and their cytokine production. In HCV infection, dendritic cells are functionally impaired, which hinders stimulation of $CD4^+$ and $CD8^+$ T cells. Upregulation of programmed death-1 (PD-1) and increased secretion of IL-10 was reported to impair T-cell function that enables HCV immune evasion (Park *et al.*, 2015). The outcome of HCV infection is determined by a complex set of interactions between the host and the virus. HCV infection clears spontaneously in 20% of infected individuals, attributed to the innate and adaptive immune responses, whereas the majority (80%) progress to chronic infection (Thomas *et al.*, 2009). Interferons α and β secretion increases during the AHCV infection followed by the high production of antiviral interferon stimulated genes (e.g. viperin) which disrupt viral replication via interaction with NS5A (Helbig *et al.*, 2011). Early proliferation of activated $CD4^+$ and $CD8^+$ cells in response to HCV infection (Chang *et al.*, 2001), high expression of interferon stimulated genes in the liver (Su *et al.*, 2002) and activation of natural killer (NK) cells (Golden-Mason et al. 2007) were reported as high predictors of HCV spontaneous resolution. In vitro models of HCV infection

suggested that cellular immune responses promote liver injury but mechanisms of tissue injury are unclear. However, understanding of the liver disease indicates that hepatocellular injury is not caused by direct cytolysis, because HCV does not possess cytopathic factors. Viral interaction with cellular proteins induces a T cell immune response that activates hepatic stellate cells, which leads to liver inflammation and fibrosis, subsequently affecting liver hepatocyte survival (Benhamou *et al.*, 1999). Investigation of the effect of host immune response in CHCV progressive liver disease is difficult due to slow chronicity progression in humans and a lack of fibrosis development in chimpanzee models.

1.13.2 Innate immune response to HCV infection.

Available evidence shows that HCV induces an innate immune response that sometimes arrests virus replication. Conversely, the HCV sometimes evades the immune responses that results in establishment of chronic infection. The HCV encodes PAMPs that are recognised by the host PRRs such as TLRs and RLRs resulting in the activation of downstream signalling pathways that activate secretion of pro-inflammatory cytokines, and chemokines such as types I and III IFNs (Thomson, Smith and Klenerman, 2011).

The IFNs elicit their antiviral activity through induction and upregulation of IFN-stimulated genes (ISGs). The HCV PAMPs bind the RIG-I through the 5′- terminal triphosphate on the viral RNA that induces production of type-I IFNs and antiviral ISGs (Kowalinski *et al.*, 2011). The type I and III IFNs are produced by host cells infected with HCV and by sentinel cells of the innate immune system as well such as the DCs, and macrophages. The DCs and macrophages regularly secrete IFNs even in the absence of interactions with viruses. Although both type-I IFNs and type-III IFNs (IFN-λ) are produced upon innate recognition of HCV, they can also trigger downstream signalling cascades through phosphorylation of signal transducers and

activators of transcription 1 (STAT1) and STAT2 suggesting induction of the same group of ISGs (Heim, 2013). The STAT1 function was also reported to be inhibited by HCV (Sun, Rajsbaum and Yi, 2015).

The HCV NS3/4A protease was also reported to inhibit the RIG-I response (Gale and Foy, 2005) as well as the TLR3 signalling by cleaving to IPS-1 (Meylan *et al.*, 2005) and TRIF (Gale and Foy, 2005) respectively. Other researchers reported that HCV NS5A also induces IL-8 production that inhibits the IFN-α secretion. Again, HCV NS3, NS5A and E2 proteins inhibit IFN-α induction through blocking expression and transcription of IFN-α/β induced genes (Polyak *et al.*, 2001).

Despite several mechanisms employed by HCV to inhibit the host response, the induction of the innate response by HCV is achieved prior to accumulation of sufficient HCV particles. When HCV infects some hepatocytes, the uninfected hepatocytes heighten their type-I IFN response as evidenced by upregulated nuclear factor kappa B (NF-κB) in uninfected cells and downregulation in infected cells in response to HCV infection (Joyce and Tyrrell, 2010; Sun, Rajsbaum and Yi, 2015).

Overall, HCV recognition is achieved by TLRs 3, 7, and RIG-I through the adaptor molecule TIR (toll/interleukin receptor) domain-containing adaptor protein inducing IFN-β (TRIF), IPS-1, and Myeloid differentiation factor 88 (MyD88). This signalling process leads to phosphorylation of the transcription factors interferon regulatory factor (IRF)-3, IRF-7 and NF-κB that induces secretion of type-I IFNs (Joyce and Tyrrell, 2010).

1.13.2.1 Dendritic cells.

The DCs are a special type of APCs of the innate immune system known as professional APCs. Two subsets of the DCs exist namely: the plasmacytoid dendritic cells (pDCs) also known as $CD123^{+}$, and myeloid DCs (mDCs) also called $CD11c^{+}$ that sense pathogens through recognition of TLRs. The mDCs express several TLRs such as TLR3 and TLR7/8 that recognised dsRNA, and ssRNA viruses respectively. Likewise pDCs express several TLRs including TLR7 that enables recognition of ssRNA virus (Hespel and Moser, 2012). The DCs have a high phagocytic capacity but upon recognition of HCV PAMPs through TLRs, the DCs become activated into mature immunologically competent DCs and express high levels of MHC and co-stimulatory molecules. The activation and maturation of DCs induces multiple signalling pathways that prime naïve T cells (Hespel and Moser, 2012). The DCs process viral antigens and present them to immune cells in complex with MHC-II or MHC-I molecules leading to production of different types of cytokines such as IFN-α, IL-10, IL-12, and TNF- α that regulate the response of neighbouring cells.

The DCs also play a key role in adaptive immune response adequate enough to enable viral clearance. The DCs are the only cells known to have the capacity for initiation of immune response in inactive T lymphocyte. The DCs function by capturing viral particles/antigens which they process and present on their cell surface, thus activating the T cell response to initiate an antiviral cell-mediated immune response. Immature DCs are specialised for antigen capture, whereas the mature DCs serve as the APCs by activation of an antigen-specific naïve CD8+ T cells. Therefore, the DCs main functions are antigen presentation, T cell stimulation, and secretion of cytokines. The DCs are found in tissues that have contact with the outside environment but immature forms of DCs are circulated in blood stream. The mature DCs exit the liver following

viral epitope collection and enter the lymphatic system for activation of T cells (Bauvois *et al.*, 2009).

In HCV infection the DCs are functionally impaired, which hinders stimulation of $CD4^+$ and $CD8^+$ T cells that resulting in chronic viral persistence (Torresi, Johnson and Wedemeyer, 2011). In vitro studies demonstrated that HCV replicates in DCs, therefore HCV infection of dendritic cells can impair their function that leads to chronicity. The DCs function in concert with HCV proteins such as Core and NS3 leading to changes in cytokine secretion that consequently results in increased production of IL-10 and TNF-α (Pachiadakis *et al.*, 2005).

1.13.2.2 Natural killer cells.

Following HCV exposure, a primary cell defence mechanism includes the activation of natural killer (NK) and natural killer T (NKT) cells (Yokota, Okabayashi and Fujii, 2010). Since infected hepatocytes release type-I IFNs, they in turn activate NK cells. The NK cells are a subset of lymphocytes that interact directly with virus-infected cells, hence they are considered the principal innate immune effector cells. The activated NK cells kill infected cells either directly via the release of cytotoxic factors (e.g. perforins, granulysin and granzymes) (both stored in granules) or via secretion of cytokines such as IFN-γ. The activated NK cells also exert their antiviral activity indirectly by triggering the activation and/or trafficking of other key immune cell populations, including T cells to promote adaptive immune responses (Shawa, Felmlee, *et al.*, 2017). Despite being a subset of lymphocytes, NK cells are more highly enriched in the liver than in the vascular compartment (Doherty *et al.*, 1999).

The NK cell activation is regulated through a complex balance between activating and inhibitory receptors. The inhibitory NK receptors (NKRs) signalling dominate over

activating receptors to safeguard from NK cell reactivity towards normal, healthy cells (Shawa, Felmlee, *et al.*, 2017). The NK cells express regulatory cell surface proteins called killer-cell immunoglobulin-like receptor (KIR) that recognize MHC-I to inhibit the NK cell activity (Middleton, Williams and Halfpenny, 2005). The main classes of NKRs are the predominantly inhibitory KIR, the natural killer group 2 (NKG2) family of inhibitory (NKG2A) and activatory (NKG2C/D) isoforms, and the activating natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46 (Shawa, Felmlee, *et al.*, 2017). Previous genetic studies of the NK cell KIR receptors identified homozygosity for KIR human leucocyte antigen class 1(KIR2DL3:HLA-C1), which is linked to a readily activated NK cell phenotype (Khakoo *et al.*, 2004), to be associated with the resistance to HCV infection seen in EU cases (Knapp *et al.*, 2011). Functional and phenotypic differences in NK cells was described among PWIDs where increased $KIR2DL3^{+}NKG2A^{-}$ NK cells levels were observed in seronegative and aviraemic PWIDs as opposed to those who developed CHCV infection or who cleared HCV infection spontaneously. Such NK cells were not susceptible to HLA-E-mediated inhibition (Thoens *et al.*, 2014). Other researchers reported a sustained NK cell activation as a contributing factor for protection against HCV infection. In the same study the highly exposed uninfected cohort showed high numbers of both activated and cytotoxic cells, coupled by increased frequencies of IFN-γ secreting NK cells (Sugden *et al.*, 2013).

The NK and NKT cells were reported to secrete sufficient amount of IFN-γ and TNF-α that are responsible to inhibiting viral replication (Rehermann, 2013). The DCs secrete IL-12 that activates NK cells, therefore NK cells also play a role in inducing DC partial or total maturation (Marcenaro *et al.*, 2005). The HCV also blocks NK cells function through interaction of HCV E2 and the NK cell CD81 molecule (Brimacombe *et al.*, 2014).

1.13.3 Adaptive immune response to HCV infection.

1.13.3.1 T-cell mediated response.

The activation of both humoral and cytotoxic T cell-mediated adaptive responses is dependent on the help from CD4 T cells that play a crucial role is activating arms of adaptive immune responses. The CD4+ T cells secrete Th1 cytokines including IFN-γ that recruit leucocytes involved in providing inflammatory response. CD4+ T cells also secrete Th2 cytokines such as IL-4 and IL-10 that restrict the Th1 cytokine-mediated response and favour initiation of the humoral response (Larrubia *et al.*, 2014). Although the HCV is capable of interfering with a wide range of the host physiological and pathophysiological processes, the recruitment of an effective adaptive response takes several weeks (Chang *et al.*, 2001). Such a swift immune response has the potential to resolve the viral infection, but it is ineffective in 50% of cases leading to the progression of chronic infection. The CHCV infection is characterised by decreased activities of CD4+ T helper and CD8+ cytotoxic T cells (Gremion *et al.*, 2002; Thimme *et al.*, 2002) with reduced type 1 IFN cytokine production.

Despite the host's robust coordinated antiviral activity, HCV develops genetic mutations that evade immune surveillance resulting in persistent viraemia in the absence of memory T cell help (Grakoui, 2003). The HCV persistence is associated with increased frequency of CD4+ regulatory T cells (Treg) that suppress the activity of HCV-specific CD8+ cytotoxic T cells (Sugimoto *et al.*, 2003). In brief, virus specific CD8+ T cell response fails due to two important mechanisms. Firstly, mutational escape of MHC-I epitopes that prevent viral recognition, and secondly, functional exhaustion of the virus specific CD8+ T cells due to persistent antigen stimulation (Wieland and Thimme, 2016).

Previous studies have reported the role of T cell response, and early multispecific T cell responses that target multiple viral epitopes in spontaneous clearance of HCV infection (Gremion *et al.*, 2002; Thimme *et al.*, 2002). Following successful resolution of HCV infection, partial protective immunity develops with lower rates of reinfection (Midgard *et al.*, 2016) than would be expected in a matched uninfected cohort of PWIDs (Grebely *et al.*, 2006). Up to 60% (Thurairajah *et al.*, 2008) of the EU population have demonstrable weaker T cell responses to multiple viral antigens as compared to spontaneous resolvers (Mizukoshi *et al.*, 2008; Zeremski *et al.*, 2009; Knapp *et al.*, 2010). The cytokines secreted by the T cells also play a crucial role in regulation of the humoral immune response.

1.13.3.2 Humoral response.

Anti-HCV neutralizing antibodies (nAbs) have been reported to support viral clearance in acute infection (Osburn *et al.*, 2014) as well as spontaneous resolution after establishment of CHCV infection (Raghuraman *et al.*, 2012) (de Jong *et al.*, 2014). Since the mechanisms that offer protection for the EU population from HCV infection still remain a mystery, we previously speculated that the effect of robust nAb responses hardly detectable by conventional serological assays may contribute to the existence of the EU phenotype (Shawa, Felmlee, *et al.*, 2017). Following establishment of CHCV infection, broad HCV nAb responses appear in the circulation but HCV continuously escapes from nAbs (von Hahn *et al.*, 2007). Several mechanisms may be involved in the viral evasion of humoral immune response that include epitope masking by interfering antibodies, the shield provided by lipoproteins (Cashman, Marsden and Dustin, 2014), and genetic mutations (von Hahn *et al.*, 2007) amongst others. Other researchers have also described the cell-to-cell viral transmission as another nAb escape strategy employed by HCV (Mothes *et al.*, 2010).

There is evidence supporting the theory that HCV nAbs have a limited influence on the outcome of HCV infection since there is a delayed antibody development and the antibody titre wanes rapidly. Other reports indicated viral clearance in the absence of detectable anti-HCV antibodies (Thimme *et al.*, 2001; Post *et al.*, 2004). On the contrary, more evidence supports the role of nAbs in spontaneous resolution of HCV infection (de Jong *et al.*, 2014).

1.14 Summary of cytokines and chemokines secretion in HCV infection.

Cytokines are soluble proteins secreted by immune cells that provide intercellular communication by binding to specific cellular receptors in order to induce or inhibit cytokine regulated genes. Different cytokines that play several roles have been reported and are classified into subgroups in accordance with their functions as follows:

a) Pro-inflammatory cytokines (e.g. IL-1, IL-6, TNF- α) (Bowen and Walker, 2005).
b) Th1 type cytokines (e.g. IFN- γ, IL-12, IL-18) (Bowen and Walker, 2005).
c) Th2 type cytokines (e.g. IL-4, IL-5, IL-10, IL-13, IL-14, IL-15) that downregulate the Th1 responses (Marcenaro *et al.*, 2005). However, IFN-λ has been shown to favour production of Th1 responses activity by decreasing secretion of Th2-type cytokines (Jordan *et al.*, 2007; Srinivas *et al.*, 2008).
d) Th17 type cytokines (e.g. IL17, IL-21, IL-23) that induce differentiation of Th17 lymphocytes (Rowan *et al.*, 2008).

On the other hand, chemokines are known as chemotactic cytokines that belong to multifunctional family of cytokines that recruit leucocytes to migrate to the site of infection. The chemokines are described as homeostatic or pro-inflammatory depending on their functions (Yokota, Okabayashi and Fujii, 2010). The chemokines

are structurally related cell signalling proteins containing four invariant cysteine residues that result in four subfamilies as follows: alpha (CXC), beta (CC), gamma (C), and delta (CX3C) that function by activation of their specific receptors namely CXCR, CCR, CR, and CX3CR respectively (Heydtmann and Adams, 2009) (Zeremski, Petrovic and Talal, 2007). Chemokines and pro-inflammatory cytokines are some of the proteins released by macrophages and dendritic cells as a result of activation of receptors (Fejer *et al.*, 2008; Lauterbach *et al.*, 2010; Yokota, Okabayashi and Fujii, 2010).

One important cytokine family essential in the control of HCV infection is IFN-λ. Discovered in 2003 (Kotenko *et al.*, 2003; Sheppard *et al.*, 2003), the IFN-λ family enabled understanding of potential genetic and immunologic resistance mechanisms conferred by HCV infection. There are three IFN-λ genes that encode IFN-λ1, -λ2, and -λ3 proteins also designated IL-29, IL-28A, and IL-28B, respectively (Kelly, Klenerman and Barnes, 2011). They all belong to type III IFN subsets. The type-I and type-II IFN subsets have different signal transduction receptor complexes to type-III IFN, but they activate similar intracellular signalling pathway that induce antiviral and immunomodulatory immune responses (Kelly, Klenerman and Barnes, 2011). The expression of IFN-α and IFN-λ genes are inducible as a result of cellular recognition of different viruses. The IFN-α receptors are expressed on leucocytes and all nucleated cells whereas IFN-λ receptors are expressed on fewer cell types of epithelial origin (Pagliaccetti and Robek, 2010).

The complex cell signalling process inducing the cytokine production during initial steps of viral infection allows recruitment of coordinated and effective innate and adaptive immune responses. However, HCV interferes with cytokines at various levels including escaping the surveillance of the immune responses by inducing a Th2 cytokine profile.

1.15 Potential mechanisms of resistance for HCV infection.

Given the importance of host lipid, and immune responses in HCV infection these factors may be considered as putative mechanisms of resistance. This section describes some potential mechanisms that confer resistance to HCV infection.

1.15.1 Genetic: IL-28B gene polymorphism.

Genetic polymorphism is one of the suggested factors that confer resistance to establishment of HCV infection (Rauch *et al.*, 2009). Different sub-groups of individuals respond differently to microbial exposures due to genetic variations among human populations that determine resistance or susceptibility to establishment of infection.

Several studies have documented the role of host genetic factors such as SNPs in IL-28B IFNL3 that may be relevant in EU phenotypes that was reported to be associated with spontaneous viral clearance (Rauch *et al.*, 2009; Thompson *et al.*, 2010; Prokunina-Olsson *et al.*, 2013). The IL-28B genotype is used to predict HCV treatment response to PEG-IFN/RBV (Suppiah *et al.*, 2011). The IL-28B SNP rs8099917 ''G'' was associated with absence of treatment-induced resolution and absence of spontaneous resolution (Suppiah *et al.*, 2011). Genome-wide association studies (GWAS) reported SNPs in IL-28B gene that allowed prediction of ≤64% for failure to clear virus during therapy in cross-sectional cohorts (Suppiah *et al.*, 2009, 2011). Knapp et al, investigated the presence or absence of IL-28B protective genotype rs12979860-CC in EUs, and reported that the EUs had a significantly lower frequency of IL-28B genotype rs12979860-CC as opposed to spontaneous resolvers, but a similar frequency to CHCV patients (Knapp *et al.*, 2011).

Our previous work demonstrated that a small proportion of HCV exposed uninfected individuals carried a protective C allele of the IL-12B gene (Hegazy *et al.*, 2008). The IL-12B is a heterodimeric cytokine composed of 35-KDa (p35) and 40-kDa (p40) subunits that promote anti-viral Th1 responses. The IL-12B gene encodes the IL-12 p40 subunit, and the production of this subunit was reported to be facilitated by variant C allele of the 1188A/C polymorphism (Seegers *et al.*, 2002). We reviewed detailed potential mechanisms that offer protection for HCV infection (appendix D).

1.15.2 HCV-lipid interaction.

The HCV utilises lipid and lipoprotein metabolism at all stages of its lifecycle from attachment, entry, replication, assembly and transport with lipoproteins in the circulation. HCV has evolved strategies to escape immunological selection pressures to establish chronic HCV infection in the majority of those exposed (Knapp *et al.*, 2010). Since HCV circulates as an infectious LVP in the vascular compartment bound to plasma lipoproteins, it is suggested that maximum levels of HCV LVPs are associated with early and persistent infection (Sheridan *et al.*, 2016).

The close association of HCV with lipids and lipoproteins contributes to the virus' ability to evade the host's immune surveillance. Given this co-dependency on host lipid pathways, it is plausible that any mechanism that would disrupt the LVP formation would potentially reduce the viral infectivity and influence outcome following HCV exposure (Shawa, Felmlee, *et al.*, 2017). Thus defective HCV-host lipid interactions may represent alternative pathways of HCV resistance that warrant further investigation.

1.15.3 Suppression of HCV T cell response by sub-infectious HCV dose.

Several lines of evidence indicate that the host's cellular immune response to multiple HCV epitopes is also vital in controlling early HCV infection, with successful early immune responses leading to spontaneous resolution. Conversely, an ineffective early cell-mediated response may lead to the development of chronic HCV infection (Bowen and Walker, 2005). The EU population exhibits HCV-specific T cell mediated responses that suggests recognition of viral peptides such as NS proteins by the immune system (Mizukoshi *et al.*, 2008; Thurairajah *et al.*, 2008). Repeated exposure to sub-infectious HCV particles can induce HCV specific T cell response in nonhuman primates, but failed to offer protection against subsequent acute infection (Park *et al.*, 2013). Similar T cell immune responses were also described in aviremic and seronegative family members of HCV infected individuals (Scognamiglio *et al.*, 1999; Al-Sherbiny *et al.*, 2005). Furthermore, several studies have proposed that repeated sub-infectious exposure primes and maintains HCV specific T cells that confer protective immunity (Scognamiglio *et al.*, 1999; Al-Sherbiny *et al.*, 2005; Park *et al.*, 2013). An early HCV specific T cell response was induced in healthcare workers following needle stick occupational exposures to HCV; but they did not exhibit detectable HCV antibodies and HCV RNA (Heller *et al.*, 2013). Thus, sub-infectious HCV exposures suppress T cell responses against subsequent acute infection (Park *et al.*, 2013). These data imply that the frequency and dose of exposure may influence the result degree of protection. T cell immune response increases in CHCV infection (Rivière *et al.*, 2012). Thus the presence of HCV specific T cell responses in EUs supports the evidence that they have indeed been exposed to HCV antigens, but does not fully explain why they do not exhibit detectable HCV RNA and HCV antibodies.

Repeated exposure to HCV without development of infection among PWIDs suggests the presence of a resistant phenotype to HCV infection. Exposure to HCV was determined by history of risk behaviours that include current sharing of needles and other injecting equipment as described in chapter 2. Probability of HCV sero-prevalence increases with duration of injection history (Law *et al.*, 2003; Roy *et al.*, 2007).

1.15.4 Epigenetic.

Epigenetics is a term that refers to all stable heritable changes that are not due to any alteration in the DNA sequence itself (Horsthemke, 2017). DNA methylation, post-translational modifications of histone proteins, chromatin remodelling, and noncoding RNAs are the four main arms of epigenetic mechanisms (Rongrui *et al.*, 2014). The epigenetic silencing of IFN-stimulated genes was reported to be responsible for the acquisition of a partially IFN-resistant phenotype of HCV replicon-harbouring cells (Naka *et al.*, 2006). Some studies have shown that HCV viral proteins may actively participate in epigenetic regulation of hepatic cancer stem cell phenotypes, that result in epigenetic alterations associated with HCC (Herceg and Paliwal, 2011; Rongrui *et al.*, 2014). Host genes that may be epigenetically regulated in exposed uninfected sub-population are unknown.

This thesis has however described the immunological (innate) and lipidomic factors that could potentially offer protection for HCV infection. The sequential patterns in which these mechanisms work affecting the HCV resistance phenotype remain speculative. It is more likely that there is a combination of different factors that are involved in the existence of the EU phenotype, whose mechanisms are still unknown.

1.15.5 HCV vaccine development.

It is important to understand the mechanism for HCV resistance to inform vaccine design strategies. Vaccination has been the most successful strategy for prevention of other viral infections such as hepatitis B virus (Zanetti, Van Damme and Shouval, 2008) that induce nAbs that could counter highly antigenically diverse viruses (Burton *et al.*, 2012). Currently, there is no licenced HCV vaccine available; however, research into HCV vaccine development is ongoing. An important explanation for lack of efficacy of vaccine candidates to protect from HCV may relate to the dynamic and complex interactions of HCV with host lipid metabolism (Shawa, Sheridan, *et al.*, 2017). Over the years, efforts to develop HCV vaccine have been affected by different factors such as: hypervariability of HCV proteins (Sabet *et al.*, 2014), genetic diversity (Dahari, Feinstone and Major, 2010), different viral genotypes and quasispecies, since 30% – 50% viral sequence diversity exist (Shi and Ploss, 2013). The meta-analysis of HCV vaccine trials conducted in chimpanzees has shown good efficacy, and was reported to contain part or all of the HCV structural envelope protein that induced sufficient neutralising response (Meunier *et al.*, 2011). The HCV prophylactic recombinant E1E2 vaccine development derived from HCV genotype 1a has reached phase II clinical trial in humans (Young *et al.*, 2015). The recombinant E1E2 vaccine successfully elicited broad cross-genotype nAb and cellular responses (Law *et al.*, 2013).

Although the DAAs have shown significant advancement in HCV therapy, they have fewer efficacies against hepatic cirrhosis and HCC cases. Therefore there is a need for continued efforts to develop prophylactic as well as therapeutic vaccine to prevent persistent HCV infection (Walker and Grakoui, 2015). A vaccine that can be used as an adjunct to DAAs treatment that could reduce viral persistence or reinfection is the desirable target (Swadling *et al.*, 2016). Previous approaches for vaccine development

based on induction of virus-specific CD8+ T cell by therapeutic vaccination did not succeed to significantly suppress viraemia, but HCV-specific T cell response was partially primed. It is still unclear why vaccine-induced T cells failed to control viraemia (Wieland and Thimme, 2016).

Likewise, another study on chimpanzees that were repeatedly infected with HCV resolved the infection following DAAs treatment; and had demonstrable CD8+ T cell responses that were incapable of preventing persistent infection when re-challenged with HCV (Tarr, Urbanowicz and Ball, 2012). Thus, an HCV vaccine will be essential to both health individuals as well as those who cleared the infection either spontaneously or following therapy. Therefore, there is an urgent need for a prophylactic vaccine that could prevent development of CHCV infection following exposure to all HCV genotypes. Since HCV is genetically diverse, the broadly effective prophylactic vaccine candidates must target conserved B cell and T cell epitopes of the virus.

Other researchers previously developed a T cell vaccine for HCV by utilising a recombinant chimpanzee-derived adenovirus 3 (ChAd3) and a human adenovirus 6 (Ad6) in a prime boost regimen (ChAd3-NSmut/Ad6-NSmut regimen), but this regimen had some limitations such as poor induction of HCV-specific T cells in a healthy control group (Barnes *et al.*, 2012). The limitations of a heterologous ChAd3-NSmut/Ad6-NSmut regimen were overcome by utilisation of a ChAd3-NSmut prime and a MVA-NSmut boost vaccination. The ChAd3 and a modified vaccinia Ankara (MVA) that encodes the HCV non-structural proteins (NSmut) in a heterologous prime/boost regimen previously optimised in healthy individuals (Swadling *et al.*, 2014) was able to induce HCV-specific T cell responses in HCV infected patients (Swadling *et al.*, 2016).

The HCV displays a narrow species tropism with humans and chimpanzees as the only hosts susceptible to infection; which affects progress in understanding the mechanistic analysis of viral pathogenesis and vaccine development. Significant progress has recently been made in relation to HCV treatment following the licencing of DAAs, however factors such as high cost, risk of reinfection, heterogeneity of the virus, narrow host tropism and lack of an immunocompetent small animal models all hamper vaccine development.

1.16 Metabolic profiling.

There are different approaches to investigate HCV mechanisms of resistance; either following a hypothesis or generating one through a non-hypothesis driven approach. Metabolomics is one of the non-hypothesis driven approaches, used in analytical chemistry for identification and quantification of smaller molecules (cellular metabolites) less than 1 KD in a sample by use of sophisticated and sensitive analytical technology coupled by biostatistics and multivariate applications for data acquisition and processing (Dettmer, Aronov and Hammock, 2007). Metabolic profiling refers to the global metabolic responses to physiological, genetic or environmental stimuli (Nicholson and Lindon, 2008). Four main approaches applied in metabolomics are: target analysis, metabolite profiling, metabolomics, and metabolic fingerprinting (Fiehn, 2002). Target analysis involves identification and quantification of specific known metabolites using a method suitable for that particular target. Metabolite profiling analyses a large set of compounds present in a mixture to identify known or unknown metabolites. In general, metabolomics approaches employ 'complementary analytical methodologies' such as MS and NMR for determination and quantification of known or unknown compounds. Metabolic fingerprinting is the fourth conceptual approach that involves generation of metabolic signatures (mass profiles) of a sample in comparison

to a large data to identify the specific differences between samples (Roessner and Bowne, 2009). The utilisation of metabolomics tools is essential in understanding an organism's response to environmental changes, genetic alterations, and immunological stimuli by observation of perturbations to specific metabolic signatures in biofluids such as urine (Williams *et al.*, 2009), intact tissues (Yang *et al.*, 2007), and serum (Chen *et al.*, 2013). Furthermore, dietary and geographical differences may be highly discriminatory, above that of gender and ethnicity which do affect the metabolic profiles (Slupsky *et al.*, 2007; Holmes *et al.*, 2008).

1.16.1 Mass spectrometry (MS).

Liquid or gas chromatography mass spectrometry (LC-MS, GS-MS) provide full mass spectral analysis in conjunction with biostatistics analysis software (Fitian *et al.*, 2014). Since 1970, MS has been utilised to identify metabolic profiles (Pauling *et al.*, 1971). Characterisation of metabolites is achieved by molecular weight and ionic charge. Serum lipidomic profiling requires chromatographic separation for reduction of ion suppression, capillary electrophoresis (CE) is carried out prior to sample analysis in MS. LC-MS and/or GC-MS are the common separation methods employed before mass spectrometric analysis; whereas Ultra Performance LC (UPLC) utilises separation columns that permit small size particles (1.4 – 1.7µm) (Boisen, 2009). The application of other techniques such as GC-MS analysis is usually limited to thermally stable compounds; and enough vapour pressure is required for volatilization during sample injections. The serum lipidome profiling studies in HCV exposed uninfected intravenous drug users have not yet been conducted. Therefore, determination of metabolic alterations associated with HCV resistance may provide a considerable amount of useful information that could explain factors that confer resistance for HCV infection.

1.16.2 Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy.

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy detects the magnetic properties of atomic nuclei to identify a wide range of hydrogen-containing compounds in a mixture. The number of nuclei measured in a mixture determines the intensity of NMR signal (peak) (Lindon, Nicholson and Everett, 1999). The ^{1}H NMR spectroscopy is preferred because hydrogen is the most abundant atom present in living organisms, so can identify peaks of small molecules. Resonances of unknown compounds present in a mixture are observed by the position and splitting patterns of signals in NMR spectra. The NMR deals with atoms that possess the property of 'spin'; such as ^{1}H, ^{13}C, ^{15}N and ^{31}P, with ^{1}H NMR spectroscopy as a preferred NMR method in profiling of biofluids such as urine. The ^{1}H NMR spectroscopy is the common method because of the abundance of hydrogen in organic molecules (reviewed in (Patel *et al.*, 2012)). Metabolomics investigations in biofluids have been conducted in a variety of diseases including liver disease, but have not been investigated in exposed uninfected PWIDs.

1.16.3 NMR studies in HCV.

The urinary NMR profiling was performed by Godoy and colleagues to compare the urinary metabolomics profiles between healthy individuals without prior history of HCV exposure and chronic HCV patients (Godoy *et al.*, 2010). Their findings indicated that HCV infected patients were correctly identified by urinary metabolomics; with a sensitivity of 94% and specificity of 97% (Godoy *et al.*, 2010). Shariff and colleagues also characterised the urinary metabolomics biomarkers of hepatocellular carcinoma in two etiologically and ethnically distinct populations (a Nigerian population infected with hepatitis B virus; and an Egyptian population infected with hepatitis C virus) (Shariff *et al.*, 2010, 2011). In a Nigerian population study, metabolomics analysis distinguished hepatocellular carcinoma patients from healthy

controls, and from the cirrhosis cohort. Creatinine, creatine, and acetone were some of the metabolites that contributed to the differences in multivariate models (Shariff *et al.*, 2010). A validation study was conducted in hepatitis C virus infected Egyptian patients with hepatocellular carcinoma. Multivariate statistical analysis identified glycine, trimethylamine N-oxide (TMAO), hippurate, citrate, creatinine, and carnitine as the discriminatory metabolites that distinguished hepatocellular carcinoma cases from cirrhotic patients and healthy controls (Shariff *et al.*, 2011). This shows that multivariate analysis can offer distinction of urinary NMR metabolites among different study populations.

1.16.4 Comparison of MS and NMR.

The generation of human metabolic profiles in biofluids such as serum and urine are performed by two commonly used platforms (NMR and MS) in metabolomic investigations. There are some differences that exist between these two robust technologies (Table **1.3**).

MS has higher sensitivity levels than NMR in metabolite detection (MS picomolar versus NMR nanomolar concentrations; some GS-MS can reach femtomolar levels) and sample degradation during run as opposed to ^{1}H NMR (Want, Cravatt and Siuzdak, 2005; Want *et al.*, 2007). Validation of identified unknown metabolites on MS is used by tandem MS as well as accurate mass (time of flight) methods. Alternatively, Fourier transform (FT) MS is used, it has a greater resolution and mass accuracy better than 1 ppm (Brown, Kruppa and Dasseux, 2005). The MS requires sample pre-separation and purification and then run into liquid or gas chromatography. The sample preparation procedure for the MS is laborious and time consuming. After sample run and processing, the sample is used and unavailable if needed for further investigations using

different techniques. Therefore the MS is considered destructive when it comes to sample processing and data acquisition.

Statistical heterospectroscopy is an approach that has recently been developed by Crockford and colleagues (Crockford *et al.*, 2006) that combines the MS and NMR data processing. This approach provides a unique and useful tool to researchers who require a combination of both platforms for identification and comparison of metabolic profiles.

In contrast, NMR sample processing is non-invasive, and has an advantage over MS due its non-destructive nature (Dunn and Ellis, 2005). Therefore the same samples can be used for separate different experiments when necessary. The NMR has a high throughput, and requires minimal sample preparation protocols (Chen *et al.*, 2006). The NMR is known to have limited a metabolite coverage which could result in same set of metabolites being reported in a variety of different pathological conditions. Such a limitation in NMR can be overcome by MS technology. Comparison of the NMR data processed at separate external laboratories for external quality assurance shows that NMR technique has a high degree of inter-laboratory reproducibility of results. Recently, the sensitivity of NMR has been improved by use of higher magnetic field strengths and cryogenic probe technology (Keun *et al.*, 2002).

Table 1.3 LC-MS/GS-LC versus Proton NMR.

Category	**LC/MS, GS/MS**	**^{1}H NMR**
Sensitivity	Higher than NMR (picomolar). Some forms of GS-MS reach femtomolar levels.	Lower than MS (nanomolar at high fields using new cryoprobes) – with limits of detection on the order of 10μM.
Sample preparation	Requires separation and purification before directing samples into mass analyser, which makes sample analysis time consuming.	Suitable for samples in different conditions.
Sample degradation	Degrades.	Does not degrade.
Metabolite identification	Labour intensive and not fully categorised.	Less laborious and well categorised.
Reproducibility	Moderate.	Highly quantitative and reproducible.
Throughput	Approximately 60 samples per day.	High (500 samples per day with the assistance of flow-injection probes and automated liquid handlers) (Pan and Raftery, 2007).

LC-MS, GS-MS and ^{1}H NMR technologies complement each other, however there are obvious differences as summarised in Table **1.3**. Profiling and analysis of the entire metabolome using a single technology is difficult due to diversity of compounds and differences in their chemical structures.

1.17 Rationale of the study.

Increasing evidence suggests that the EU population is distinct from those who become infected but spontaneously clear HCV, and is a true but rare population highly pertinent in the study of mechanisms of resistance to HCV infection to facilitate rational development of vaccine and alternative therapeutic avenues. The HCV lifecycle is dependent on host lipid pathways for production of infectious virions. A hallmark of HCV infection is altered lipid and lipoprotein metabolism. Differences in lipidomics profiles may indicate unique protective mechanisms against infection by HCV. There's growing evidence suggesting that HCV utilizes lipid and lipoprotein metabolism at all stages of its lifecycle. Therefore, an 'omics' approach was employed to assess if there are any unique lipidomic signatures that can separate the EUs from other comparator groups.

The main aim was to determine metabolic alterations associated with HCV resistance to gain mechanistic insight into HCV protection. In this study, serum lipidomics analysis was performed to characterise the lipidomic profiles of EU cases compared to HCV susceptible cases. I sought to use UPLC-MS to identify distinguishing factors in serum lipidomics of the EU cohort. Differences in lipidomics profiles may indicate unique protective mechanisms against infection by HCV.

I also employed the urinary NMR-based metabolic phenotyping to identify metabolic unique fingerprints that could emerge to distinguish the exposed uninfected cohort from the comparator groups. Cellular metabolic products perform different important functions that maintain physiological state of an organism. Any genetic or environmental perturbations in metabolites will change their functional properties which will determine how an organism responds to different stimuli. Analysis of both serum lipidomics and/or urine metabolomics provides useful insights for resistance to HCV.

The innate immune response plays a crucial role in providing the first line of defence against the establishment of HCV infection, and subsequently coordinates the HCV-specific adaptive immune responses. Understanding the peculiar interplay of HCV with host innate immunity could reveal mechanistic insights into the outcomes following HCV exposure. The activation of host immune signalling pathways leads to the induction of highly orchestrated responses that are designed to prevent establishment of HCV infection. The contributions and interactions of these factors in protection from HCV are unknown. Protection from viral infection can arise as a consequence of an adaptive immune response able to prevent an infection becoming established, or it may be innate in that an individual has genetically determined resistance and is not susceptible to infection. The available evidence suggests the involvement of both innate and adaptive responses in spontaneous clearance of acute HCV infection however, evasion of the innate immune response in early stages of HCV infection results in HCV persistence and potential progression to CHCV disease. The HCV is also capable of disrupting cellular immune signalling pathways and subsequently blocks expression of interferon-stimulated genes to partly or completely limit their antiviral activities. Therefore this work attempted to investigate the host immune responses following repeated exposure to HCV through injecting and sharing of needles and other equipment in PWIDs.

It is now accepted that there is a subset of PWIDs who remain uninfected despite repeated long-term exposure through sharing of contaminated needles and other paraphernalia. Detection of true exposure to HCV and determination of infective dose is difficult to estimate; but there is a relatively high risk of exposure when sharing contaminated injecting equipment. This thesis will address fundamental key questions in order to establish the existence of the EU phenotype among PWIDs. Such important questions expounded included:

a) Does the exposed uninfected phenotype exist?
b) If yes, what are the potential mechanisms of resistance?
c) Why are some people susceptible to HCV infection than others?
d) Why do some individuals clear HCV infection more efficiently than others?
e) Do the EUs have unique lipidomics features that could potentially protect them from HCV infection?
f) Do the EUs exhibit potent multi-faceted immune responses that possibly work in concert with the HCV-lipid interactions?

1.18 Hypothesis.

The hypothesis is that there are host metabolic determinants involved in interrupting the HCV life cycle or that are involved in innate immunological response that confer resistance to the HCV exposed uninfected cohort.

1.19 Overall aim.

The overall aim of the project is to identify aberrations in lipid profiles or metabolic pathways that point to the protection from HCV infection; and to analyze the involvement of innate factors responsible for conferring resistance to intravenous drug users who are exposed to HCV but remain seronegative and aviraemic.

2 CHAPTER TWO

2.1 Methodology.

2.2 Study population: exposed uninfected intravenous drug users.

Individuals with a history of high risk parenteral exposure to HCV who have no evidence of infection, testing negative for both HCV antibody and HCV RNA by sensitive assays, were studied.

2.2.1 Ethical approval.

The study was approved by the National Research Ethics Service (NRES) Committee South West - Cornwall & Plymouth; through the health research authority (HRA) (REC number 1703; Grant reference number PUPSMD-0004R1) for collection of blood and urine samples from exposed uninfected IDUs. The study was carried out by adhering to ethically acceptable standards.

2.2.2 Case identification and recruitment procedure.

After obtaining ethical approval, I set off for case identification and recruitment. Fliers (appendix A) were distributed in various pharmacies and drug and alcohol rehabilitation centres; and posters (appendix A) were placed in selected centres in Plymouth. The study information sheet was included in the drug injection pack given to clients on their routine visits to inform potential study participants.

Interested study participants were booked via telephone, and were given adequate time to consider their decisions for participation without coercing them. The blood-borne virus Nurse (Ms Lynsey Opara) and my colleague (Dr Paraskevi Mandalou) also offered their help in blood sample collection. The intravenous drug users studied for this thesis

were identified and recruited amongst over 2000 clients attending drug services at the Harbour Drug and Alcohol Service in Plymouth, United Kingdom (UK). HCV exposed uninfected (EU) cases were selected based on the high probability of HCV exposure as determined from their injection history. The degree of exposure was assessed using a standard questionnaire detailing duration of drug use. EU study participants fulfilled the following criteria: substantial long (more than 6 months) and repeated history of injection drug use, currently sharing of needles and other injection paraphernalia, Caucasian adults who gave a written informed consent, and those who were screened to be negative for their last HCV test. A structured questionnaire was used to collect drug injection history, and assess the risks of HCV exposure (refer to the questionnaire in appendix A 9.1.2). The questionnaire covered the following aspects: frequency of drug injection; sharing of needles and other paraphernalia with friends, and history of imprisonment amongst others. Other HCV transmission risk factors such as blood transfusion, tattooing, and sexual contact with an HCV positive individual were also assessed using the questionnaire to ascertain the extent of viral exposure from other sources other than drug injection. Serum blood samples were sent to Microbiology laboratory at Derriford hospital for HCV antibodies and RNA testing. Seronegative aviremic cases were defined by serial negative testing for anti HCV antibody and HCV RNA by conventional assays (Biorad monolisa HCV Ag/ab test; and Roche Ampliprep HCV PCR viral load - COBAS® TaqMan® HCV Test v2.0). No subjects recruited tested positive for *Human immunodeficiency virus* (HIV), and/or *Hepatitis B virus* (HBV) surface antigen (HBsAg) prior to and/or after recruitment. Blood samples (both stored and freshly collected) from chronic HCV infected patients, and those who cleared the virus spontaneously, served as comparators. Stored blood samples from healthy volunteers who had no history of HCV infection were used as controls.

2.2.3 Comparison cohorts.

Four cohorts were studied in this project. The exposed uninfected cohort was compared to three distinct groups as follows: (1) IDUs who were categorized as HCV spontaneous resolvers (HCV antibody positive but HCV RNA negative), (2) Chronic HCV cases (individuals who tested positive for both HCV RNA and antibodies), and (3) healthy volunteers who had no history of drug injection or any risk factors for HCV transmission. For the lipidomic work additional samples were obtained from the HCV Research UK (HCVRUK) biobank (Glasgow) from 150 CHCV patients, HCV RNA negative patients who cleared the virus following treatment (sustained viral response (SVR – 100)), and spontaneous resolvers (20).

Exposed uninfected cases were recruited as described in Section 2.2.2; whereas stored serum samples for healthy individuals were used as comparator group in lipidomics investigations. Additional healthy volunteers were recruited among researchers and postgraduate students in John Bull building research laboratories in Derriford. To maintain confidentiality, all recruited cases were anonymised by assigning a unique study number (South West number – SW) and data protection protocols were observed. No personally identifiable information (such as names) was kept in the study documentation. All hard copies were stored in a safe-locked filing cabinet, and soft copies were stored in encrypted external hard drives.

2.2.4 Sample collection procedure.

2.2.4.1 Serum.

Serum is the fluid part of the blood without fibrinogen and other clotting components. Blood was collected in a 5mL serum separating tubes (BD vacutainer gold-topped) (BD Diagnostics,UK) containing silica to activate blood clotting; but no anticoagulant; and incubated in an upright position at room temperature (to allow clotting) for two hours. Samples were transported under ambient conditions to John Bull research laboratories. The blood was centrifuged at 2000g (Labofuge 400R centrifuge (Thermo Scientific) for 10 minutes to allow the separation of serum. Brakes were not used to stop the centrifuge. Under a level II biosafety cabinet, the serum was carefully aspirated using clean pipette tips; and aliquoted into cryovials (SARSTEDT, Germany). The labelled aliquots were stored at – 20°C.

2.2.4.2 Plasma.

Plasma is the liquid component of blood (usually yellowish in colour) where blood cells are suspended. Blood was collected in a 3mL Ethylenediaminetetraacetic acid (EDTA) BD vacutainer plastic tubes (BD Diagnostics,UK). Tubes were inverted 8 – 10 times to mix the blood with the anticoagulant. Samples were stored at room temperature for up to 4 hours until centrifugation. Samples were transported under ambient conditions to Peninsula School of Medicine, John Bull research laboratories. Samples were centrifuged at 2000g for 10 minutes, with no brakes activated. The plasma was carefully aspirated into clean labelled cryovials (SARSTEDT, Germany), and stored at – 20°C.

2.2.4.3 Urine.

Mid-stream urine samples were collected from each participant in a 20 mL screw-capped container (Sterilin, UK) between 14:00 and 16:00 hrs. Early urine samples were shown to exhibit greater inter-individual variations as opposed to random urine samples collected after first-void urine samples (Lenz *et al.*, 2003; Walsh *et al.*, 2006). Samples were transported under ambient conditions to Peninsula School of Medicine, John Bull research laboratories. Samples were centrifuged at 2500 rpm for 20 minutes to remove any precipitates, following an adopted protocol from Williams (Williams *et al.*, 2009). The samples for cases collected on early stages of recruitment were transferred to a clean 15 mL container (Greiner Bio-One, Germany) and stored at –20ºC; the rest were stored in siliconized microcentrifuge polypropylene vials (Sigma-Aldrich Corporation, UK) at –20ºC in preparation for metabolomics studies. No preservatives were used in stored urine.

2.3 Reagents and materials for peripheral blood mononuclear isolation.

2.3.1 Reagents:

a) RPMI-1640 (Lonza Verviers, Belgium).
b) Phosphate Buffered Saline (PBS) (Lonza Verviers, Belgium).
c) Foetal Bovine serum (FBS) (Sigma-Aldrich Corporation, UK).
d) Histopaque-1077 (Sigma-Aldrich Corporation, UK).
e) HEPES 1M (Lonza Verviers, Belgium).
f) Cell Culture Freezing Medium, dimethyl sulfoxide (DMSO) (Sigma-Aldrich Corporation, UK).
g) Mr. Frosty (cell freezing apparatus).
h) Freezing medium (absolute isopropyl alcohol).
i) Pen-Strep 10,000 U (Lonza Verviers, Belgium).
j) L-Glutamine (Lonza Verviers, Belgium).
k) Penicillin-Streptomycin (Pen/Strep) (Lonza Verviers, Belgium).
l) 0.4% Trypan Blue (Sigma-Aldrich Corporation, UK).

2.3.2 Peripheral blood mononuclear cell extraction.

Heparinized (Sodium heparin) whole blood collected in a 9 mL vacutainer tubes (Greiner Bio-One, Austria), was diluted in equal volumes with 1X Phosphate Buffer Saline (PBS) without calcium and magnesium. The peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque-1077 that helps to aggregate red cells by polysucrose. The histopaque separates the blood into layers during centrifugation; where lymphocytes and monocytes lie under a layer of plasma (at a plasma/histopaque interface) in a separate buffy coat. The blood mononuclear cells were separated through density gradient centrifugation at 2100g for 10 minutes with brake activated to provide rapid rotor deceleration. The isolated PBMCs were washed three times with Roswell Park Memorial Institute medium (RPMI)-1640, supplemented with 1M HEPES buffer, 10,000 U/mL Penicillin-Streptomycin (Pen/Strep), 200mM L-Glutamine and 10%FBS. The cells were prepared for counting as described in Section 2.3.3.

2.3.3 Cell counting and apoptosis assay (cell viability).

Following cell centrifugation as described in Section 2.3.2, the pellet was re-suspended in 1 mL of supplemented RPMI (sRPMI) and counted using the haemocytometer (Superior, Marienfield, Germany). The cells were diluted 1:20 using trypan blue dye exclusion method i.e. 0.4% trypan blue (0.4 g of trypan blue into 80mL of PBS). The 1 mL of 0.4% trypan blue was added to 4mls of RPMI to prepare counting solution. The haemocytometer was assembled by affixing the micro cover slip (22 x 22mm) ensuring absence of Newton's rings to achieve desirable optical conditions allowing visualisation of cells. The 10µL of diluted cells were carefully loaded into the haemocytomer and counted using a low power phase contrast microscope objective (x10). Four outer squares of the counting chamber were counted and the average was obtained, and then multiplied by the dilution factor using the following formula:

Cell count/ml = Average x dilution factor x 10^4

Since dead cells do not exclude trypan blue, they stain blue but viable cells do not allow trypan blue in the cellular cytosol. Cell viability percentage was calculated by counting the number of viable cells divided by the total number of cells (both viable and non-viable) multiplied by 100.

$$\frac{Viable\ cells}{Non - viable\ cells}\ x\ 100 = Percent\ viability$$

2.3.4 Cryopreservation of PBMCs.

The isolated counted PBMCs were re-suspended in 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich Corporation, UK) in foetal bovine serum (FBS) (product of Brazil, SIGMA, Dorset UK); and stored in 1 mL aliquots of between 2 $x10^6$ – 7 $x10^6$ cells per mL. Cryoprotectant such as DMSO was used to reduce the amount of ice that forms during freezing. Since DMSO is a hypertonic compound and can cause osmotic injury to the cells, DMSO was added gently dropwise. The cryopreservation of cells was done by placing the cryovials (Sarstedt, Germany) in a freezing container (1^oC/min Mr Frosty) with isopropyl alcohol and kept at – 80^oC overnight; before being transferred to liquid nitrogen tanks for longer storage.

2.4 Laboratory testing of collected samples.

2.4.1 Serum lipid profiling.

Stored non-fasting serum samples from chronic HCV patients, spontaneous resolvers, healthy volunteers, and exposed uninfected cases were analysed for apolipoproteins A1 (apoA1), apoB, and apo E using automated rate nephelometric methods (Siemens Dade Behring, BNII Nephlometer, Germany) in collaboration with University of Newcastle (Newcastle Upon Tyne Biochemistry department). The samples were sent shipped to Newcastle on dry ice for apolipoprotein profiling, which was done by their biochemistry department and results were analysed by this student researcher.

A total of 61 stored serum samples from three different study groups were analysed to determine their apolipoprotein levels. Out of the 61 samples, 22 (36.1%) were HCV exposed uninfected, 8 (13.1%) cleared the infection spontaneously, whereas the other 31 (50.1%) were health volunteers previously recruited from research staffs and postgraduate students. The Plymouth samples were compared with 124 CHCV samples generated in Newcastle Upon Tyne.

2.4.2 Serum apolipoprotein E.

The apolipoprotein E (apoE) testing only was performed at Peninsula School of Medicine, John Bull research laboratories using sandwich ELISA; on ABCAM ab108813 –apoE human *in vitro* ELISA kit that recognizes apoE-2, apoE-3, and apoE-4 isoforms. The specific apoE antibodies were pre-coated onto a 96-well plate, and blocked by the manufacturer. The serum samples were diluted 1:400 with diluent supplied together with the kit. The standards were serially diluted from an initial 2μg/mL apoE standard concentration. 50μL of diluted duplicate samples and standards were added to appropriate wells and incubated at room temperature for 2 hours.

Following steps of washes, 50 µL of biotinylated detection antibody was added and incubated at room temperature for an hour. After further washing, 50µL of streptavidin-peroxidase (SP) conjugate complex was added and incubated for 30 minutes. A chromogen substrate tetramethylbenzidine (TMB) was added to visualize SP enzymatic reaction, and incubated for 15 minutes. Once the optimal blue colour was formed, the reaction was stopped by adding 50µL of stop solution. The plate was read at 450nm wavelength using GENios; Firmware: V 4.62 - 07/01, XFLUOR4 Version: V 4.51 reader.

2.5 PBMC stimulation for innate immune response.

Cryopreserved PBMCs were thawed in ice as described in Section 2.6.1, cultivated and stimulated by pattern recognition receptor ligands modelling viral infection (such as the RIG-I, TLR3 and TLR7/8 ligands, Polyinosinic-polycytidylic acid (PolyIC), and R848) or infected with Influenza A virus to determine the up-regulation and production of specific cytokines in response to viral stimulation. A total of 200 x 10^3 cells were seeded in each well for 18 hours. The cells were co-cultured (stimulated) with PolyIC (2.5µg/mL), PolyIC:Lyovec complex (2µg/mL), R848 (4µg/mL), and different doses of Influenza A virus (Perth strain, subtype H3N2) in 200µL medium; cultured in a 96-well plate (Greiner-Bio-One, Germany), and incubated overnight at 37°C in a 5% IR Sensor CO_2 incubator (SANYO Model MCO-17A, Japan). The changes in concentrations of IFN-α and multiplex panel were analysed using sandwich ELISA and Luminex.

2.5.1 Ligands for stimulation of immune cells (optimisation).

The ELISA optimisation protocols were performed to ensure reliability of the test results. ELISAs were performed on triplicate wells to reduce inter-assay variation and possibly increase the sensitivity. The innate immune response to single or a combination of TLR ligands as a model for viral PAMP as well as Influenza A virus, was determined by ELISA analysis. Type 1 interferons were discovered to have an antiviral activity against Influenza virus (Lindenmann, 1982).

The following ligands were used:

a) Single PRR ligands as a model for relevant antiviral signalling pathways:
- Single stranded viral nucleic acid sensor ligands such as gardiquimod (TLR7) – R848 (TLR8) – TL8-506; supplied by Invitrogen, San Diego, USA.
- Viral replication intermediate nucleic acid sensors such as PolyIC (TLR3 ligand).

b) PolyIC/ Lyovec complex that serves as RIG-I/MDA-5 ligand.

c) Influenza A virus subtype H3N2 (A/Human/µ/16/09 strain) supplied by Public Health England as a representative of RNA viruses. This stimulus triggers TLR3, TLR7/8 and RIG-I in infected cells.

The seeded cells were co-cultured overnight with varying volumes of stimulants. PolyIC/Lyovec complex, R848, and PolyIC (InvivoGen, San Diego, USA), were used at a final concentration of 2µg/mL, 4µg/mL, and 2.5µg/mL respectively. Different concentrations of 20µg/mL, 50µg/mL, and 100µg/mL were used for TLR8-506 in optimisation experiments. The Influenza A virus stimulants had 9.68×10^6 PFU/mL stock; therefore different Influenza A virus doses were prepared as follows: 9.68×10^3 PFU, 19.36×10^3 PFU, and 29.04×10^3 PFU; and were added to the seeded cells for the intracellular activation of cytokine production and secretion.

PFU is defined as a measure of infectious virus particles; whereas multiplicity of infection (MOI) is the average number of virus particles infecting a single cell. The following formula was applied: $\mathrm{MOI} = \frac{\text{PFU used to infect the cells}}{\text{Number of cells}}$

200,000 cells were infected by 1μL of Influenza A virus with a titre of 9.68 x 10^6

PFU/mL, the MOI was calculated as follows: $\mathrm{MOI} = \frac{0.001 * 9.68 \times 10^{\wedge}6}{2 \times 10^{\wedge}5} = 0.048$ (i.e approximately 1 virus particle in 20 cells).

Unstimulated PBMCs and medium only from seeded cells were used as negative controls. The EU samples were finally stimulated with Poly:IC/Lyovec complex (2μg/mL), R848 (4μg/mL), and the lower doses (9.68x10^3PFU and 9.68x10^3PFU) of Influenza virus.

2.6 Detection of interferons type-I using ELISA.

2.6.1 Cell thawing.

The cryopreserved PBMCs were transferred from the liquid nitrogen tank on ice to a 37°C water bath until a small piece of ice remained. The cells were transferred into a 15 mL Falcon tube, and 1 mL of warm medium (containing 20% FBS) was added gently dropwise to fully liquefy the frozen solution. 2 mL of medium was added again after shaking, then the last 6 mL of medium. The tube was shaken and placed in water bath at 37°C for 20 minutes. The cells were centrifuged at 2000g for 10 minutes with break activated. The cells were then resuspended with 1 mL of sRMPI containing 10% FBS. Cell count and viability was assessed by trypan blue stain as described in Section 2.3.3.

2.6.2 Interferon-α ELISA.

Following an overnight (18 hour incubation) cell culturing as described in Section 2.5, the PBMC cell culture supernatants were harvested and either stored at –20°C or used immediately to run ELISA assay for the detection of interferon-α.

The IFN-α capture antibodies (Mabtech, Sweden) were added to a high-membrane binding 96-well ELISA plate in a final concentration of 4μg/mL, and incubated overnight at 4 – 8°C. After overnight incubation, the plate was washed twice with PBS and blocked by 0.1% concentration of bovine serum albumin (BSA) containing 0.05% Tween20, and incubated for 1 hour at room temperature. The 50μL of samples, and human IFN-α standard (serially diluted from stock solution of 1μg/mL) were added to appropriate wells and incubated overnight at 4 – 8°C. The highest IFN-α standard concentration was 1000pg/mL. Following all necessary washing steps, 100μL of biotinylated detection antibodies for IFN-α in concentration of 1μg/mL was added to each well and incubated for 1 hour. Horseradish peroxidase (HRP) conjugated streptavidin diluted in 1% BSA (1:1000) was added and incubated for 1 hour. No sodium azide was added to the washing buffer to avoid inhibiting HRP activity. To enhance detection of HRP activity, chromogenic substrate (Super Aqua Blue) (eBioscience California) was added and allowed to incubate for 45 minutes. The ELISA data acquisition was performed on triplicate wells. The innate immune response to single or a combination of TLR ligands as a model for viral PAMP; as well as Influenza A virus, was determined by ELISA analysis (VersaMax PLUS ELISA microplate Reader ROM v1.23; Molecular Devices, USA).

2.6.3 Principle of sandwich ELISA technique.

The technique developed in 1977 (Kato *et al.*, 1977) is designed for detection and quantification of an analyte of interest (e.g. antigen) between two layers of antibodies (capture and detection antibody). The capture antibody is coated on a polystyrene microtiter plate through the antibody Fc region. The microtiter plates must have a minimum protein binding capacity of 400ng/cm^2. All unbound antibodies are washed using appropriate washing buffer. A blocking buffer is added that passively adsorbs to the remaining binding sites of the microtiter plate to reduce the nonspecific binding and eliminate background. A sample containing antigens is added and the specific antigen is immobilised by binding to the coated antibody. The plate is incubated and washed to remove unbound antigens. A detection antibody tagged with an enzyme specific to the antigen is added, and incubated. Following a washing step, all unbound antibodies are washed. A secondary enzyme-linked antibody (coupled to substrate-modifying enzyme) is added that binds to the detection antibody. After incubation and washing, the substrate is added, and is converted by the enzyme to produce a chromogenic signal which is detected by a spectrophotometer. The colour production reveals an enzyme activity, whereas lack of colouration indicates lack of enzyme activity or negative result (Aydin, 2015). The colour intensity is directly proportional to the levels of the measured analyte. The analyte is quantified by measuring the amount of light absorbed or transmitted (depends on the analyser) through the coloured liquid in the wells.

2.7 Luminex magnetic bead-based multiplex Assay.

In magnetic multiplex bead-based assay, beads of discrete fluorescence intensities and wavelengths detect multiple analytes in a single sample. This detection method is based on flow cytometry and allows repeatability. The measurements were performed on a Luminex system comprising the Luminex LX100/LX200 analyzer, Luminex XYP plate handler, Luminex SD sheath fluid delivery system, and Luminex xPONENT software. Data analysis was performed on Microsoft Excel 2010, and GraphPad Prism version 5.01 (GraphPad software Inc. USA).

2.7.1 Cell preparation and assay protocol.

The samples were prepared following the manufacturer's instructions. In brief, frozen cell culture supernatants (stimulated with stimulants described in Section 2.5.1) were thawed at room temperature. In this protocol, culture supernatants that were stimulated with lower dose of Influenza A virus (9.68×10^3 PFU) were used in a 6-plex assay. The reagents for the immunoassay were prepared according to the manufacturer's instructions. The 200µL of the assay buffer was added into each well of the plate; and left on a plate shaker at room temperature for 10 minutes. The buffer was removed by tapping onto absorbent towel. The 25µL of neat samples, prepared controls and standard were added to appropriate wells in triplicate. The wells for controls and standard were added with 25µL of assay buffer whereas samples wells were added with cell culture media (sRPMI plus 20% FBS). 25µL of premixed fluorescent-coded magnetic beads were added to each well (beads were mixed intermittently to avoid settling). The plate was sealed, wrapped with aluminium foil, and incubated with agitation on a shaker for 18 hours at 4°C in the dark. Following overnight incubation, the beads were washed twice and 25µL of biotinylated detection antibody was added

into each well and incubated on a plate shaker sealed with foil for 1 hour at room temperature. After washing, 25µL of detection conjugate (streptavidin-phycoerythrin) was incubated with the beads for 30 minutes at room temperature in a sealed plate covered with foil. The beads were washed again, and the wells were filled with 150µL of sheath fluid (pre-filtered, pH balanced PBS concentrated solution for transporting samples in a flow cytometer). The beads were resuspended on a plate shaker for 5 minutes. The levels of fluorescence from the cytokines tested in a single bead were determined with the Luminex LX100/LX200 analyzer (software version: xPONENT for LX100/LX200 3.1.971.0 USA) and expressed as median fluorescence intensity (MFI) using a 5-parameter logistic method to determine concentrations of cytokines in samples.

2.7.2 Principle of luminex magnetic bead assay.

The fluorescent colour-coded beads (microspheres) are pre-coated with cytokine-specific capture antibody. When samples are added, the cytokines of interest are captured by the analyte-specific antibodies on the beads. Biotinylated detection antibodies specific to the cytokines of interest are introduced to each well and an antibody-antigen complex (sandwich) is formed. The reaction mixture is then incubated with Phycoerythrin (PE)-conjugated streptavidin. The colour-coded beads are read by a dual-laser flow-based detection Luminex analyser which excites the internal dyes marking the beads and a second laser excites PE, the fluorescent dye on the reporter molecule. The Luminex flow-cytometry-based instruments integrate detection components (lasers, optics, fluidics, and digital signal processors). The quantification of each microsphere bioassay is based on fluorescent reporter signals.

Figure 2.1: Principle of Luminex multiplex assay.

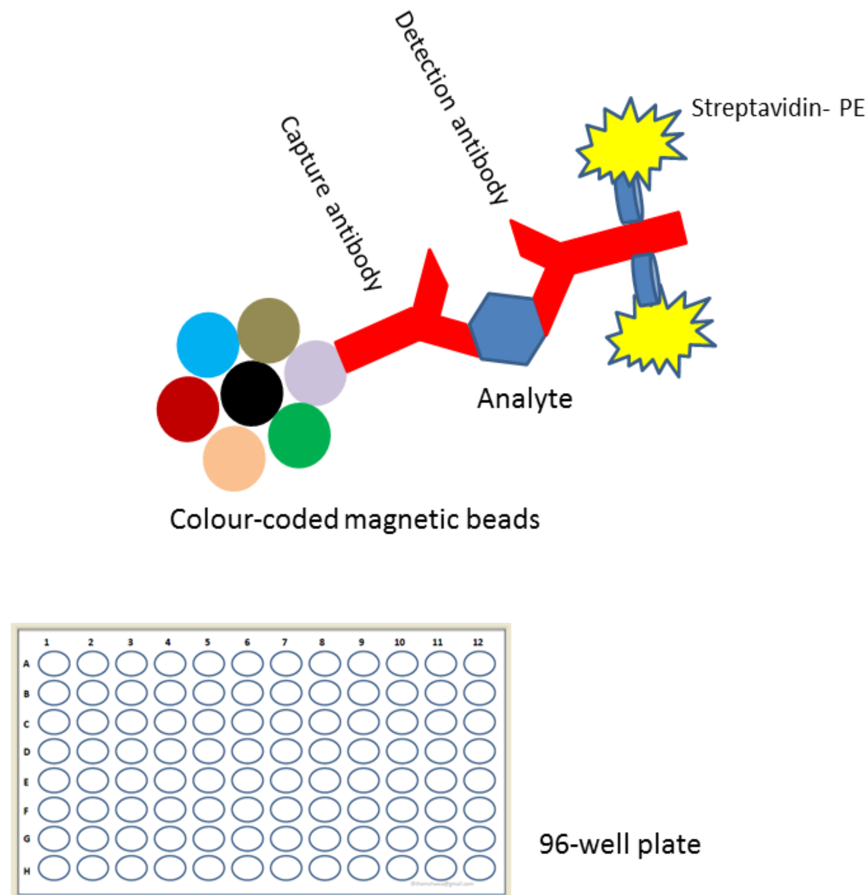


Figure 2.1 shows a diagrammatic representation of the principle of Luminex magnetic assay. The magnetic beads (coloured) contain fluorescent dyes. The beads are coated with capture antibody. The antigens present are captured by the primary antibody. The detection antibody is introduced that binds to the antigen to form an antigen-antibody complex. The secondary antibody is tagged with phycoerythrin (PE)-conjugated streptavidin (a reporter molecule that completes the reaction on the surface of microspheres) allowing detection of the cytokines by the Luminex analyser.

2.8 Interferon-gamma (IFN-γ) Enzyme-Linked Immunospot (ELISpot) assay.

The HCV-specific T cell responses were studied using frozen PBMCs. The T cell reactivity to a range of recombinant HCV proteins and peptides encompassing both structural and non-structural regions were characterised by detection of IFN-γ in co-cultured cells. The IFN-γ releasing cells were enumerated by ELISpot assay using an AID ELISpot Reader version 4.0 (AID GmbH, Strassberg, Germany).

2.8.1 Sample preparation for ELISpot assay.

The frozen PBMC samples were thawed and counted following protocol described in 2.5.2. The cell culture media (RPMI) was supplemented with 50 mL of off-the-clot human AB serum (Gemini, UK supplied by Seralab). The counted cells were adjusted to 2.0 x 10^6 cells/mL by addition of sRPMI/10% AB Serum so that 100µL should have 200 x 10^3 cells. Each well in a 96-well plate contained 200 x 10^3 cells in 200µL.

2.8.2 Cell culture with recombinant HCV peptides.

The following antigens were used to stimulate the PMBCs: Lectins such as phytohaemagglutinin (PHA) from phaseolus vulgaris (Red kidney bean) (Sigma-Aldrich Corporation, UK) are potent polyclonal T cell activators that were used as positive control in a final concentration of 1µg/ml. The PHA was used as a non-specific T cell mitogen to confirm cell viability in each assay. An antigen-specific T cell stimulation was measured in response to overlapping peptide pools spanning the entire HCV genome and 'CEFT' – (JPT Peptide Technologies, Berlin, Germany) consisting of immunodominant peptides from Cytomegalovirus, Epstein Barr virus, Influenza virus,

and Tetanus toxin. The CEFT peptide pool was used as a positive control (recall antigen response) and consists of 14 viral peptides which stimulates T cells to produce IFN-γ responses in approximately 90% of all Caucasians (MABTECH, 2016). The majority of Caucasians would have been immunised in childhood against different viruses, therefore it was expected that the positive controls would induce a T cell response. The CEFT was used in a final concentration of 3µg/mL per well in a cell culture plate. Positive controls were used to assess cell viability as well as functionality of the immunoassay. The wells with unstimulated PBMCs with sRPMI/10% AB serum only were used a negative control. The HCV overlapping peptides were from HCV genotype 3 (the dominant genotype PWIDs in Plymouth) and were obtained from BEI Resources, USA. The lyophilised HCV peptides were reconstituted in 50µL DMSO to make a stock concentration of 20mg/mL. Each HCV peptide was pooled by pipetting 10µL from each vial into a clean tube, aliquoted and stored at -20°C. HCV genotype 3 peptides used in a final concentration of 3µg/mL per well, were: Core (spanning aa 1 – 29 in a single pool), NS3 (aa 1 – 98 single pool), NS5A (aa 1 – 71 single pool), and NS5B (aa 1 – 91 single pool) all from BEI Resources, USA. A total of 200 x 10^3 cells/well were co-cultured in triplicate with the peptides in a flat-bottom 96-well plate (Greiner-Bio-One, Germany) at a final concentration of 1µg/mL (Lectin) and 3µg/mL (CEFT, HCV peptides) and incubated for 20 hours at 37°C in a 5% IR Sensor CO_2 incubator (SANYO Model MCO-17A, Japan). Our previous optimisation experiments determined that a cell density of 200 x 10^3 cells/well was adequate to enhance probability of cell contact between stimulation cells and responding cells. Therefore to achieve an optimal T cell stimulation, 200 x 10^3 cells/well were co-cultured overnight with cell stimulants.

2.8.3 Interferon-γ ELISpot procedure.

A high-membrane-binding 96-well ELISpot plate (MAIPS4510; Millipore, Ireland) was coated with 100µL of 5µg/mL primary/capture IFN-γ antibody (human IFN-γ, BD Bioscience UK) in sterile PBS; and incubated at 4ºC for 16 hours. After overnight incubation, the ELISpot plate was washed once with sRPMI/10% AB Serum. 200µL of the same medium (sRPMI/10% AB Serum) was added into each ELISpot well to block the membrane (in order to reduce cross reactivity), and left at room temperature for 2 hours. After 2 hour incubation the blocking solution was discarded from the plate, and blotted gently. The activated PBMCs were transferred from the culture plate to the ELISpot plate in respective triplicate wells; and incubated for 24 hours at 37ºC in a 5% IR Sensor CO_2 incubator (SANYO Model MCO-17A, Japan).

After a 24 hour incubation, the ELISpot plate was washed several times using 1 x PBS+0.05% Tween 20 as the main washing solution; allowing soaking for 1 – 2 minutes between washes. After cell incubation on the ELISpot plate the stimulated cells become sticky, therefore 0.05% Tween 20 was used to ensure complete removal of the cells and secondary antibodies during washing. 2µg/mL of secondary biotinylated (detection) antibody in PBS + 10% FBS was added to the wells to ensure maximum degree of labelling; and incubated at room temperature for 2 hours.

Following washing, a streptavidin protein that was covalently conjugated to horseradish peroxidase (HRP) enzyme (Avidin-HRP) was diluted 1:100 and used as a conjugate in this protocol. The 100µL of the Avidin-HRP conjugate (prepared 45 minutes before use) was added to the wells and incubated at room temperature for 1 hour. The streptavidin binds to biotin in secondary antibody, and the enzymatic activity is provided by the conjugated HRP. The ELISpot plates were developed using an HRP substrate 3'-amino-9-ethylcarbazole (AEC) (BD Bioscience, USA) for 15 minutes for detection of IFN-γ

secreting cells. The AEC dilution was 1 drop of AEC chromogen in 1 mL of substrate; prepared not more than 15 minutes before use; to ensure that it forms intense red colour spots. The reaction was stopped by immersion of plates in tap water. The plates were rinsed thoroughly with running water ensuring that each well was filled and emptied at least five times. The plates were left to dry inverted for more than 3 hours, or overnight before reading on ELISpot reader.

2.8.4 ELISpot parameters and plate reading.

Spot forming units (SFU) were counted using an AID ELISpot plate Reader version 4.0 (AID GmbH, Strassberg, Germany). The spot definition settings were as follows: Thresholds – Intensity (minimum 20, maximum 255), Size (minimum 20, maximum 5000), gradient (minimum 1, maximum 90); Basic algorithm settings: Emphasis (Small), Algorithm C, Invert recognition was left blank, well saturation was set at 60%. Stage calibration was performed before reading each plate. SFU counts were transferred from the reader to excel spread sheet. A positive IFN-γ response to HCV peptides was determined if the calculated mean number of spot-forming cells in stimulated wells was greater that the calculated mean number of spot-forming cells plus 2 standard deviations (SD) (Thurairajah *et al.*, 2008).

Figure 2.2: ELISpot 96-well plate layout.

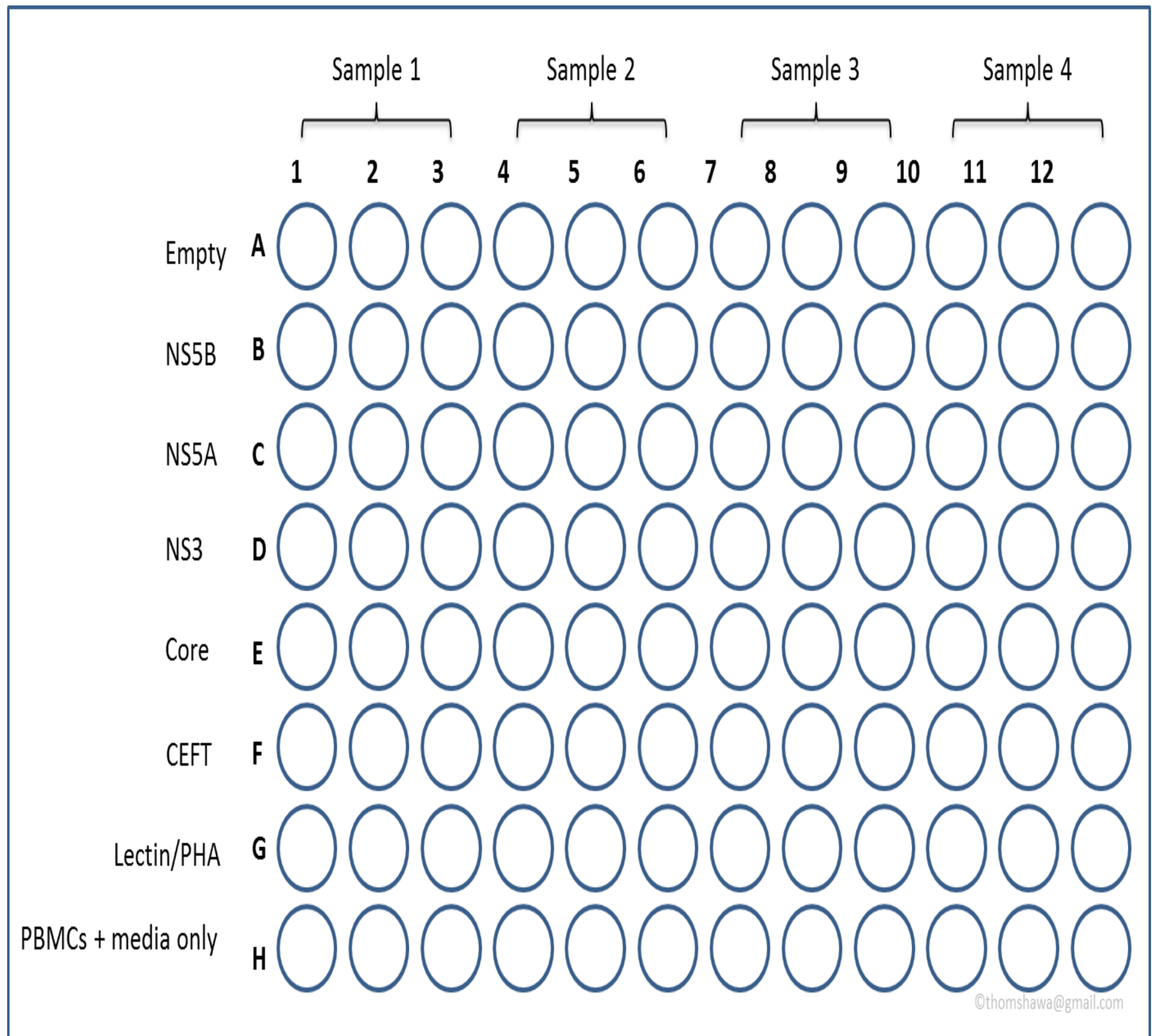


Figure **2.2** shows a schematic diagram of a 96-well ELISpot plate showing the layout of cell stimulants. The 100µL of cell stimulants were added to another 100µL of PBMCs (2 x 10^5 cells). The total volume in each well was 200µL in a final antigen concentration of 1µg/mL (Lectin), and 3µg/mL the rest of the antigens. Negative control wells (PMBCs + media only) were added with 200uL of cells and media only without stimulants. The lectin/PHA and CEFT were used as positive control, with CEFT preferentially as a recall antigen.

2.8.5 Principle of ELISpot technique.

ELISpot is a method used for quantification of frequency of cytokine-secreting T cells at a single cell level. PBMCs are cultured overnight in the presence of antigen-specific stimuli. The stimulated cells are then transferred to an ELISpot plate coated with cytokine-specific monoclonal capture antibodies immobilised on a polyvinylidene difluoride (PVDF) or nitrocellulose membrane; and then incubated appropriately to allow cytokine secretion. Secreted cytokines by stimulated cells are captured by the specific antibodies on the surface of the 96-well plate. The cells are removed through washing with appropriate solution, and secreted cytokines are detected by addition of biotinylated cytokine-specific detection antibodies. Spot formation on the membrane is enabled by streptavidin-HRP conjugate. The cytokine secreting cells are detected by addition of precipitating substrate (AEC); and the cells appear as visible red spots. Each spot corresponds to the 'foot-print of an individual cytokine secreting cell. The colour intensity and size of the spots depends on the levels of secreted cytokines. Artificial spots form as a result of incomplete removal of cells from the plates during washing or due to aggregation of antibodies in the process of coating and detection (MABTECH, 2016).

Figure 2.3: Schematic diagram of ELISpot principle.

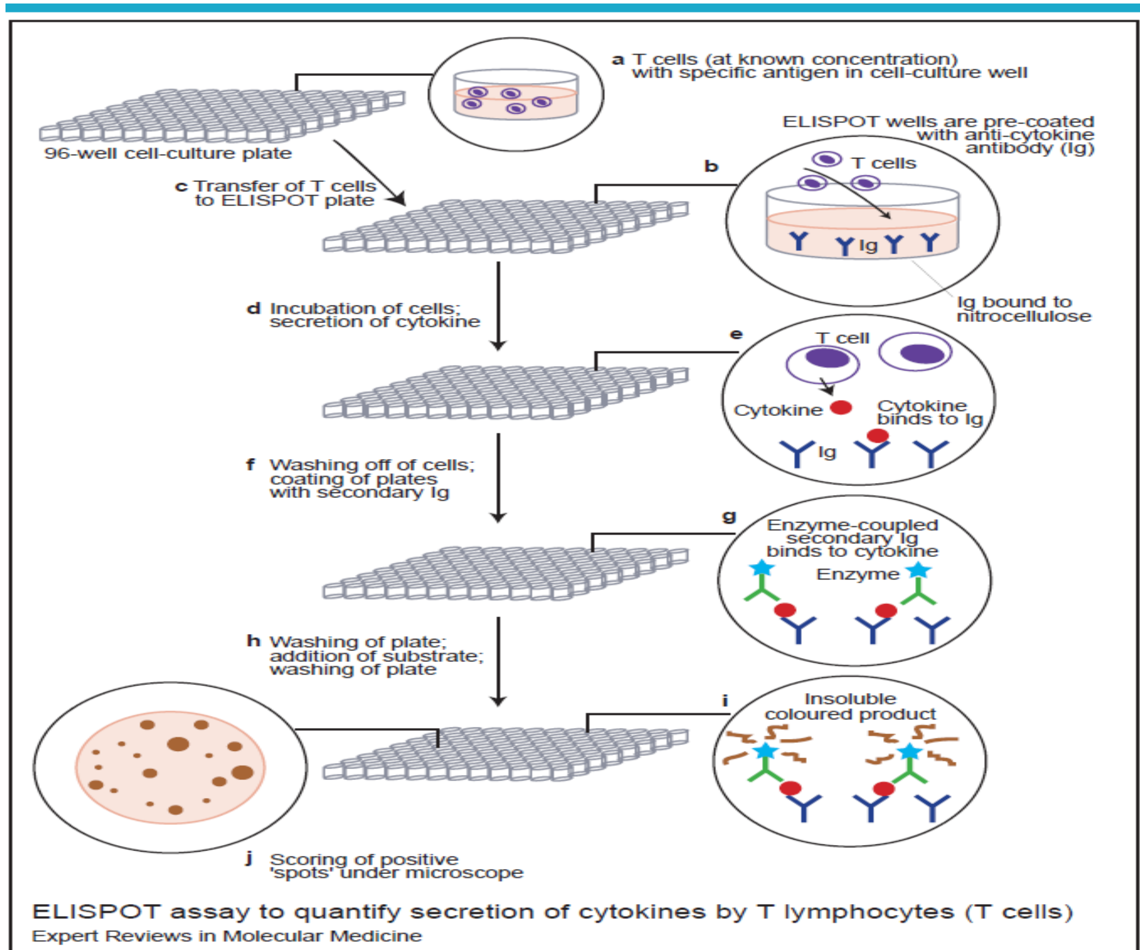


Figure **2.3** shows a schematic illustration of the principle of ELISpot protocol (Hickling, 1998).

2.8.6 A positive IFN-γ response definition criteria for ELISpot assay.

Positive IFN-γ responses were determined based on the mean + 2SD of the healthy volunteers. A positive IFN-γ response in other studies was determined by subtracting the average number of spot-forming T cells in negative control triplicate wells from that in antigen-stimulated triplicate wells and expressed as spot forming unit (SFU) per million cells (Tang *et al.*, 2005). Others determined an IFN-γ response by observing the probability of a spot appearing in the stimulated well if it was significantly different from the probability of a spot appearing in the negative control well (Barnes *et al.*, 2009). There is no consensus on criteria for defining ELISpot positive or negative responses (Moodie *et al.*, 2010), therefore lack of such agreement may affect the number of positive responses. Reference/precise research laboratories established individual in-house criterion; therefore usage of a consensus average in assessing the accuracy is appropriate in dealing with the inter-assay variability. A cut-off of 5 SFU in unstimulated control wells were acceptable in this assay (Alexander *et al.*, 2013). The cut-off values were defined with reference to criteria commonly known as empirical methods based on responses in known ELISpot negative population (Moodie *et al.*, 2010).

2.9 Metabolomics.

2.9.1 Shipment of samples.

All serum and urine samples were collected as described in Section 2.2.4 and shipped on dry ice from Plymouth to London where they were stored at – 80°C at Imperial College London, and Institute of Hepatology, London until required for preparation and analysis.

2.9.2 Mass spectrometry lipidomics profiling.

I performed serum lipidomics using MS at Imperial College London South Kensington London in Prof. Simon Taylor-Robinson and Prof. Elaine Holmes research groups with the help of Dr. Maria Gomez-Romero and Dr. Alexandros Pechlivanis. The lipidome of 386 serum samples were analysed comprising 60 EU, 36 SR, 100 SVR, 159 CHCV patients and 31 healthy controls, utilising EU / SR cases collected locally and additional CHCV samples from the HCV Research UK biobank. An ACQUITY UPLC/MS system (Waters) in both positive and negative electrospray ionisation modes (ESI+ and ESI-) was used following established protocols. A composite QC sample defined the system suitability, analytical stability, and sample repeatability.

2.9.2.1 UPLC-MS mobile phases.

Chromatographic separation of lipid profile in serum samples using UPLC utilises mobile phases/solvents A and B; using Acquity CSH_{18} column. The mobile phases were prepared with LC-MS grade solvents, formic acid, and ammonium formate (NH_4HCO_2) (Sigma-Aldrich Corporation (Dorset, UK). The mobile phase A consisted of high purity water, 10mM NH_4HCO_2, and 0.1% formic acid (CH_2O_2) mixed in that order. Acetonitrile (C_2H_3N) was added slowly while mixing until the solution was clear. The mobile phase B consisted of isopropanol (C_3H_8O), C_2H_3N, CH_2O_2, and NH_4HCO_2 all mixed at the same time and sonicated until complete dissolution of ammonium formate. Refer to Table **2.1** for details of solvents preparation.

Table 2.1 Mobile phase solvents preparation.

	Chemicals	**Amount**	**Rationale**
Solvent A (1 litre)	Acetonitrile (C_2H_3N)	600mL	(60 x A) / 100, mL
	Ultra-pure Water	400mL	(40 x A) / 100, mL
	Formic acid (CH_2O_2)	1mL	(0.1 x A) / 100, mL
	Ammonium formate (NH_4HCO_2)	0.6306g	A ml x 10e-3 L/mL x 10mmol/mmo, g
Solvent B (1 litre)	Isopropanol (C_3H_8O)	900mL	(90 x B) / 100, mL
	Acetonitrile (C_2H_3N)	100mL	(10 x B) / 100, mL
	Formic acid (CH_2O_2)	1mL	(0.1 x B) / 100, mL
	Ammonium formate (NH_4HCO_2)	0.6306g	B mL x 10e-3 L/mL x 10mmol/mmol, g

2.9.2.2 Sample preparation for UPLC-MS: protein precipitation and lipid extraction.

All the samples were thawed at 4°C, and transferred to 2ml safe-lock eppendorf tubes (Eppendorf AG, Hamburg, Germany), and prepared for UPLC-MS analysis by isopropanol protein precipitation (Sarafian *et al.*, 2014). In order to precipitate the proteins and extract the lipids (Pfaender *et al.*, 2015), 150µL of cold isopropanol was added to each 50µL serum sample (ratio 3:1). Composite (QC) sample was generated by pooling 10µL of each sample into a single 2mL safe-lock Eppendorf tube to create a single QC. All the samples and composite QC were homogenised by vortexing for 30 seconds, and incubated at 4°C for 2 hours. The virally inactivated serum samples (by cold isopropanol) (Pfaender *et al.*, 2015) were centrifuged at 4°C for 15 minutes at 13,000 rpm. The 100µL of supernatant was transferred into a glass insert with poly spring, in a glass vial. The prepared samples were kept in the fridge at 4°C or transferred to the auto-sampler where they were kept at 8 °C throughout the analysis.

2.9.2.3 UPLC-MS conditions.

The serum lipid UPLC-MS profiling was performed using an ACQUITY UPLC system (Waters Ltd., Elstree, UK) coupled to a Q-ToF Premier mass spectrometer (Waters MS Technologies Ltd, Manchester, UK) using an electrospray ion source operated in both positive and negative modes.

The LC conditions have been previously described (Shockcor *et al.*, 2011). Separation was done in a Waters Acquity UPLC HSS CSH column (1.7 µm, 2.1 × 100 mm) maintained at 55^{0}C. The mobile phases consisted of ACN/H20 (60:40) (A) and isopropanol/acetonitrile (IPA/CAN) (90:10) (B), both containing 10 mM ammonium formate and 0.1% (v/v) formic acid. The flow rate was set at 0.4 mL/min. Injection

volume was 5μL and 15 μL for positive (ESI +ve) and negative (ESI –ve) modes, respectively.

The ESI conditions were as follows: capillary voltage for ESI- 2500V, for ESI +ve 3000V, cone voltage 25V for ESI -ve and 30V for ESI +ve, source temperature 120^0C, desolvation temperature 400^0C, cone gas flow 25L/h, and desolvation gas 800L/h. The MS data were collected in centroid mode. For mass accuracy, leucine enkephalin (555.2692 Da calculated monoisotopic molecular weight) was used as a lock mass. The lockmass scans were collected every 30 seconds and averaged over 3 scans to perform mass correction. Instrument calibration was performed using sodium formate prior to each ESI mode.

To equilibrate the system, ten conditioning QC samples were performed at the start of acquisition. The QC samples were run periodically after 10 sample injections to monitor instrument performance. Data-dependent acquisition (DDA) and MSE analysis of the QC sample was performed to obtain MS/MS information for metabolite annotation.

Refer to Table 2.2 for mobile solvents gradient conditions. High organic wash step duration was adjusted for elution completion of lipids, preventing their accumulation on the column. Three cycles of weak solvents (H_2O/ C_3H_8O, 90:10) and strong C_3H_8O solvent were performed in tandem with sample analysis to reduce the injection carry-overs.

Table 2.2 LC conditions.

<table>
<tr><th>Variable</th><th colspan="4">Conditions</th></tr>
<tr><td>Column</td><td colspan="4">Acquity CSH C_{18} (2.1 x 100 mm, 1.7 µm)</td></tr>
<tr><td>Column temperature</td><td colspan="4">55ºC</td></tr>
<tr><td>Sample temperature</td><td colspan="4">8ºC</td></tr>
<tr><td>Flow rate</td><td colspan="4">0.4 mL/min</td></tr>
<tr><td>Mobile phase A</td><td colspan="4">Acetonitrile/water (60:40 with ammonium formate and 0.1% formic acid)</td></tr>
<tr><td>Mobile phase B</td><td colspan="4">Acetonitrile 90:10 with 10mM ammonium formate and 0.1% formic acid</td></tr>
<tr><td>Injection volume</td><td colspan="4">5 µL (full loop)</td></tr>
<tr><td>Run time</td><td colspan="4">20 minutes</td></tr>
<tr><td rowspan="9">Gradient</td><td>Time (min)</td><td>% A</td><td>% B</td><td>Curve</td></tr>
<tr><td>Initial</td><td>60</td><td>40</td><td>Initial</td></tr>
<tr><td>2.0</td><td>57</td><td>43</td><td>6</td></tr>
<tr><td>2.1</td><td>50</td><td>50</td><td>1</td></tr>
<tr><td>12.0</td><td>46</td><td>54</td><td>6</td></tr>
<tr><td>12.1</td><td>30</td><td>70</td><td>1</td></tr>
<tr><td>18.0</td><td>1</td><td>99</td><td>6</td></tr>
<tr><td>18.1</td><td>60</td><td>40</td><td>6</td></tr>
<tr><td>20.0</td><td>60</td><td>40</td><td>1</td></tr>
</table>

2.9.2.4 MS data pre-processing.

The data set was separated into subgroups according to their outcomes following exposure to HCV. The UPLC-MS raw data was acquired using MassLynx software version 4.1 (Waters, Manchester, UK) and converted in NetCDF files using Databridge; a module within MassLynx software 4.1. The CDF files were pre-processed using XCMS package within the R statistical software version (Rx64 3.2.5), and in-house developed scripts.

2.9.2.5 Multivariate statistical analysis.

The data was subjected to multivariate statistical analysis using SIMCA-P (version 14.1, Umetrics, Umeå, Sweden). Principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) were performed on all data after pareto scaling and log transformation for detection of patterns, trends and outliers; and construction of discriminant models for classification and the discovery of potential biomarkers respectively.

2.9.2.6 Filtration and peak identification.

The generic ‘findpeaks’ function in XCMS was used to find peaks and processed them in batch mode, stored, aligned and grouped them for further analysis. Table **2.3** below provides a summary of different parameters used for peak picking in centroid mode to enable sensitive detection of low intensity peaks. Filtering of peak intensity for different lipids allowed exclusion of unnecessary signals (drifts and noise) derived from the data.

Table 2.3 Data pre-processing, and adjustment of parameters.

Parameter	Abbreviation used	Description	Values set (ESI +)	Values set (ESI -)
CentWave	centWave	Peak detection algorithm in centroid mode. It detects features based on their intensity and wavelet. Detects overlapping peaks and close-by-peaks as well	Default	Default
Noise	noise	centroids with intensity < noise are omitted from regions of interest (ROI) detection	50	5
Parts per million	ppm	The maximal tolerated error or *m/z* deviation between actual mass and measured mass in consecutive scans	35	50
Prefilter =c	c(k,l)	Mass traces are only retained if they contain at least x scans with intensity y	(8, 100)	(5, 30)
Signal/Noise ratio	snthresh	Signal/Noise ratio: ([maximum peak intensity] - [estimated baseline value]) / standard deviation of local chromatographic noise	10	10
Peakwidth =c (min, max)	peakwidth	Minimum and maximum chromatographic peak width detectable (seconds)	4, 20	4, 25
Integrate	integrate	Integration type (1=on bounds decided by waves, 2=on raw data)	2	2
nSlaves	nS	Number of computer core processors	3	3
minfrac	minfrac	Minimum fraction of samples necessary in at least one sample group for it to be a valid group	0.2	0.2
minsamp	minsamp	Minimum number of samples necessary in at least one of the sample groups for it to be a valid group	0	0
Bandwidth	bw	Bandwidth (standard deviation or half width at half maximum) of gaussian smoothing kernel to apply to the peak density chromatogram	2	6
mzwid	mzwid	Width of overlapping m/z slices to use for creating peak density chromatograms and grouping peaks across samples	0.01	0.01

The identified peaks were matched and grouped using a default 'density-based approach' algorithm in XCMS. This density algorithm groups is capable of grouping peaks in all samples using *m/z* bins, calculates smoothed peak distributions in chromatographic time. Some of the parameters that were adjusted included: minfrac, minsamp, bw, and mzwid as presented in Table **2.3**. The XCMS software has the ability to identify well behaved peaks and differentiate them from missing peaks, and improperly grouped multiple peaks from a large fraction of samples, in order to calculate the median retention time, and a deviation from that median. 'Rector' generic method and 'loess'default algorithm within XCMS was applied on smoothed deviations for correction/alignment of retention time. Furthermore, normalisation technique helps to remove sources of systematic variations between samples as a result of factors that are not related to the biological processes. Finally XCMS generated files in .csv format which was transposed on SIMCA-P for multivariate analysis.

2.9.2.7 MS/MS metabolite annotation.

The candidate metabolites were annotated using accurate *m/z* values, fragmentation patterns, retention times, and the METLIN database (https://metlin.scripps.edu/). The database contains information related to the *rt* and *m/z* value of the precursor ion. The lipidomics moieties annotation procedure involved selection of a valid OPLS-DA model in pareto scaling.

The lipidomics features of chromatographic peaks were extracted in order to further identify the lipid compounds that distinguished the EUs from the comparator groups. Following OPLS-DA analysis, the results of S- and Variable Importance for the Projection (VIP)-plots were assessed and the database provided for retention time (rt), m/z (mass over charge ratio), and MS/MS data and was verified by the raw data. Significant lipid features were generated from the VIP plots using a VIP cut-off of 1.5

that showed confidence intervals for the VIP values at the 95% level. The VIP plot summarizes the importance of the variables both to explain X and to correlate to Y. VIP-values larger than 1 indicated “important” X-variables, and values lower than 0.5 indicated “unimportant” X-variables. The VIP plot was sorted from high to low, and showed confidence intervals for the VIP values, normally at the 95% level. In this study VIP features from 1.5 and above indicated important variables. All unsorted VIP values were copied to an Excel spread sheet ensuring that the VIP values were greater than VIP (cvSE), and data ranked according to VIP values. The data was filtered and sorted according to the retention time. Using the raw chromatogram data organised in peak intensity of lipids eluted at the same retention time, the MS/MS data was derived by mining the peak intensity. The MS/MS fragmentation pattern was observed using the data dependent analysis (DDA), SURVEY, and MSE. Peak detection was based on their intensity and wavelet. The peak height and area were defined following conditions described in Table **2.3**.

2.9.2.8 Principle of LC-MS.

Figure 2.4: Schematic diagram of the principle of ESI LC-MS.

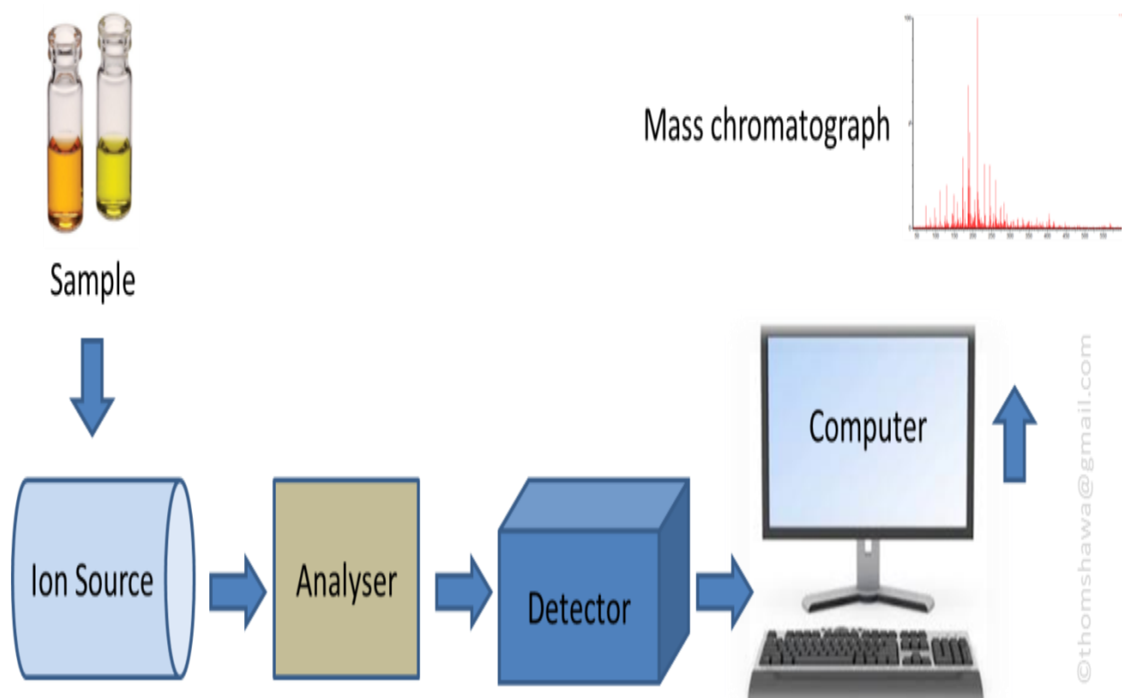


Figure **2.4** shows a schematic representation of the basic components of a mass spectrometer.

An ESI LC-MS is used for production of ions in a sample by using electrospray and is composed of three basic components namely: Ion source, mass analyser, and a detector. The ion source is kept at atmospheric pressure, and uses a pressure gradient and voltage gradient to help pump out ions from the source to the detector. When the sample is injected in the instrument, and high electric voltage is applied, the molecules are bombarded with a beam of energetic electrons from the ion source. The ESI accelerates the movement of ions from the liquid solution to gaseous phase ions (electron ionisation). Ionisation requires solvent evaporation; therefore two different mobile

phase solvents are prepared by addition of water, acetic acid to decrease the droplet size and increase conductivity, as well as providing protons to facilitate the ionisation process. The ions are separated according to their mass-to-charge ratio (*m/z*) by the mass analyser via ion optics that help the ion beam stream to maintain a stable flight (trajectory) of the ions. The ESI enables molecular ions to undergo fragmentation. The electrical/magnetic field is created that accelerates the ions through the flight tube toward the detector systems to measure their concentrations. The magnetic field subjects samples of same masses to deflect with the same magnitude according to their *m/z*; big ions travel slowly whereas light ions arrive at the detector first. Since ions are very reactive and have short life, ion optics for MS are kept in high vacuum achieved by use of oil diffusion pumps or turbomolecular pumps. The sample flow rate is enhanced by usage of nitrogen gas (nebulising gas). Due to evaporation of mobile phases, the charged droplets in a mist reduce their size, that result in reduction in their radius, and increased surface charge density (Konermann *et al.*, 2013). The charged particles are detected by the instrument systems detector that converts the ions into electrical signal. The signals are processed and transmitted to the computer that acquires and compares the spectra data to reference library. The results are displayed as a mass chromatographic spectrum. Mass spectrum is a graphical plot of signals of ions in relation to their *m/z*, which is used to determine the mass of metabolites in a sample, and helps to predict the structural arrangement of the molecules. Each ion's ratio of mass to charge (*m/z*) determines the molecular mass of the ion.

2.9.3 Nuclear Magnetic Resonance metabolomics.

The urine samples were collected in Plymouth as described in Section 2.2.4.3, and was transported on dry ice to the Institute of Hepatology in London.

2.9.3.1 NMR data acquisition.

The NMR sample preparation was performed by Dr Antonio Riva of the Institute of Hepatology according to the literature (Dona *et al.*, 2014). The NMR sample processing was performed in the department of Chemistry at University College London (UCL). All data acquisition and analysis was performed by this student researcher.

2.9.3.2 Proton NMR spectral acquisition.

The samples were prepared into 5mm NMR tubes for NMR study based on previously published standard methodology (Beckonert *et al.*, 2007; Dona *et al.*, 2014). The urine samples were analysed in a random order at the Department of Chemistry, University College London. The NMR spectra were recorded on a Bruker Avance III 600 NMR spectrometer operating at proton NMR frequency of 600.13 MHz equipped with a 5 mm DCH cryoprobe. The samples were placed in a sample queue at 21 °C on the auto sampler and some samples may have remained in the queue for up to 6 h before NMR analysis.

The data acquisition and processing were performed using standard TopSpin (version 3.2) software. The NMR spectra were recorded at 300 K using a standard pulse sequence *noesygppr1d* with water presaturation during relaxation delay (Dona *et al.*, 2014). Four dummy scans were used for equilibration followed by 64 scans collected into 144K points with a total repetition time of 8.0s at each scan (acquisition time = 4.0 s; relaxation delay = 4.0s). The NMR spectra were processed using the Bruker AMIX

data processing package and the KnowItAll Informatics System v9.0 (Bio-Rad, Philadelphia, PA). The Free Induction Decays were zero-filled and an exponential 0.3 Hz line-broadening function was applied before Fourier transformation.

All the NMR spectra were automatically phased and a baseline correction was applied. The trimethylsilyl propanoic acid (TSP) peak was assigned to be at δ 0.00 ppm for an internal chemical shift reference. The NMR peaks in the range δ 0.50-9.50 ppm were analysed, although the region δ 4.50-6.40 ppm was excluded to remove the residual water signal and also the signal from urea. The urinary NMR peaks were assigned to metabolites on the basis of chemical shifts and coupling patterns and with reference to the published literature (Wishart *et al.*, 2007, 2013, Shariff *et al.*, 2010, 2011; Heinzmann *et al.*, 2012; Bouatra *et al.*, 2013; Ladep *et al.*, 2014).

2.9.3.3 Multivariate data analysis.

The resonances in the urinary NMR spectra were assigned using online databases (for example the Human Metabolome Data Base (HMDB) http://hmdb.ca/) (Wishart *et al.*, 2007) and according to published literature (Holmes *et al.*, 1997; Lindon, Nicholson and Everett, 1999; Trygg, Holmes and Lundstedt, 2007). The multivariate analysis was performed by BioRad KnowItAll® Informatics System version 17.0. The water and urea region (δ 4.50 to 6.40 ppm) was excluded from the analysis as this region was distorted by the water suppression technique. The complex urinary NMR spectra were sub-divided into 'Buckets/Bins' (smaller regions) using 'intelligent bucketing' algorithm. The following regions were specifically identified prior to principal component analysis (PCA): 7.80 – 7.88 ppm (hippurate), 7.60 – 7.67 ppm (hippurate), 7.53 – 7.58 ppm (hippurate), 4.04 – 4.05 ppm (creatinine), 3.04 – 3.05 ppm (creatinine), 3.97 – 4.01 ppm (hippurate), 2.65 – 2.73 ppm (dimethylamine (DMA)), and 2.51 – 2.55 ppm (citrate). The PCA was used to identify outliers and group clustering.

2.9.3.4 Statistical comparison of discriminating metabolites.

The comparison between creatinine, hippurate and trimethylamine N-oxide (TMAO) for study groups was assessed by performing One-way analysis of variance (ANOVA) to analyse the differences of urinary metabolites among EU, SR, CHCV and HC groups. Bonferroni's Multiple Comparison Test was performed using GraphPad Prism-5, and statistical significance was accepted at $p < 0.05$.

2.9.3.5 Principle of NMR technique.

Figure 2.5: Basic principle of H NMR spectroscopy.

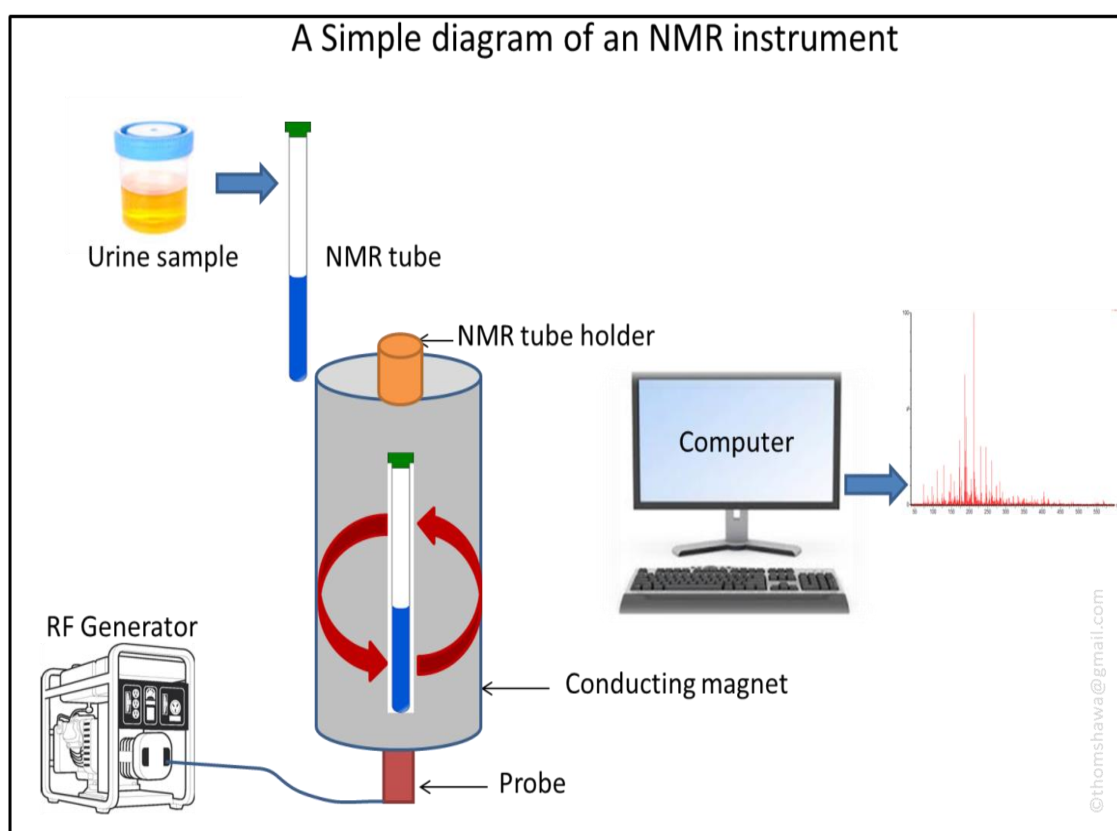


Figure **2.5** shows the basic illustration of an NMR instrument. The conducting magnet generates an external magnetic field rotating around the sample. The probe subjects the urine sample to RF frequencies generated externally by the RF generator. The continuous excitation and relaxation of the sample creates NMR signals that are forwarded to the computer that processes the data and presents NMR spectrum.

The NMR spectroscopy is an analytical chemistry technique used for determining the concentration and molecular structure of metabolites in a sample. The principle for NMR is that nuclei of elements or compounds have spin and all nuclei are electrically charged. When the sample is placed in an NMR tube and subjected to an external magnetic field, the protons spin and align in one direction. The radiofrequency (RF) electromagnetic radiation is applied that sends electromagnetic waves to the sample that excites the protons to move from the lower energy level (α-state) to the higher energy level (β-state) between a single energy gap. When the RF is reduced, the protons relax back to the base level (relaxation) that emits energy generated as an NMR signal. Atoms with even mass number and even atomic number do not possess nuclear spin and become NMR invisible; whereas atoms with odd mass number have spin I and they behave like a magnet and will give NMR signal. The nuclei when they are in magnetic field absorb and emit electromagnetic radiation. The energy that drives the vibrating atoms in oscillation is referred to as resonance. Therefore the frequency of resonance depends on the magnitude properties of the atoms as well as the strength of the magnetic field created. Since distinct protons exist in different chemical environments, thus a different amount of energy is required to bring nuclei in different environment into resonance. This results in different protons generating different NMR signals. The energy transfer generates some signals or peaks that are processed to produce NMR spectra to represent the amount of energy that brought the nuclei in the sample into resonance (reviewed in (Schanda and Ernst, 2016).

2.10 Statistical analysis.

Statistical calculations were performed using GraphPad Prism version 5.01 (GraphPad software Inc. USA) and ‘R’ statistical software version (Rx64 3.2.5). To further evaluate the significance of these findings, One-way analysis of variance (ANOVA - Kruskal-Wallis test), and the t-test (Mann Whitney test and/or Wilcoxon signed rank test) were used to determine the differences among the means when comparing several groups or just two groups respectively. A p.value of <0.05 was considered significant.

3 CHAPTER THREE

3.1 Urinary metabolic profiling of HCV exposed uninfected injection drug users.

3.2 Background.

The liver serves as the main metabolic and biosynthetic organ (Mitra and Metcalf, 2009) and therefore infection by HCV may cause a range of metabolic changes that can be identified not only in the liver itself, but also in blood and urine (Bundy, Davey and Viant, 2009; Nicholson *et al.*, 2012). A highly detailed metabolic profiling of body fluids using either nuclear magnetic resonance spectroscopy (NMR) or mass spectrometry (MS) techniques is an emerging scientific discipline and there remains considerable scope for improving the definition of the metabolic phenotype in liver disease (Wishart *et al.*, 2013).

The urine is a sterile biofluid produced by the kidneys and useful for metabolic profiling studies (Bujak *et al.*, 2011) as it is the primary route for elimination of water-soluble waste products (Bouatra *et al.*, 2013). Urine has been recognised for centuries as an easily accessible human sample for the identification of certain disease-specific compounds (Nicholson and Lindon, 2008; Echeverry, Hortin and Rai, 2010), but it is only recently that highly detailed chemical analysis has become possible using high-field NMR spectroscopy or liquid chromatography mass spectrometry (LC/MS) (Law *et al.*, 2008; Navarro-Muñoz *et al.*, 2012). Urinary metabolome identification may be further improved by performing a combination of these analytical methods (Yang *et al.*, 2008). The urinary metabolites represent end products of both normal or pathological cellular process, and such metabolites are closely linked to the subject's phenotype (Bouatra *et al.*, 2013). It is an advantage that urine sample collection is non-invasive and requires little, if any, subject preparation (Zhang *et al.*, 2012) and may therefore

provide real-time information about the disease state (Beckonert *et al.*, 2007). Urinary metabolic profiling studies can be affected by external confounding factors such as diet, lifestyle and medication (Lenz *et al.*, 2003) and, importantly, may also reflect alterations in gut microbiota. A number of statistical tools provide rapid and reliable analysis of complex NMR spectra that allow extraction of trends and patterns (Lindon *et al.*, 2000).

To date little is known about urinary metabolomic profiling in HCV infection. The urinary NMR profiles able to discriminate cirrhosis and hepatocellular carcinoma (HCC) have been described in an African population (Shariff *et al.*, 2010, 2011; Ladep *et al.*, 2014) and more recently in a UK population where multivariate analysis models (with a sensitivity and specificity of 53.6% and 96% respectively) reported reduced creatinine, citrate and hippurate and elevated carnitine that were comparable with the African studies (Shariff *et al.*, 2016).

I therefore hypothesise that there are host metabolic determinants involved in interrupting the HCV life cycle that could potentially confer resistance to HCV exposed uninfected cohort. Therefore, determination of systemic metabolic alterations associated with HCV exposed but uninfected cases may provide information on factors that confer resistance to HCV infection. The urine metabolic profiling studies in the EU cases may provide such insight.

3.3 Results.

3.3.1 Demographics of the study subjects.

Baseline demographics and risk behaviour characteristics for the recruited IDU cases are summarised in Table **3.1**. There were no significant differences in age or duration of injection use between IDU groups. All subjects were Caucasian and the majority were male. The median age for the healthy control group was 26.1 years and the age range was 21 – 39 years.

Table 3.1 Demographics and injection history for study groups.

Variable	**EU (n=38)**	**SR (n=8)**	**CHCV (n=9)**
Gender (Female)	9	3	1
Gender (Male)	29	5	8
Age, Range (years)	23 - 58	33 - 53	29 - 50
Age, Average (years)	33.5	37.6	36.3
Age, Median (years)	32	36	36
Caucasian	38	8	9
Duration of injection use, Range (years)	1-34	2-25	13-30
Duration of injection use, Average (years)	11.7	13.1	17.5
Currently injecting	34	6	7
Sharing behaviour (n)			
Sharing around others	36	7	9
Shared needles	31	7	9
Shared syringes	30	7	8
Shared other drug injecting paraphernalia	35	7	9
Shared with someone known to have HCV	10	3	2
Other risk factors:			
Had blood transfusion before	4	1	3
Had tattoos	32	7	8
Had body piercing	29	6	7
Had acupuncture before	8	4	3
Sex with HCV infected person	6	2	1
Family member injects drugs	22	5	3
Imprisoned before	28	6	7
HCV testing results:			
HCV antibody testing	Negative	Positive	Positive
HCV RNA results	Negative	Negative	Positive

3.3.2 Urinary NMR multivariate analysis.

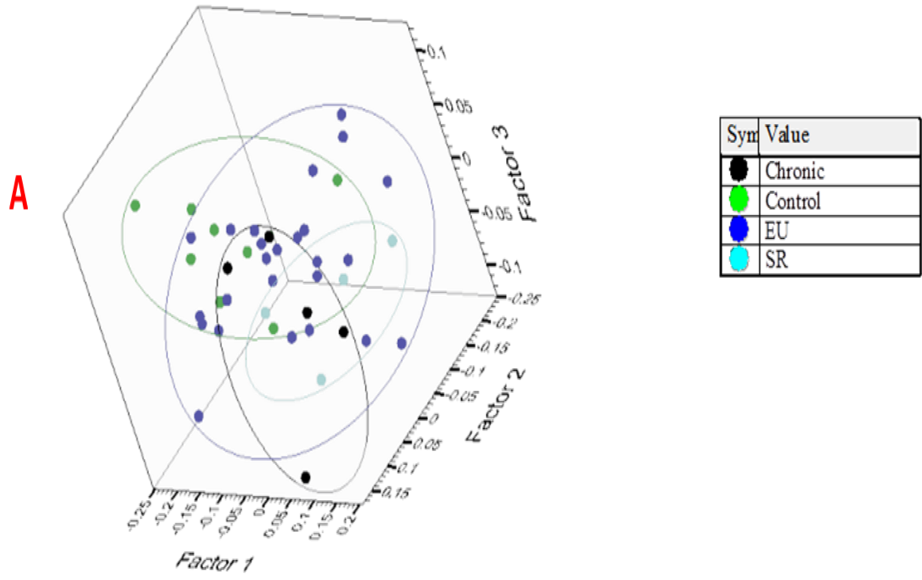
The total number of the EUs cases were 38 but 2 individuals (study identification numbers SW575 and SW584) did not give urine samples. Ten urine samples from healthy volunteers were provided by the University College London. Thus, a total of 63 samples comprising the 36 EUs, 8 SRs, and 9 CHCV (all recruited in Plymouth) and 10 healthy control (from UCL) samples were analysed for the urinary NMR metabolomics.

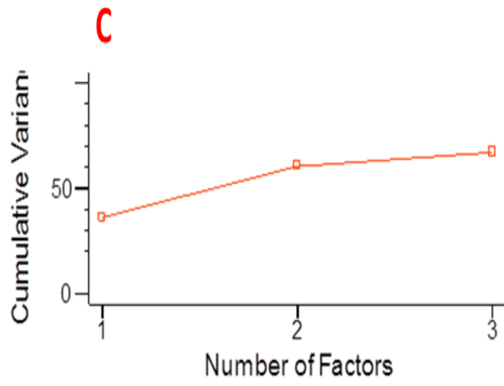
Whilst urinary NMR data from all subjects showed good spectral resolution (linewidths of $\leq$1Hz) and quality, 21 of 63 HCV data sets (20 cases from Plymouth and one control from UCL) were excluded for various confounding factors (dominant signals from ethanol (5 EU, 1 SR, 3 CHCV); comparatively strong signals from taurine (3 EU); multiple peaks from mannitol (3 EU, 1 SR); resonances from 2-hydroxybutyrate (1 CHCV); high levels of paracetamol metabolites (1EU); extremely high TMAO levels in a fish eater (1 healthy control), and unassigned peaks (1EU, 1 SR).

The final cohort for multivariate NMR analysis therefore comprised 23 EU, 5 SR, 5 CHCV, 9 controls. The NMR spectra showed wide range of metabolites resonances observed in the spectra. The principal component analysis was performed to assess the clustering and presence of outliers.

Figure 3.1 below shows the clustering of study groups in a 3D format.

Figure 3.1: Urinary NMR class separation.





B

EU	SR	Chronic	Control	
	0.53	0.42	0.89	EU
0.53		0.15	0.92	SR
0.42	0.15		2.62	Chronic
0.89	0.92	2.62		Control

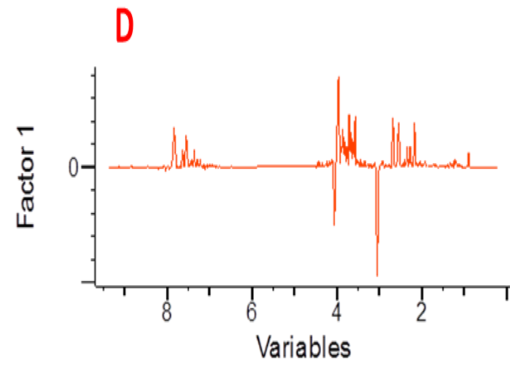


Figure 3.1 (**A**) shows a 3D factor scores plotted based on the ^{1}H NMR spectra of urine obtained from the EUs, SRs, CHCV patients, and healthy control groups. Factor analysis is a variable reduction method used for the identification of observed computed variables. In this 3D plot, the factor analysis assumes that the covariation in the observed variables is due to the presence of one or more latent urinary metabolic variables (factors) that exert causal influence on these observed variables. The factors 1, 2, and 3 estimations are mathematically computed (done by the statistical software), presented in a 3D format and estimated by a linear combination of the observed variables (e.g. hidden metabolites). (**B**) shows the urinary NMR class separation ranked statistically by the KnowItAll Informatics System v9.0 (Bio-Rad, Philadelphia, PA) software. The higher the number in class separation the greater the possible difference between groups (e.g. the EUs vs SR class separation is 0.53). The high difference was observed when the EUs were compared to the HCs followed by SRs and CHCV. (**C**) shows the number of factors scored in a 3 D format. (**D**) shows an example of a chromatogram for factor 1 variables with creatinine peaks (3.03s – 4.03s) as reference points.

3.3.3 The Urinary NMR spectra.

The forty two NMR spectra were of good quality and were usable. To measure the quality of the data, the line with NMR reference standard (TSP) (δ 0.00 ppm) was measured (0.5 – 1.0 Hz width). The normal urine NMR spectra contain the following metabolites: the creatinine (3.05s – 4.06s), hippurate (3.98d, 7.55t, 7.64t, and 7.84d), creatinine (3.03s – 4.03s), glycine (3.55s), trimethylamine N-oxide (TMAO) (3.27s), dimethylamine (2.72s), citrate (2.55d and 2.7d), acetate (1.92s); all are endogenously synthesised.

Figure 3.2: Urinary NMR spectrum.

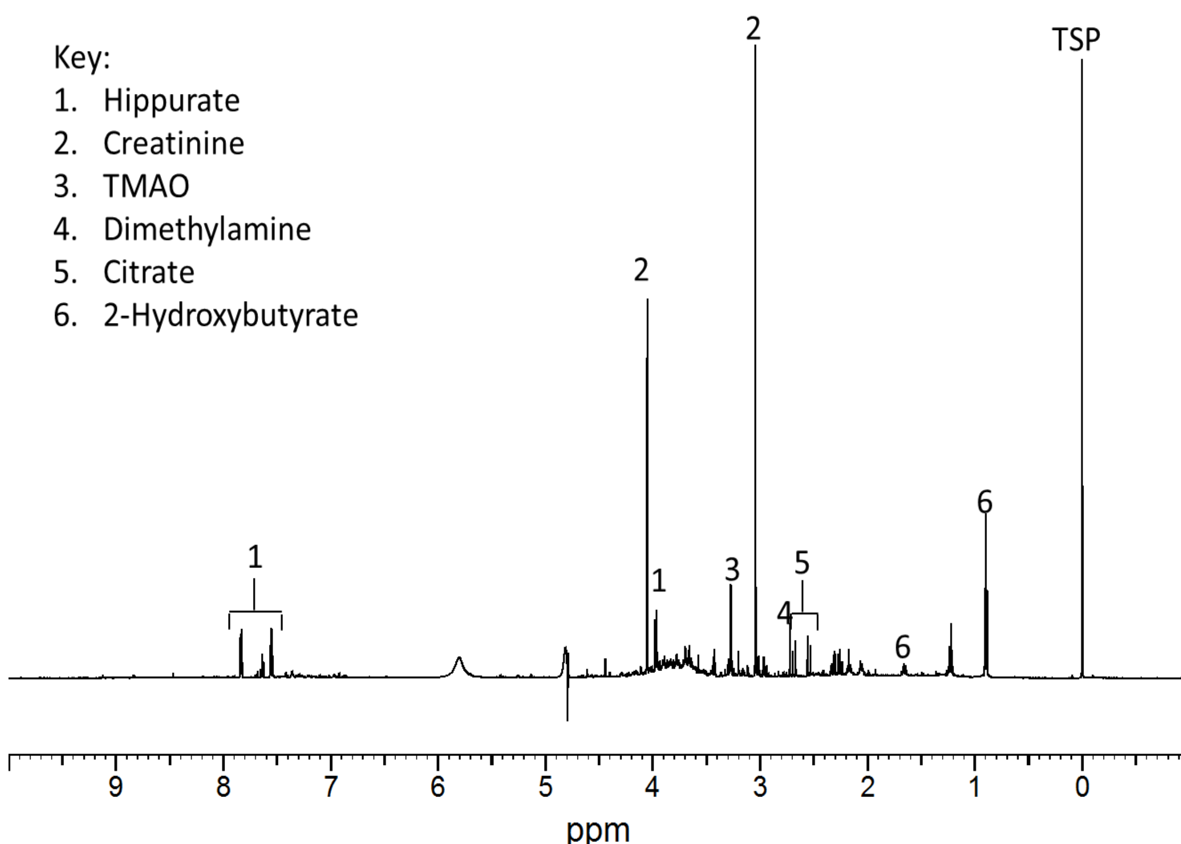


Figure **3.2** shows the urinary NMR spectrum for subject SW580. Apart from the normal urine constituents observed in the NMR spectra, additional components were observed from the whole dataset such as; ethanol (1.2m and 3.7m), paracetamol metabolites (7.00 – 7.40m and 2.10 – 2.30t ppm), mannitol (3.60m –3.95 ppm), taurine

(3.40s – 3.44 ppm), 2-hydroxybutyrate (0.85s – 0.95 ppm), scyllo-inosotol (3.35 – 3.4 ppm), and unassigned peaks (1.10 – 1.20s ppm and 1.15d ppm). The peak position(s) depends on the chemical structure of the metabolite. The peak height relates to concentration of the metabolite. The numbered peaks represent the matbolites on the NMR spectrum (e.g. creatinine fingerprint has two peaks on separate positions). ppm = parts per million (in relation to the ^{1}H chemical shift). The line with NMR reference standard (trimethylsilyl propanoic acid (TSP) was assigned δ 0.00 ppm for an internal chemical shift reference. **s** = singlet, **d** = duplet, **t** = triplet, **m** = multiplet.

3.3.4 Urinary NMR confounding metabolites.

Assignment of the candidate urinary metabolites was based on the web-accessible reference databases of authentic compounds such as Human Metabolome Data Base (HMDB)–http://hmdb.ca/ and urine metabolome database (UMDB) – http://www.urinemetabolome.ca.

Out of 63 samples, 21 (33.3%) had possible confounding metabolites (appendix B); that were excluded from the analysis. The following were the confounders: (dominant signals from ethanol (5 EU, 1 SR, and 3 CHCV); strong signals from taurine (3 EU); multiple peaks from mannitol (3 EU, 1 SR); resonances from 2-hydroxybutyrate (1 CHCV); increased paracetamol metabolites (1 EU); one control data set from a healthy control (known fish eater) was excluded due to high TMAO; and unassigned peaks (1 EU, and 1 SR). The final study cohort for multivariate analysis therefore consisted of 42 subjects (i.e. 23 exposed uninfected cases, 5 spontaneous resolvers, 5 chronic HCV cases, and 9 cases from individuals without history of HCV exposure or liver disease). The median age for healthy control samples was 26.1, whereas the mean was 24; and the age range was 21 – 39 years.

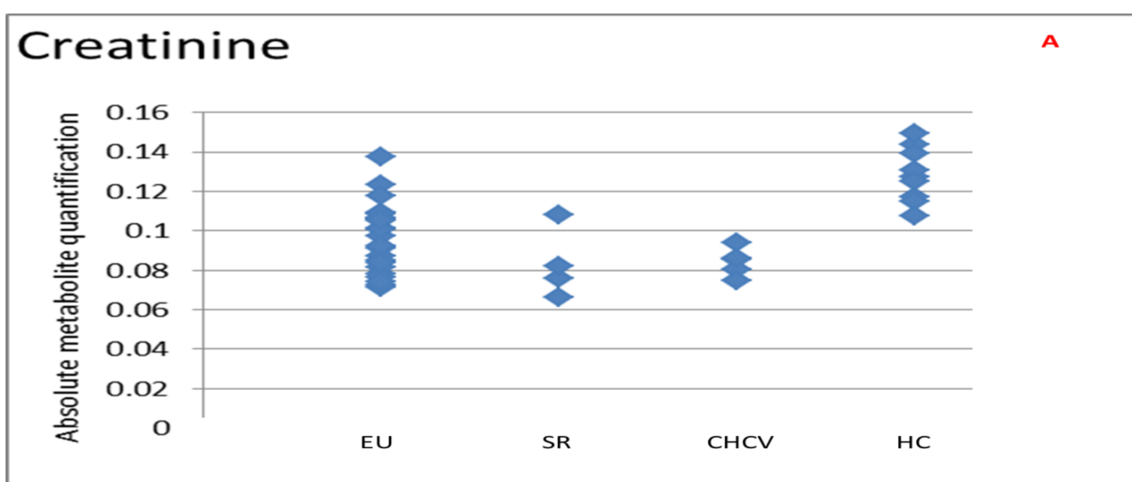
3.3.5 NMR metabolites annotation.

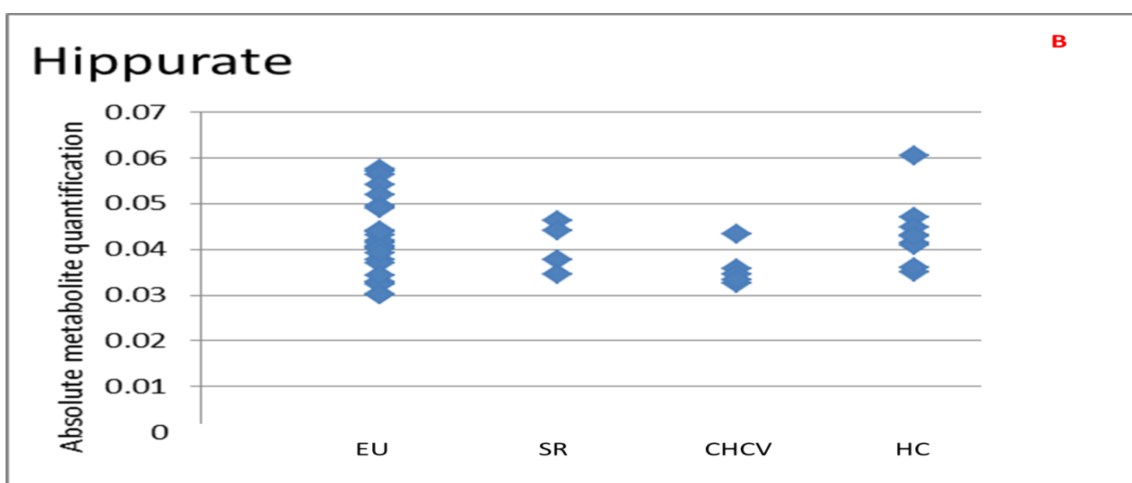
Low citrate levels were observed in 5 out of 63 subjects (7.9%) and were all predominantly males (4 EU and 1 SR). The most prominent peaks recorded in the 42 samples were creatinine, hippurate, glycine, DMA, citrate, and TMAO. Refer to Figure 3.2 for citrate position and fingerprint on the NMR spectrum.

3.3.6 Comparison of discriminating metabolites.

To investigate the influence of discriminating metabolites on the data, the concentrations of creatinine, hippurate and TMAO were determined (Figure **3.3**). The concentrations of all the three discriminating urinary metabolites were high as compared to other urinary metabolites in the NMR spectra. Creatinine levels were elevated in HC as well as in EU group. The CHCV group had the least levels of creatinine and hippurate but slightly elevated TMAO levels.

Figure 3.3: Concentrations of discriminating metabolites.





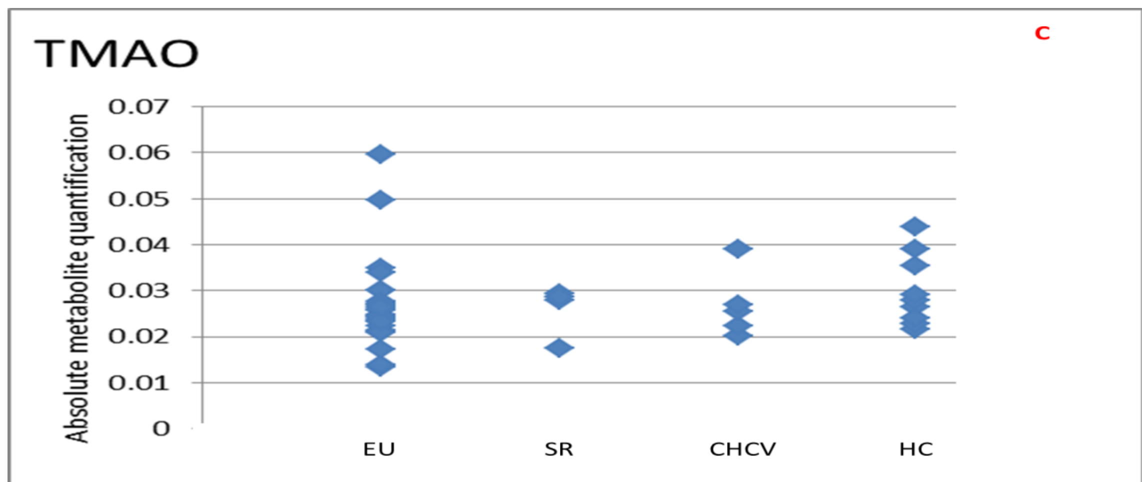


Figure **3.3** shows the different absolute levels of selected metabolites among the four study groups. Each blue block on the graph represents a sample. The differences in absolute hippurate and TMAO levels were not significant.

3.3.7 One-way analysis of variance (ANOVA).

I analysed the differences of urinary metabolites among EU, SR, CHCV and HC groups by comparing the creatinine, hippurate, and TMAO levels. Bonferroni's Multiple Comparison Test was performed and statistical significance was accepted at $p < 0.05$. The mean creatinine concentrations were significantly different between EU vs HC (95% confidence interval (CI) -0.04650 to -0.01589); SR vs HC (95% CI of difference -0.06890 to -0.02158); and CHCV vs HC (95% CI of difference -0.06606 to -0.02214).

The one-way anova t-test statistical differences were again observed for creatinine between EU vs HC (Wilcoxon signed rank test p.value < 0.0039) (Figure **3.4**).

Figure 3.4: Comparison of hippurate, creatinine, and TMAO among EU, SR, CHCV & HC study groups.

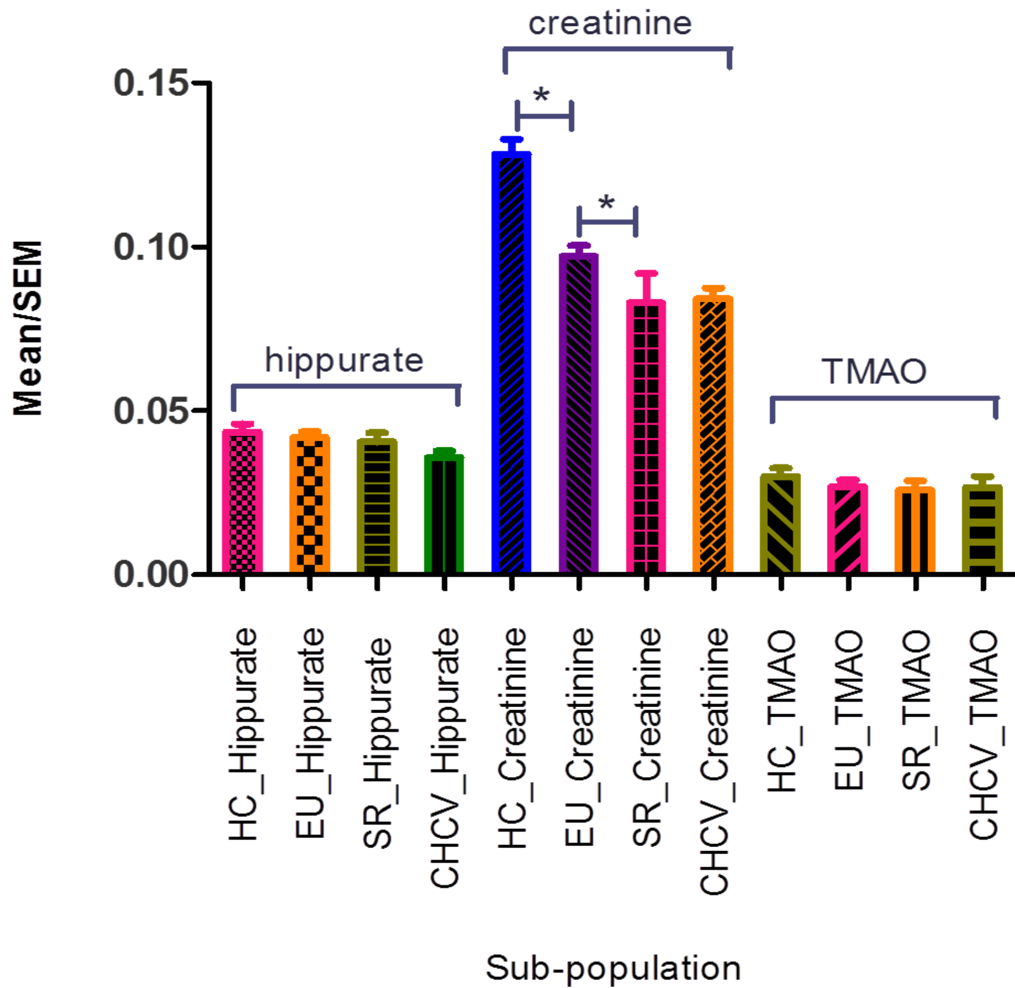


Figure **3.4** shows the different mean concentrations of hippurate, creatinine and TMAO among different study groups as analysed by one-way ANOVA. There was significant increase in creatinine levels observed between EU and HC (Wilcoxon signed rank test p.value < 0.0039), and HC vs CHCV (p.value < 0.001). No differences were observed in hippurate and TMAO levels among the four cohorts.

3.4 Discussion.

I studied differences in host metabolism by identification and quantification of urine metabolic biomarkers by panning strategy using 1H NMR spectroscopy in individuals with different outcomes from HCV exposure to seek metabolic correlates of resistance to HCV infection. This is the first report of urinary metabolomic profiling in exposed uninfected intravenous drug users and I have observed that the urinary NMR metabolic profiles have the potential to distinguish different study cohorts. Multivariate statistical analysis identified hippurate, TMAO, and creatinine as the possible discriminatory metabolites. Creatinine distinguished the EUs from the healthy control group. No differences were observed for hippurate and TMAO.

In this study, different metabolites were identified in NMR spectra that were associated with either diet, or biological metabolism. The urinary NMR data from all the subjects showed good spectral resolution (TSP linewidth of ≤ 1Hz) and were of high quality. However, 21 out of 63 data sets were excluded for a range of confounding factors.

3.4.1 Confounding metabolites.

Ethanol: The urinary NMR spectra revealed large peaks of ethanol (1.2m and 3.7m) in nine subjects (5 EU, 1 SR, and 3 CHCV). This demonstrates that ethanol can be excreted from normal humans following consumption of alcohol as much as 50 units per week. The few subjects who provided their dietary history, those who accepted drinking less than 18 units of alcohol per week did not show ethanol peaks on urine spectra. Therefore all subjects with reported high ethanol levels were excluded from the analysis.

Taurine is an amino acid synthesized endogenously in human adults, abundant in kidneys, brain, gallbladder, but largely dietary dependent (Cross, Major and Sinha, 2011)

especially consumption of food stuff derived from animal origin (Lissner *et al.*, 2007). Despite taurine's presence in animal produce, it is also an ingredient of some energy drinks. Lack of adequate dietary history in this study makes it difficult to suggest the source of taurine in this data. However, because it is strongly dietary related, it was excluded from the analysis. Excessive alcohol consumption is also related to taurine loss in urine and other metabolic disorders; this is consistent with our data where high ethanol levels were recorded in this cohort.

Mannitol is a sugar alcohol poorly absorbed by humans but its presence in urine is dietary related. It is present in fruits such as apples, pineapples, asparagus, and carrots (Kern *et al.*, 2003) and other foods such as eggs, meat, milk and fish. In this data, mannitol could possibly be an adulterant in injecting drugs. It is popularly known as 'baby laxative' among IDUs when it is used in this manner.

2-hydroxybutyrate: Urinary excretion of 2-hydroxybutyrate is usually associated with high alcohol consumption as well as physical exercise. 2-hydroxybutyric acid is also associated with ketoacidosis and lactic acidosis. Some studies reported increased 2-hydroxybutyrate as a result of microbial degradation of dietary proteins (Li *et al.*, 2008). In this study 2-hydroxybutyrate was high in two EU and CHCV male subjects that skewed the data. This did not demonstrate any specific functional differences between the study groups; therefore 2-hydroxybutyrate was excluded from the analysis since there was no control mechanism for confounders.

Paracetamol metabolites: PCA examination of the data set revealed some paracetamol peaks on urinary NMR spectra. All the samples save healthy controls showed some trace signals of paracetamol metabolites. High signals were observed in samples SW555, SW578, SW583 and SW586. All the four subjects were HCV exposed uninfected. Visual inspection of the urine spectra showed tiny peaks of paracetamol

that could not be identified as outliers by PCA analysis. Therefore, those EU samples were distinct outliers that skewed the data, and were excluded from the analysis. Paracetamol was reported as one of the commonest drugs involved in self poisoning episodes in the United Kingdom (Buckley *et al.*, 1995) which may suggest that the study participants might have used it as an analgesic. Paracetamol NMR resonances constitute four metabolites (sulfate, glucuronide, cysteinyl, and N-acetyl-cysteinyl conjugates). Glucuronides are the major paracetamol metabolites that are excreted in urine within 24 hours (Godejohann *et al.*, 2004).

Unassigned peaks: Two data sets were excluded from the analysis due to unassigned peaks (1.10 – 1.20s ppm and 1.15d ppm).

3.4.2 Discriminant metabolites on urinary NMR spectra.

Hippurate, creatinine and TMAO were the discriminant metabolites on the spectra.

Hippurate is a gut microbial co-metabolite of benzoic acid that is associated with diet and gut microbial activities. Gut microbes act upon aromatic compounds and polyphenols, and hydrolyse hippurate to benzoic acid and subsequently conjugates with glycine in mammalian mitochondria (reviewed in (Lees *et al.*, 2013)). Benzoic acid, and consequently hippurate is produced from metabolism of dietary components that are transformed through microbial and mammalian co-metabolism (Clifford *et al.*, 2000). Alterations in urinary hippurate levels have significantly been associated with gut microbial activities. Studies have reported that animals that are free from intestinal microorganisms do not excrete hippurate; but following environmental exposure hippurate becomes a dominant metabolite 2 – 3 weeks post-exposure (Nicholls, Mortishire-Smith and Nicholson, 2003; Claus *et al.*, 2008). The role of gut microbiota has been investigated through administration of oral antibiotics in animals with an

established gut microbiota. The findings suggest that antibiotic-induced suppression of gut microbiota reduce excretion of hippurate levels and its co-metabolites (Williams *et al.*, 2002; Yap *et al.*, 2008; Swann *et al.*, 2011).

Alterations in hippurate levels have also been described in HBV infection (Cox *et al.*, 2016) and as a urinary biomarker of Hepatocellular carcinoma where hippurate levels were significantly reduced in HCC subjects compared to healthy controls but was not significantly different compared to cirrhotic patients (Shariff *et al.*, 2011).

Creatinine levels were significantly increased in healthy controls compared to the three cohorts. Creatinine levels were also significantly higher in the exposed uninfected group compared to SR and CHCV patients. There was no statistical difference between SR and CHCV groups. Creatinine is formed through non-enzymatic breakdown of creatine in the muscle. Creatine is involved in muscular energetic metabolism that results in phosphocreatine. Creatinine excretion in the urine is largely dependent on the individual's physical activities and muscle mass (Baxmann *et al.*, 2008). In this data, creatinine gave rise to two singlet peaks (3.03s and 4.03s ppm) in the ratio of 3:2 according to their NCH_3 and CH_2 protons. I therefore speculate that the increased creatinine levels among healthy controls could be attributed to possible behavioural changes or increased muscle mass among healthy volunteers compared to injection drug users in whom poor nutrition is common. Objective assessment of dietary patterns among free-living individuals is challenging and has some limitations since the obtained information relies on self-reported dietary history (Garcia-Perez *et al.*, 2017). Dietary misreporting prevalence was estimated at 30 – 80% (Rennie, Coward and Jebb, 2007; Poslusna *et al.*, 2009) due to utilisation of assessment tools such as questionnaires, and dietary diaries (Garcia-Perez *et al.*, 2017). In this study I did not monitor the dietary history and body mass index (BMI).

TMAO was one of the potential metabolite that could be used in urinary NMR metabolomics to distinguish study groups. However, TMAO levels in EUs were not significantly different from the comparator groups. TMAO is a small amine oxide derived from dietary choline and phosphatidylcholine (Tang *et al.*, 2014; Kaysen *et al.*, 2015) through action of gut microbial activities. Red meat, eggs and dairy products are potential sources of TMAO because they are rich in choline, lecithin, and carnitine. Intestinal microbes metabolise dietary choline and convert it to trimethylamine (TMA) and then TMAO by the liver catalysed by hepatic flavin monooxygenases 3 (FMO3) (Koeth *et al.*, 2014). TMAO was reported to serve as a substrate in metabolism of anaerobic marine microbiota; therefore studies have reported increased TMAO levels in animals that were colonised with TMA-producing bacteria but not in animals that were colonised with bacteria that do not convert choline to TMA (Romano *et al.*, 2015). Therefore this information suggests that TMAO levels are determined by the alteration of gut microbiota, hepatic FMO enzymes, and diet.

Patients with advanced liver disease or HCC have reduced TMAO levels compared to healthy controls (Shariff *et al.*, 2011). Increased TMAO levels suggest a competent liver function through conversion of choline to TMAO. Therefore TMAO could be an important predictor of liver function through its ability to convert choline. There is no published data available about the clinical relevance of TMAO perturbation in pathophysiology of liver diseases. Gut microbial activities also affect TMAO levels in such a way that reduced microbial activities could potentially reduce TMAO levels and vice-versa.

3.4.3 Additional NMR metabolites.

Glycine fingerprints were also detected on NMR spectra from the majority of the majority of the samples. Glycine is a small non-essential amino acid found in human proteins synthesized from serine. Glycine is involved in several functions including protein synthesis; and it is an essential component of methylation reaction in cells (Ogawa *et al.*, 1998). Glycine was reported to be a bi-product of hippurate hydrolysis to benzoic acid by microbial flora which provides a useful association of this data to the involvement of gut microbial activities. Glycine conjugation of benzoic acid is one of the important factors that contribute to hippurate production (Gregus *et al.*, 1993). In this study, urinary glycine levels were increased in some subjects but not significantly raised in EU subjects to offer meaningful clustering in principal component analysis. Therefore increased glycine production in some EU subjects may have contributed to high hippurate levels observed in this study as a result of glycine conjugation of benzoate in mitochondria. This suggests the involvement of intestinal microbial flora in determination of urinary metabolites.

Dimethylamine (DMA) peaks were also detected on NMR spectra. DMA is an organic compound, a weak base that is involved in salt formation following reaction with acids. DMA endogenously inhibits nitric oxide synthase (NOS), an enzyme that catalyses the production of nitric acid. DMA levels were not significantly high or low in the study population. Therefore DMA levels could not be identified as a discriminant by PCA. However, DMA has exogenous sources related to diet and TMAO is one of the main sources that increase DMA urinary levels (Tsikas *et al.*, 2007) but in this study increased TMAO levels did not influence DMA since subjects who has high TMAO did not show increased DMA levels.

Citrate, a tricarboxylic acid, is a derivative of citric acid, a weak organic acid synthesised endogenously in mitochondria through the Krebs cycle (Krebs and Johnson, 1980); and its metabolic fingerprints were observed in the spectrum. A greater amount of citric acid is present in fruits such as lemon, orange, and vegetables. Citrate can also be transported out of the mitochondria and into the cytoplasm, where it is broken down into acetyl-CoA for production of fatty acid. Citrate levels were significantly reduced in all HCV exposed uninfected cohort, predominantly male subjects as opposed to other comparator groups. Studies in HCC patients revealed significant reduction in citrate levels in HCC patients as compared to healthy controls, non-significant reduction among healthy controls as opposed to cirrhotic patients (Shariff *et al.*, 2011). Citrate is a known factor that inhibits renal stone formation, therefore reduced citrate levels in HCV uninfected cohort may be associated with other mechanisms that could be associated with risk of kidney stone formation. The participants could have had other underlying pathological conditions that were not reported during participation of this study that implicated metabolites levels.

3.4.4 Study limitations.

Despite the interesting findings there are a number of limitations to this study. No standardised dietary history was taken and no dietary restrictions imposed before sample collection in this study; only 10 subjects submitted a dietary history for the 24 hours before sample collection. It is therefore possible that high levels of the possible discriminant urinary metabolites were dietary related rather than a marker of metabolic differences between the groups.

A large part of the original dataset was excluded from the full analysis due to presence of confounding factors; consequently reducing the sample size. As a result, our conclusions are largely observational and it is possible that they significantly concealed the trends and meaningful association between the identified metabolites and any consequence of biological differences.

3.5 Conclusion.

The urinary metabolic profiling have the potential to distinguish study groups. This study identified creatinine as the main urinary metabolite that distinguished the EUs from the control groups. There was no statistical difference observed for hippurate and TMAO metabolites. The computed analysis in multivariate analysis observed that hippurate and TMAO were slightly elevated but the differences were statistically insignificant.

The mechanism of resistance to HCV in exposed uninfected cases remains unclear, although a potential link between the gut microbiome and resistance to HCV infection using urine metabonomics approach may be of interest. At present these findings are unverified but warrant further study in additional cases with a clear dietary history and BMI evaluation. The study was limited by lack of dietary history and metabolic information. The sample numbers were reduced due to confounders; therefore the findings are observational and descriptive. Therefore this data paves the way for future studies in a bigger cohort to assess the link between gut microbial activities and resistance for HCV infection.

4 CHAPTER FOUR

4.1 Determination of serum apolipoprotein profiles and lipidomics in exposed uninfected cases compared to other groups.

4.2 Background.

The HCV life cycle is reported to be associated with lipoprotein metabolic pathways. HCV utilises virally encoded envelope glycoproteins, and cellular protein apolipoprotein E (ApoE) (Jiang *et al.*, 2012) and other apolipoproteins for attachment onto the host cellular receptors. The apolipoproteins are present on the outer surface of lipoprotein particles and play an important role in lipoprotein metabolism as well as ensuring adhesion of HCV into cellular receptors. The liver is the central platform for lipoprotein/apolipoprotein metabolism in the body. The triglycerides, cholesterol, and proteins are the main constituents of lipoproteins which transport lipids around the body. The protein component of lipoproteins is used to emulsify lipid/fat molecules. An essential stage in the HCV lifecycle is the formation of lipoviral particles that are associated with different lipoproteins (Dao Thi, Dreux and Cosset, 2011). The formation of LVPs possibly obscures the virus from host immune response, which may in turn help the virus to gain entry into the host cell (André *et al.*, 2005). Previous studies reported that reduced serum lipid levels in HCV infection are also associated with PEG-IFN treatment failure (Bassendine *et al.*, 2013). Since the ApoE is an important ligand for receptor mediated removal of triglyceride rich lipoproteins by the liver (Bassendine *et al.*, 2013), the determination of their levels in EU cohort would strongly link the purported resistance that has been observed in this unique cohort of IDUs. No published studies have reported the apolipoprotein profiles in HCV exposed uninfected cohort.

One of the strategies employed by HCV to escape immune surveillance and establish persistent infection is exploiting the lipid pathways. The production of infectious HCV is dependent on the export of VLDL by the hepatocytes and the HCV replication cycle depends on host lipid pathways (Bassendine *et al.*, 2011). The HCV interacts with host lipid metabolism at all stages of the viral lifecycle - from attachment and entry into hepatocytes, to replication and assembly of new viral particles. The circulating HCV is associated with lipoproteins as complex 'lipoviral particles' (LVP) (Piver, Roingeard and Pagès, 2010) which contain viral RNA, viral proteins, and host apolipoprotein constituents including apoB, apoE, apoA1, and apoC (Diaz *et al.*, 2006). Growing evidence suggests that the LVP lipid and apolipoprotein components facilitate viral attachment to host cells by binding to cellular lipoprotein receptors and that the exchangeable ApoE appears to be crucial for infectivity at the attachment step and at masking envelope glycoproteins from neutralising antibodies (Fauvelle *et al.*, 2016). The HCV association with lipoproteins may also be one means by which circulating HCV can avoid immunoglobulin recognition and evade the host's immune surveillance. Given this co-dependency on host lipid pathways, any mechanism that would disrupt the LVP formation would potentially reduce the viral infectivity and influence outcome following HCV exposure (Shawa, Sheridan, *et al.*, 2017).

I carried out apolipoprotein profiling to assess if there were significant differences in apolipoprotein profiles in HCV exposed uninfected cases as opposed to the comparator groups. Therefore, I considered the whole lipidomics investigation to observe if there are lipid profiles distinct in EUs.

Furthermore, LC-MS allows for a highly detailed and quantitative analysis of all the lipid constituents found in the serum; and allows distinguishing between them due to its high sensitivity and stability. The analysis of serum lipid perturbations using this

allows us to interrogate and better understand the mechanisms associated with HCV resistance. Either fasting or non-fasting blood samples are less important in this method in view of previous studies that did not show significant differences in lipids, lipoproteins and apolipoproteins profiling between non-fasting and fasting serum samples with the exception of triglycerides, which were higher in non-fasting state (Nordestgaard, 2009). Therefore I performed serum lipidomics on non-fasting samples as described in the methods chapter (Chapter 2). Given the importance of lipid pathways for all stages of the viral life cycle, we have investigated whether variation in the lipidome of apparently HCV-resistant individuals may be associated with the EU phenotype.

4.3 Results.

In this Chapter I have taken two approaches investigating the association between perturbations in lipid metabolism and HCV resistance. First, I have studied serum apolipoproteins, and secondly serum lipidomics profiling on non-fasting samples.

The lipidome of 386 serum samples were analysed comprising 60 EU cases (collected in Plymouth), 36 SR (16 cases collected in Plymouth, whereas 20 additional cases were provided by HCVRUK), 159 CHCV patients (9 collected in Plymouth, the rest were from HCVRUK), 100 SVR samples from HCVRUK, and 31 healthy controls (all from Plymouth). All samples were stored in John Bull research laboratory, and later transported to London on dry ice. Refer to Chapter 2 for details of sample collection, processing, storage, transport and analysis.

Serum apolipoprotein levels were measured by our collaborators at University of Newcastle. I went to Imperial College London for training and performing lipidomics experiments using LC-MS. There were no statistical apolipoprotein differences in EUs and the other groups. Serum lipidomics findings were relevant and offered more meaningful relationships.

4.3.1 Study subjects for apolipoprotein profiling.

Apolipoproteins A1, apoB, and apoE levels in a total of 61 non-fasting serum samples were studied. They comprised 22 HCV exposed uninfected, 8 spontaneous resolvers, and 31 healthy controls. No significant differences were observed in apolipoprotein levels among all study cohorts.

Figure 4.1: Apolipoprotein levels in different cohorts.

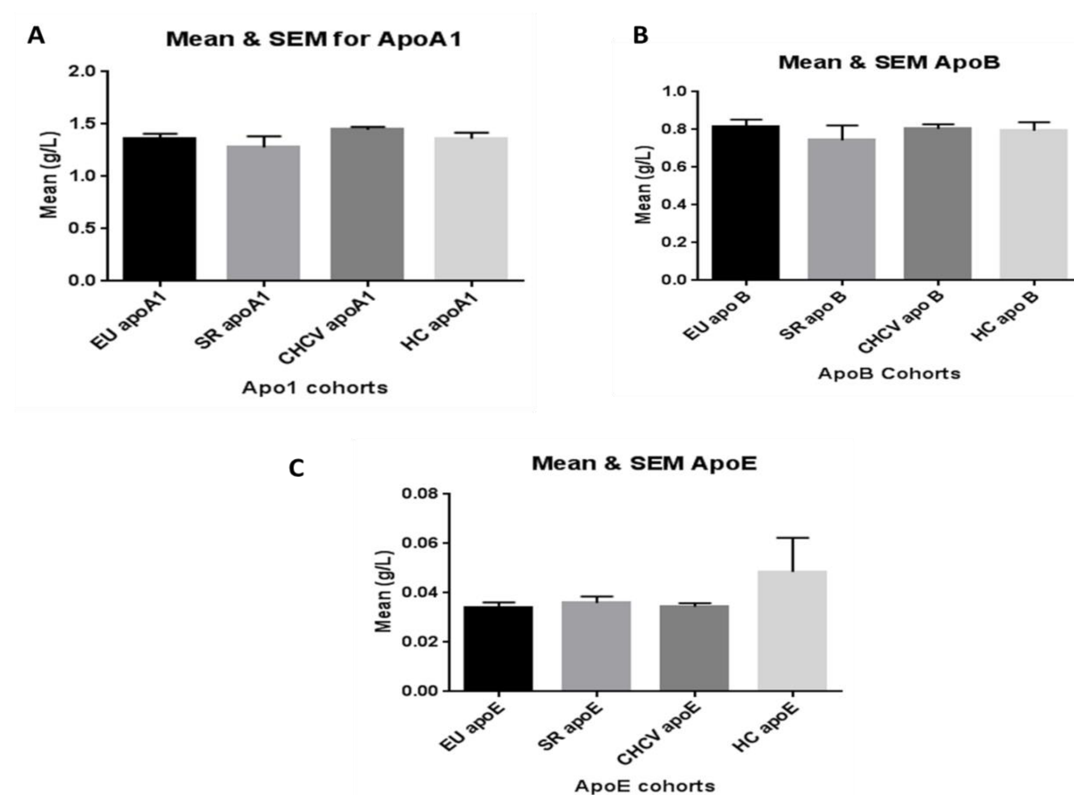


Figure 4.1 shows comparison of apolipoprotein levels in EU, SR, HC and chronic HCV patients. There were no statistically significant differences observed in apolipoproteins A1, B and E between the four groups (Figure **4.1** A, B, and C). Apo A1 Mann Whitney test: EU vs SR (p.value <0.38); EU vs CHCV (p.value <0.21); EU vs HC (p.value <0.81). ApoB Mann Whitney test: EU vs SR (p.value <0.57); EU vs CHCV (p.value <0.45); EU vs HC (p.value <0.55). ApoE Mann Whitney test: EU vs SR (p.value <0.67); EU vs CHCV (p.value <0.79); EU vs HC (p.value <0.52).

4.3.2 Study subjects for serum lipidomics.

All the study participants were Caucasians and the mean ages for EUs, SRs, and CHCV groups were 32, 36, and 36.3 years respectively. A total of 386 subjects were analysed comprising 60 EUs, 31 HC, 36 SRs, 100 SVRs, and 159 CHCV, utilising EU cases collected locally and additional SR, SVR, and CHCV samples from the HCV Research UK (HCVRUK) biobank as described in the methods section (Chapter 2). UPLC-MS of all the serum samples were acquired and analysed using multivariate methods in order to determine the differences in lipid profiles in EUs versus different comparator groups. Statistical estimations utilising XCMS package within the R statistical software and SIMCA were performed.

Table 4.1: Demographics for the serum UPLC-MS lipidomics profiling.

Characteristic	**EU (n=38)**	**SR (n=28)**	**SVR (n=100)**	**CHCV (n=159)**
HCV Ab	Negative	Positive	Positive	Positive
HCV RNA	Negative	Negative	Negative	Positive
Age (Mean) (years)	32	36	48+/-5 years	48+/-5 years
Age range (years)	23 - 58	33 - 53	43-53	43-53
Gender (M/F) %	76.3/23.7	62.5/37.5	70/30	70/30
Cirrhosis (%)	None	None	25% compensated cirrhosis	25% compensated cirrhosis
Anti-viral treatment	None	None	Peg-IFN/Ribavirin	None

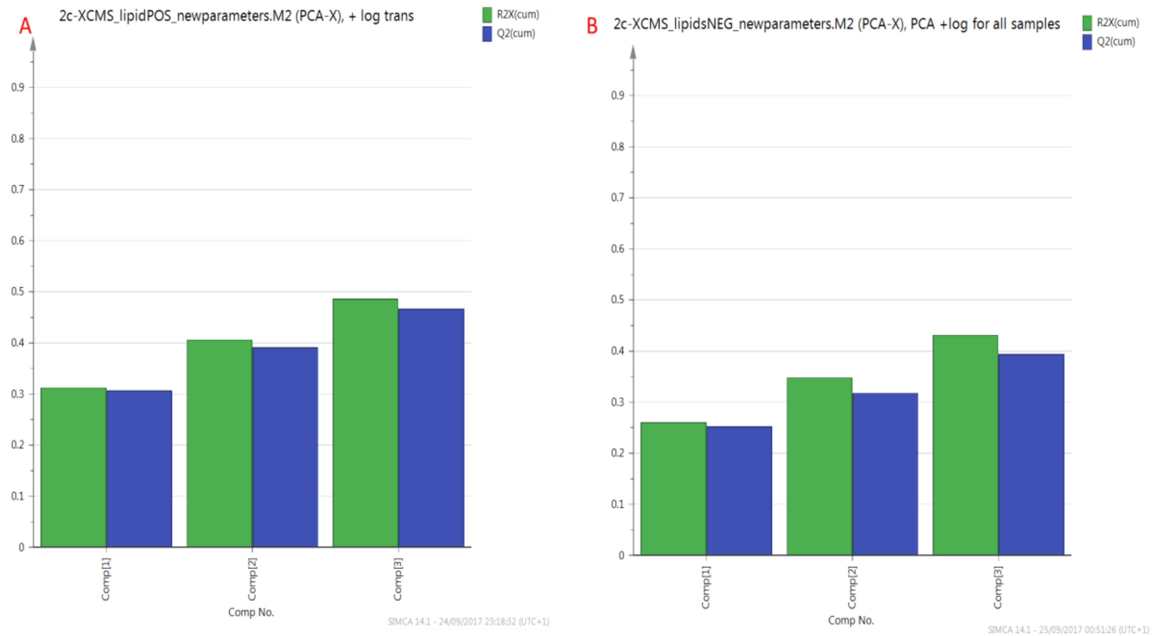
Table 4.1shows the demographics of the cases studied for the UPLC-MS lipidomics. All the EU (38) and some SR (8), and 9 (CHCV) cases were recruited from Plymouth. Additional 20 SR , 100 SVR and 150 CHCV cases were provided by the HCVRUK.

4.3.3 Initial lipidomics PCA for *all* samples including quality controls (QCs) in both ESI+ and ESI- modes.

The LC-MS lipidomics analysis generates a vast amount of data but its analysis is highly complex requiring use of a variety of statistical methods. Some lipid classes may comprise more than 100 different species, their routine quantification represents a great challenge. In this study, the approach used for data pre-processing, multivariate analysis, filtration and peak identification was performed using XCMS package within the R statistical software and SIMCA (details provided in the Methods chapter, Section 2.9.2.4). The serum lipidomics features for all the study groups were modelled together using the principal component analysis that aids visualisation of major sources of variations in the data. Different high-tech models were explored which showed that serum contains a wealth of lipidomics information that provide deeper understanding of lipid species that distinguish the groups. In this Chapter, data mining and multivariate data analysis has been presented with unsupervised PCA and supervised OPLS-DA. A combination of S- and VIP plots generated from the OPLS-DA models were carried out that selected distinct lipid features as potential markers for distinguishing the EUs from the control groups.

The PCA was created on the matrix of 386 variables comprising 60 EUs, 31 HC, 36 SRs, 100 SVRs, and 159 CHCV collected from Plymouth and some were provided by the HCVRUK as previously described in Section 2.2.3. Trends and patterns were observed in this PCA (encircled). Further interrogation revealed that the clustered samples went through increased freeze-thawing cycles. The QC samples clustered together defining the system suitability, analytical stability, and sample repeatability.

Figure 4.2: PCA for combined cohorts.



Scores plots of PCA model from UPLC-MS analyses of all the serum samples.

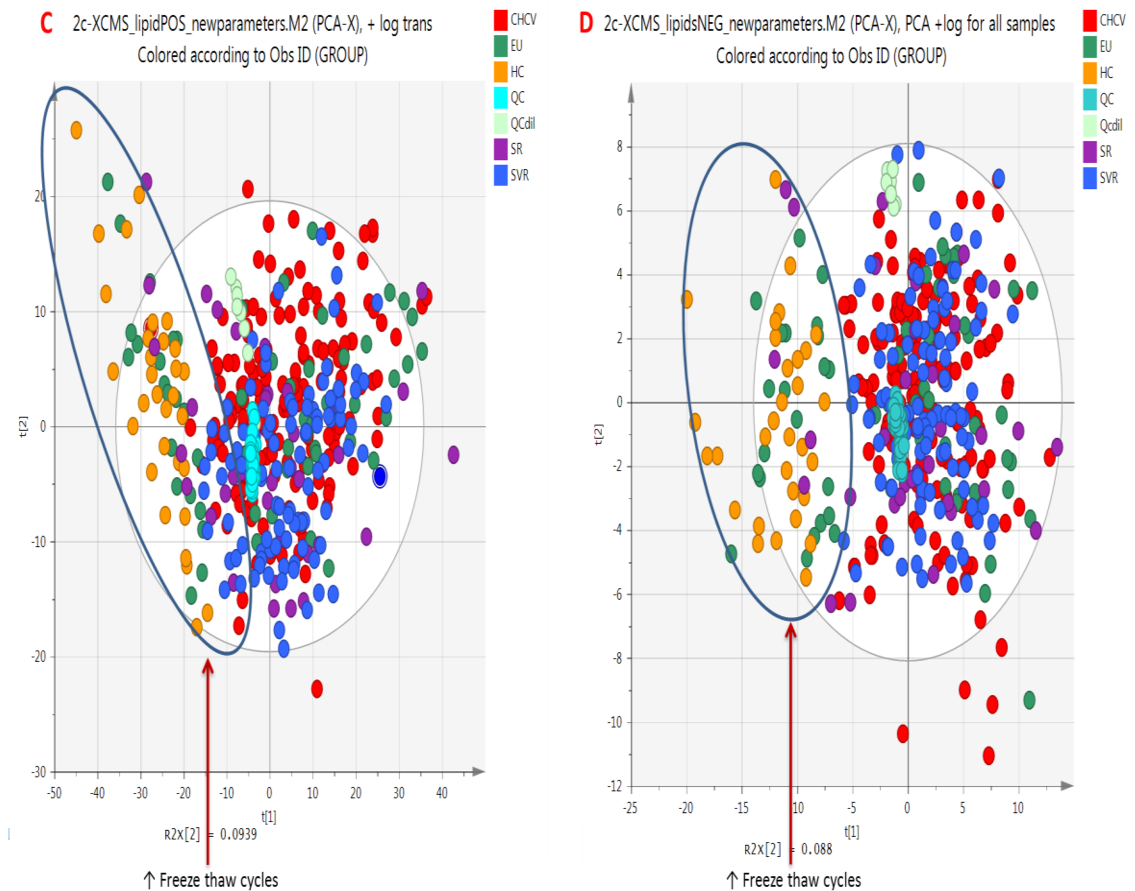


Figure 4.2 shows (A) shows the scores plots of principal components analysis (PCA) model generated from the ultra-performance liquid chromatography mass spectrometry (UPLC-MS) analysis of serum samples, and quality controls (QCs). (A) and (B) show green and blue histograms that represent R^2 and Q^2 in both positive and negative electrospray ionisation modes (ESI+ and ESI-). The R^2 is a measure of fit, i.e. how well the model fits the data and Q^2 indicates how well the model predicts new data. A large R^2 (close to 1) is a necessary condition for a good model, but it is not sufficient. Because Multivariate Analysis separates out useful information from the noise a low R^2 indicates a large amount of noise or irrelevant information in the data. The Q^2 indicates how well the model predicts new data, with a large Q^2 (Q2 > 0.5) indicating good predictivity. Both R^2 and Q^2 are established statistical data presentation methods used to confirm the validity of the models of the data. (C) and (D) show PCA for all the samples in both ESI+ and ESI- (C and D respectively). A dot represents one sample, and they are coloured according to different groups. The tight grouping of the QCs, which are run at the same time with the samples, highlights the instrument stability and reliability of the assay.

The X and Y axes are derived from the development of the PCA as orthogonal linear combinations of the factors exhibited in the dataset. The PCA are the linear combinations of the original variables. In this scores plot, the original factors were not assigned units since they were patients recognizable study numbers, therefore the PCA won't have a unit. Consequently, the units are the crude components scores. The X and Y axes in a PCA plot correspond to a mathematical transformation the X-axis (PC1 – the first principal component) and the second principal component (Y-axis) so that data can be displayed in two dimensions. In this initial analysis there was a mixed group of outliers from healthy controls, EU and SR cases that underwent increased freeze-thaw cycles.

4.3.4 PCA for all samples after removing the old samples.

This PCA plot provided good graphical overview of the entire data in multivariate analysis after exclusion of old samples. The old samples undergoing multiple freeze-thaw cycles comprised all healthy controls (31), 22 EUs, and 8 SRs. The subsequent supervised models were prepared from this PCA.

Figure 4.3: PCA model for all samples after excluding old samples.

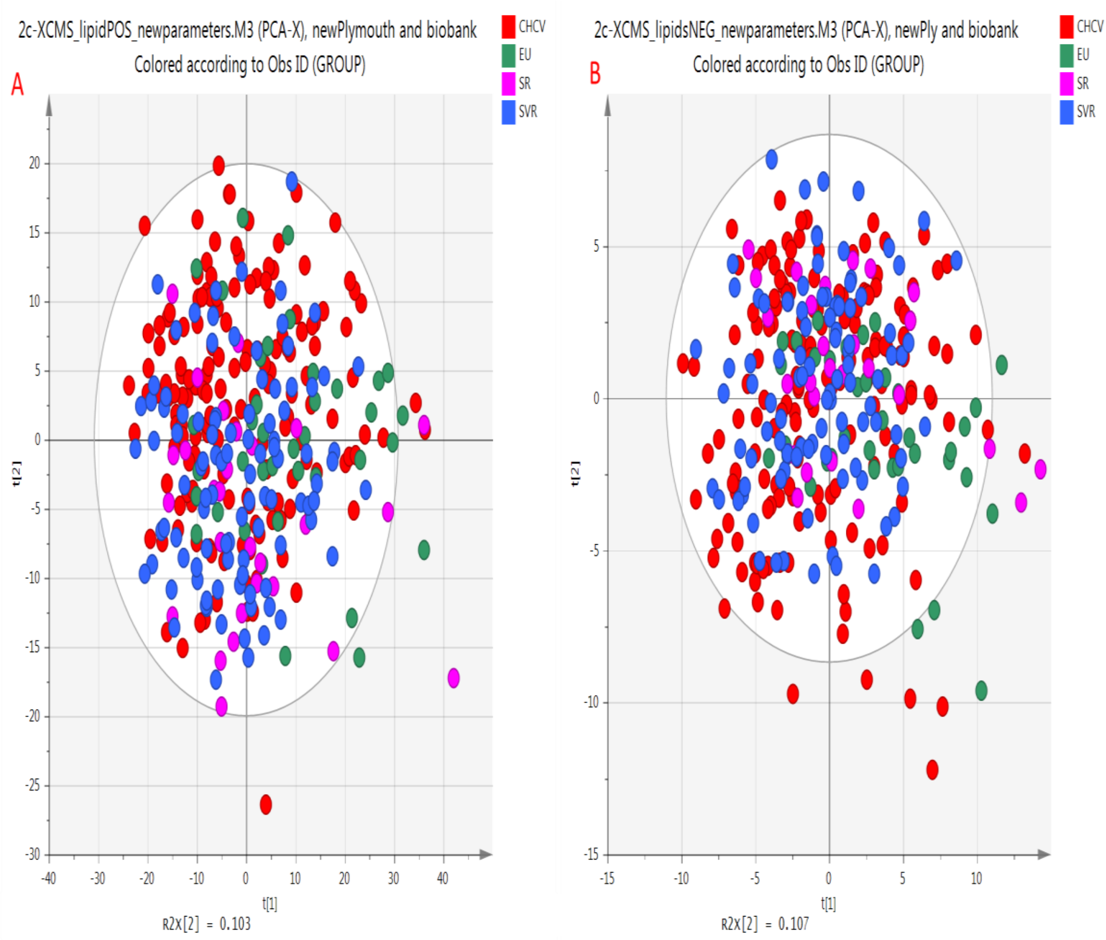


Figure **4.3** shows the scores plots of the principal components analysis (PCA) model generated from the ultra-performance liquid chromatography mass spectrometry (UPLC-MS) analysis of serum samples after excluding old samples that had increased freeze-thaw cycles. (A) and (B) show the PCA for all the samples in both ESI+ and ESI- respectively. A dot represents one sample, and they are coloured according to different groups.

The X and Y axes are derived from the development of the PCA as orthogonal linear combinations of the factors exhibited in the dataset. The PCA are the linear combinations of the original variables. In this scores plot, the original factors were not assigned units since they were patients recognizable study numbers, therefore the PCA won't have a unit. Consequently, the units are the crude components scores. The X and Y axes in a PCA plot correspond to a mathematical transformation the X-axis (PC1 – the first principal component) and the second principal component (Y-axis) so that data can be displayed in two dimensions.

4.3.5 Is the lipidome different between the EU and HCV susceptible cases?

This model shows lipidomics analysis comparing lipid profiles in serum from HCV ‘susceptible’ with HCV ‘resistant’ cases in order to observe if the HCV resistance is associated with a different lipidome than in those susceptible to HCV. The multivariate analysis was performed from HCV susceptible cases, who were all HCV antibody positive and divided into 3 categories of 1) chronic infection (HCV RNA positive) [CHCV] 2) sustained viral responders to previous anti-viral treatment (HCV RNA negative), [SVR] or 3) spontaneous resolvers (HCV RNA negative) [SR]. The lipidomes of these groups was compared to HCV ‘resistant’ exposed uninfected cases. The PCA and OPLS-DA were performed on all data and showed that the HCV exposed uninfected cohort had very distinctly different lipidomics features from all the HCV ‘susceptible’ groups including CHCV, SVR and SR in both positive and negative ionisation modes (Shawa, Sheridan, *et al.*, 2017).

Figure 4.4: EUs vs HCV Ab+ groups.

Scores plots of PCA model from UPLC-MS analyses of EU vs HCV susceptible groups.

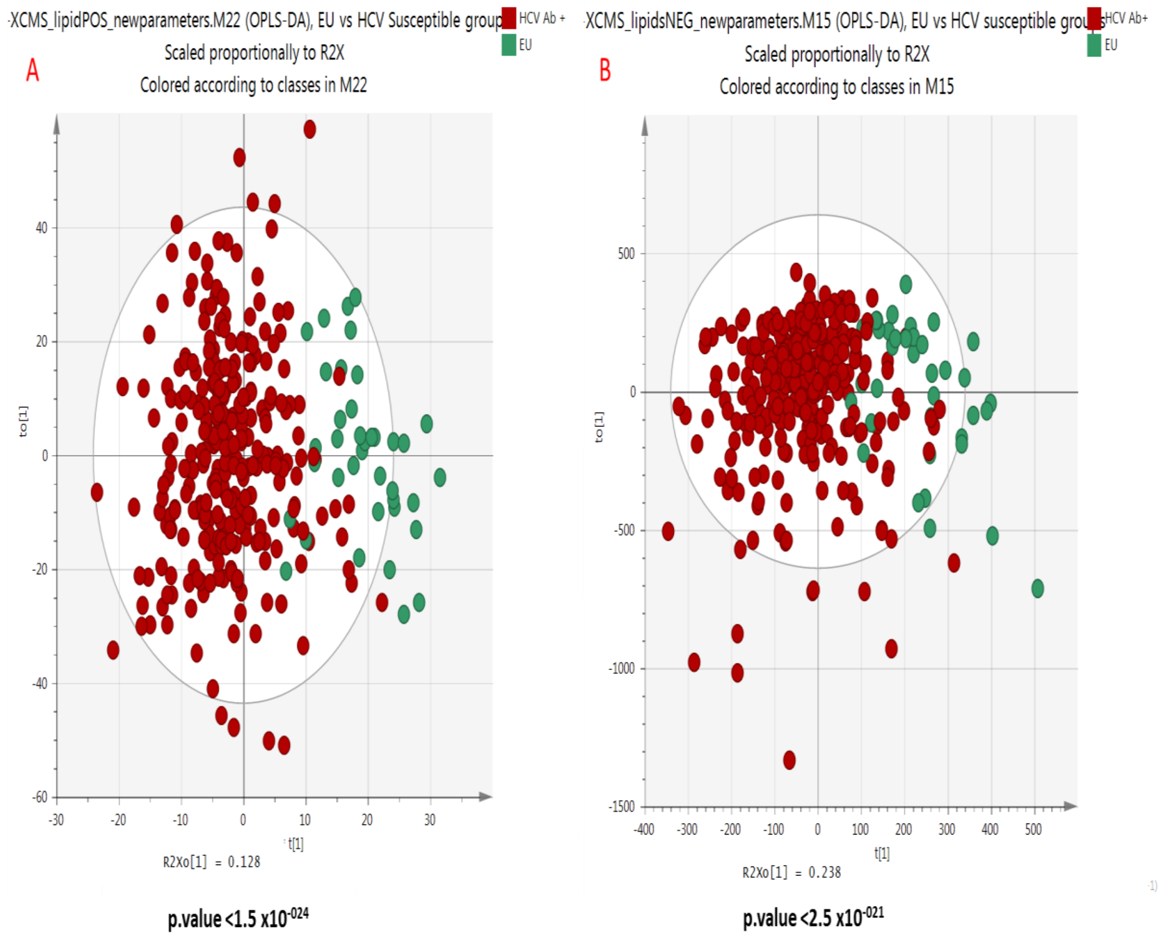


Figure 4.4 shows multivariate analysis the principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) for HCV ‘resistant’ cases [n=38]) compared to HCV ‘susceptible’ (HCV antibody positive, [n=295]), including chronic HCV patients genotypes 1 or 3 (HCV RNA positive [n=159]), sustained virological responders (SVR) [n=100] and spontaneous resolvers [n=36] (Shawa, Sheridan, *et al.*, 2017).

The scores plots (OPLS-DA) models (‘A’ and ‘B’ in both ESI+ and ESI- respectively) generated from the ultra-performance liquid chromatography mass spectrometry (UPLC-MS) analysis of serum samples. A dot represents one sample, and they are coloured according to different groups. The data was subjected to multivariate statistical analysis using SIMCA-P (version 14.1, Umetrics, Umeå, Sweden). The statistical estimations utilising XCMS package within the R statistical software and SIMCA were performed using ANOVA of the cross-validated residuals (CV-ANOVA). The serum lipidomics features distinguished the EU from the HCV Ab+ groups in both ESI+ and ESI- (p.value $<1.5 \times 10^{-024}$, and $<2.5 \times 10^{-021}$ respectively).

The X and Y axes are derived from the development of the PCA as orthogonal linear combinations of the factors exhibited in the dataset. The PCA are the linear combinations of the original variables. In this scores plot, the original factors were not assigned units since they were patients recognizable study numbers, therefore the PCA won't have a unit. Consequently, the units are the crude components scores. The X and Y axes in a PCA plot correspond to a mathematical transformation the X-axis (PC1 – the first principal component) and the second principal component (Y-axis) so that data can be displayed in two dimensions.

4.3.6 Analysis for EU vs CHCV group.

The 38 EU cases were compared to 159 CHCV cases. Figure 4.5 compares non-viremic EU cases (green dots) vs viremic (red dots) cases. The lipidomics analysis showed clear segregation of the 2 groups with highly significant differences between EU cases and CHCV patients.

Figure 4.5: EU compared to CHCV patients.

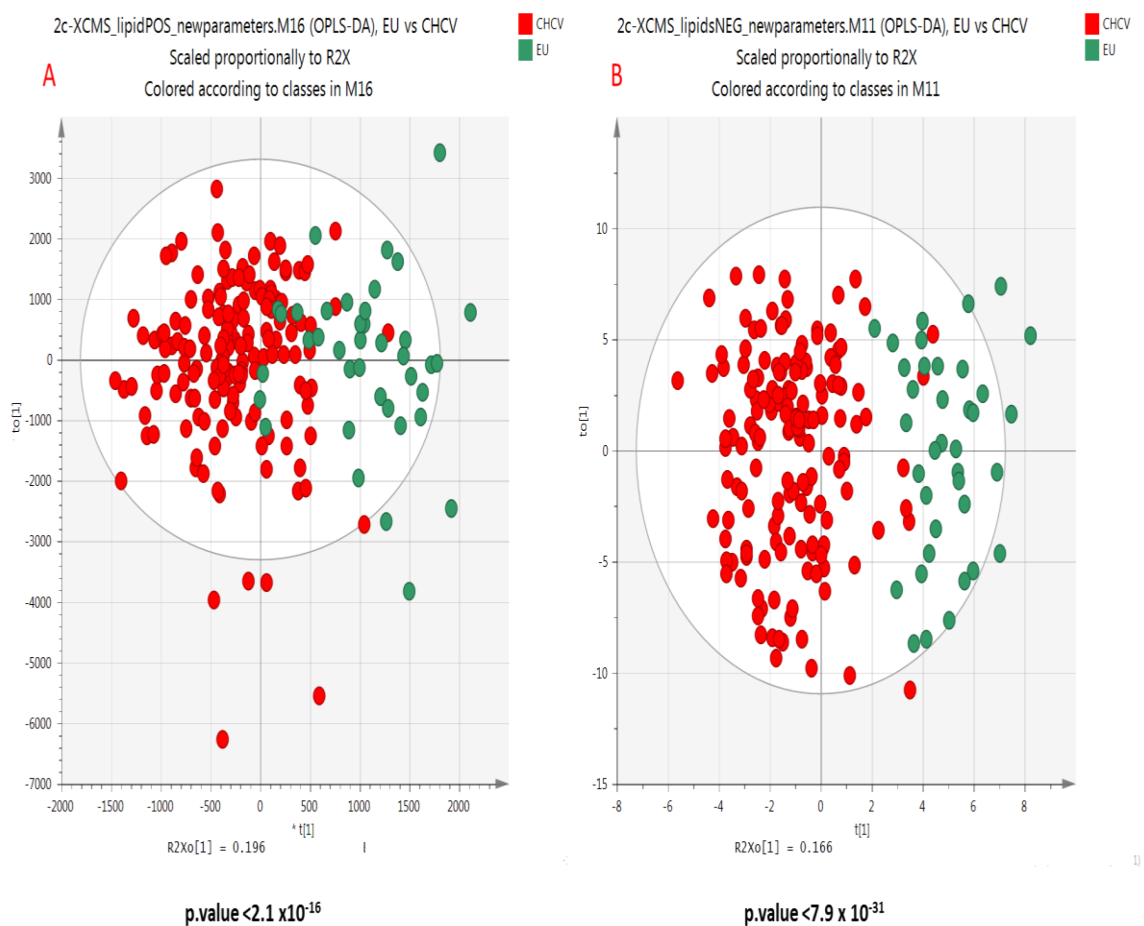


Figure 4.5 shows multivariate analysis the principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) for the EU vs CHCV patients.

The scores plots (OPLS-DA) models ('A' and 'B' in both ESI+ and ESI- respectively) generated from the ultra-performance liquid chromatography mass spectrometry (UPLC-MS) analysis of serum samples. A dot represents one sample, and they are coloured according to different groups. The data was subjected to multivariate statistical analysis using SIMCA-P (version 14.1, Umetrics, Umeå, Sweden). The statistical estimations utilising XCMS package within the R statistical software and SIMCA were performed using ANOVA of the cross-validated residuals (CV-ANOVA). The serum lipidomics features distinguished the EU from the CHCV patients in both ESI+ and ESI- (p.value $<2.1 \times 10^{-16}$, and $<7.9 \times 10^{-31}$ respectively).

The X and Y axes are derived from the development of the PCA as orthogonal linear combinations of the factors exhibited in the dataset. The PCA are the linear combinations of the original variables. In this scores plot, the original factors were not assigned units since they were patients recognizable study numbers, therefore the PCA won't have a unit. Consequently, the units are the crude components scores. The X and Y axes in a PCA plot correspond to a mathematical transformation the X-axis (PC1 – the first principal component) and the second principal component (Y-axis) so that data can be displayed in two dimensions.

4.3.7 Host's susceptibility to HCV infection and the ability to resolve spontaneously (EU vs SR).

The lipidomics multivariate analysis was also able to distinguish the two groups that were both aviremic (i.e. HCV RNA negative), the EUs who were both HCV antibody and HCV RNA negative and the SRs were HCV Ab positive and RNA negative. The LC-MS serum lipidomics analysis clearly distinguished the EUs from the SRs (p-value <0.0005 and p-value <0.005 for both positive and negative ionisation modes respectively).

Figure 4.6: The EU compared to the SRs.

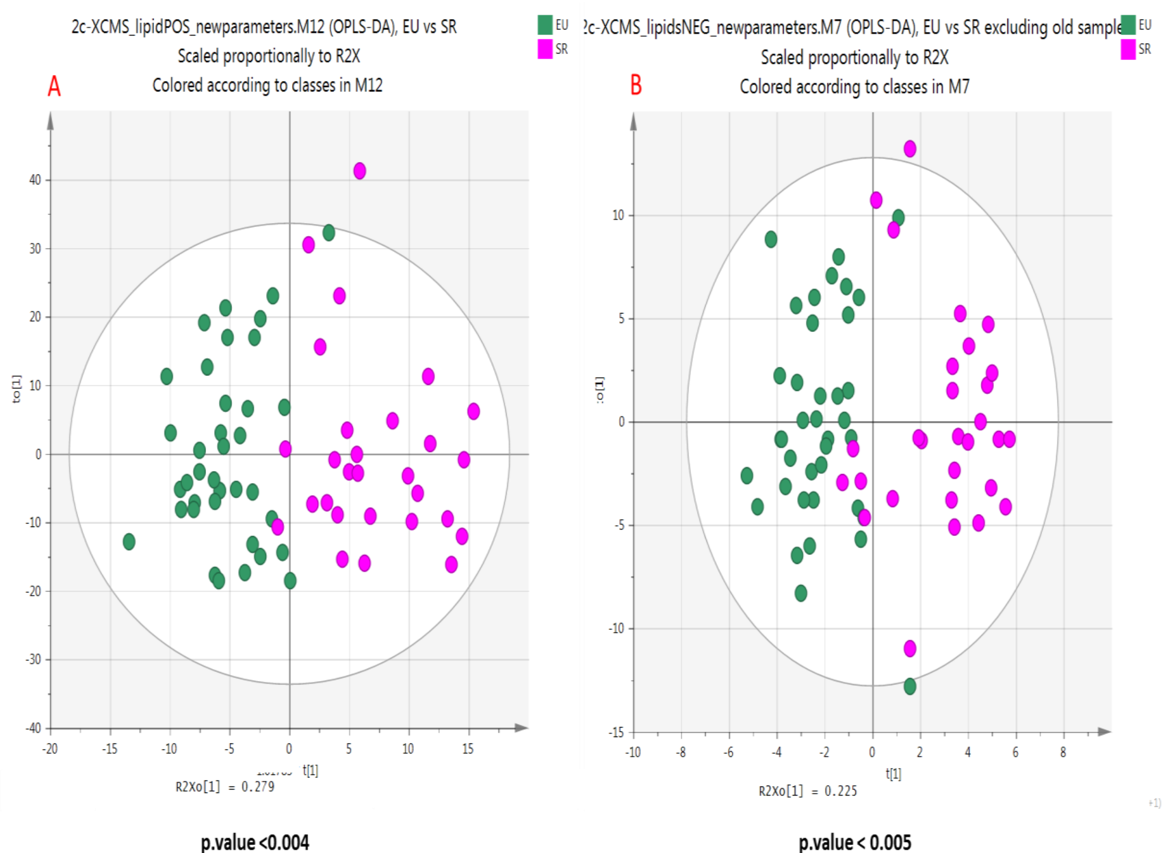


Figure 4.6 shows multivariate analysis the principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) for the EU vs the spontaneous resolvers (SRs).

The scores plots (OPLS-DA) models ('A' and 'B' in both ESI+ and ESI- respectively) generated from the ultra-performance liquid chromatography mass spectrometry (UPLC-MS) analysis of serum samples. A dot represents one sample, and they are coloured according to different groups. The data was subjected to multivariate statistical analysis using SIMCA-P (version 14.1, Umetrics, Umeå, Sweden). The statistical estimations utilising XCMS package within the R statistical software and SIMCA were performed using ANOVA of the cross-validated residuals (CV-ANOVA). The serum lipidomics features distinguished the EU from the SRs in both ESI+ and ESI- (p.value <0.004, and <0.005 respectively).

The X and Y axes are derived from the development of the PCA as orthogonal linear combinations of the factors exhibited in the dataset. The PCA are the linear combinations of the original variables. In this scores plot, the original factors were not assigned units since they were patients recognizable study numbers, therefore the PCA won't have a unit. Consequently, the units are the crude components scores. The X and Y axes in a PCA plot correspond to a mathematical transformation the X-axis (PC1 – the first principal component) and the second principal component (Y-axis) so that data can be displayed in two dimensions.

4.3.8 Host's susceptibility to HCV infection and the ability to resolve the infection following anti-HCV therapy.

Figure 4.7 shows comparison of two aviraemic groups but one group were susceptible to HCV infection but cleared the infection following IFN-base therapy (SVRs). This study has also revealed that the EUs have distinctly different lipid profiles from SVRs (p-value <0.0, and p-value $<6.9 \times 10^{-30}$ for positive and negative ionisation modes respectively).

Figure 4.7: The EU compared to the sustained viral responders (SVRs).

Scores plots of PCA model from UPLC-MS analyses of EU vs SVR.

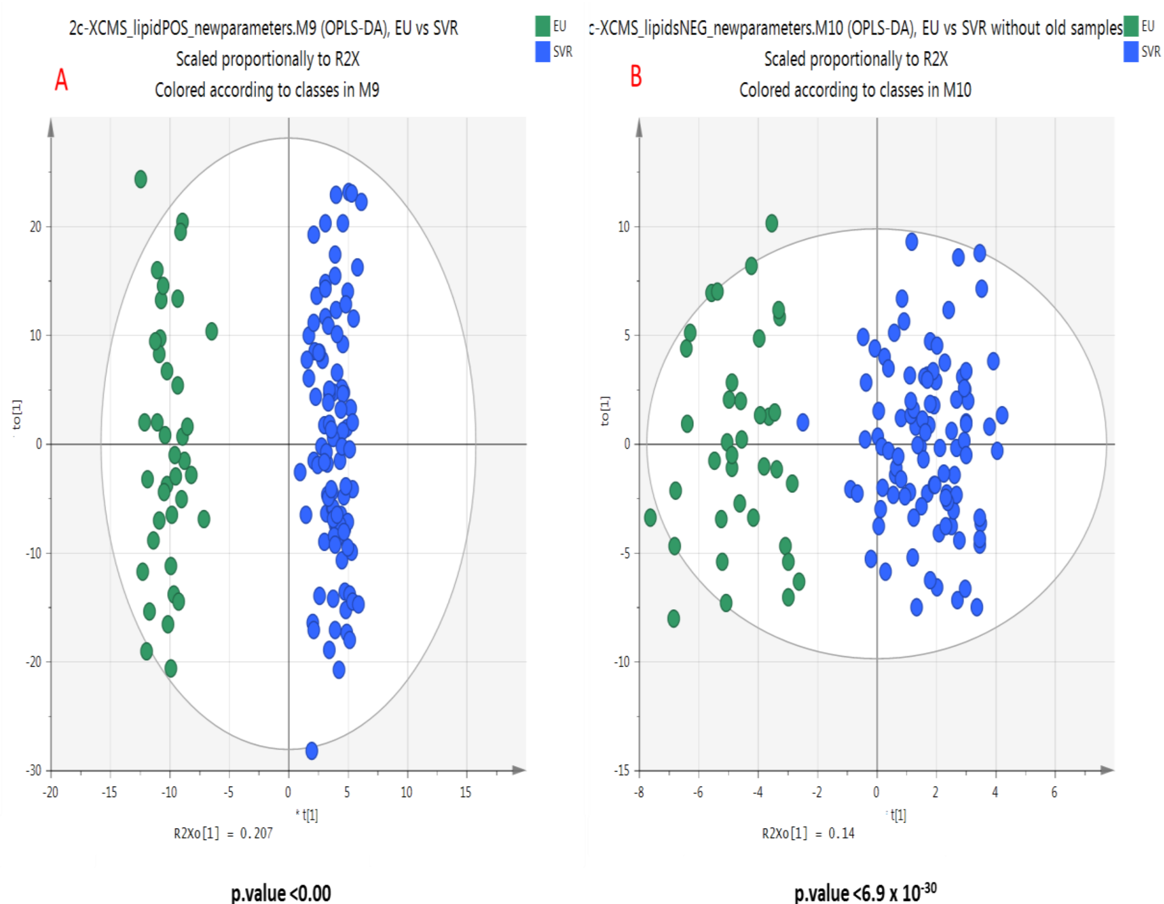


Figure 4.7 shows multivariate analysis the principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) for the EU vs the sustained viral responders (SVRs).

The scores plots (OPLS-DA) models ('A' and 'B' in both ESI+ and ESI- respectively) generated from the ultra-performance liquid chromatography mass spectrometry (UPLC-MS) analysis of serum samples. A dot represents one sample, and they are coloured according to different groups. The data was subjected to multivariate statistical analysis using SIMCA-P (version 14.1, Umetrics, Umeå, Sweden). The statistical estimations utilising XCMS package within the R statistical software and SIMCA were performed using ANOVA of the cross-validated residuals (CV-ANOVA). The serum lipidomics features distinguished the EU from the SVRs in both ESI+ and ESI- (p.value <0.00, and $<6.9 \times 10^{-30}$ respectively).

The X and Y axes are derived from the development of the PCA as orthogonal linear combinations of the factors exhibited in the dataset. The PCA are the linear combinations of the original variables. In this scores plot, the original factors were not assigned units since they were patients recognizable study numbers, therefore the PCA won't have a unit. Consequently, the units are the crude components scores. The X and Y axes in a PCA plot correspond to a mathematical transformation the X-axis (PC1 – the first principal component) and the second principal component (Y-axis) so that data can be displayed in two dimensions.

4.3.9 Resolution of HCV infection by different mechanisms.

The resolution of HCV infection separated both groups that were previously infected but cleared the infection by different mechanisms. In order to evaluate whether HCV specific variation in the lipidome persists after HCV resolution following treatment or spontaneous resolution, a supervised model was created. This OPLS-DA shows clear distinction in lipidomics species between the SRs and SVRs.

Figure 4.8: Spontaneous resolvers compared to a group that received IFN-based therapy.

Scores plots of PCA model from UPLC-MS analyses of SR vs SVR.

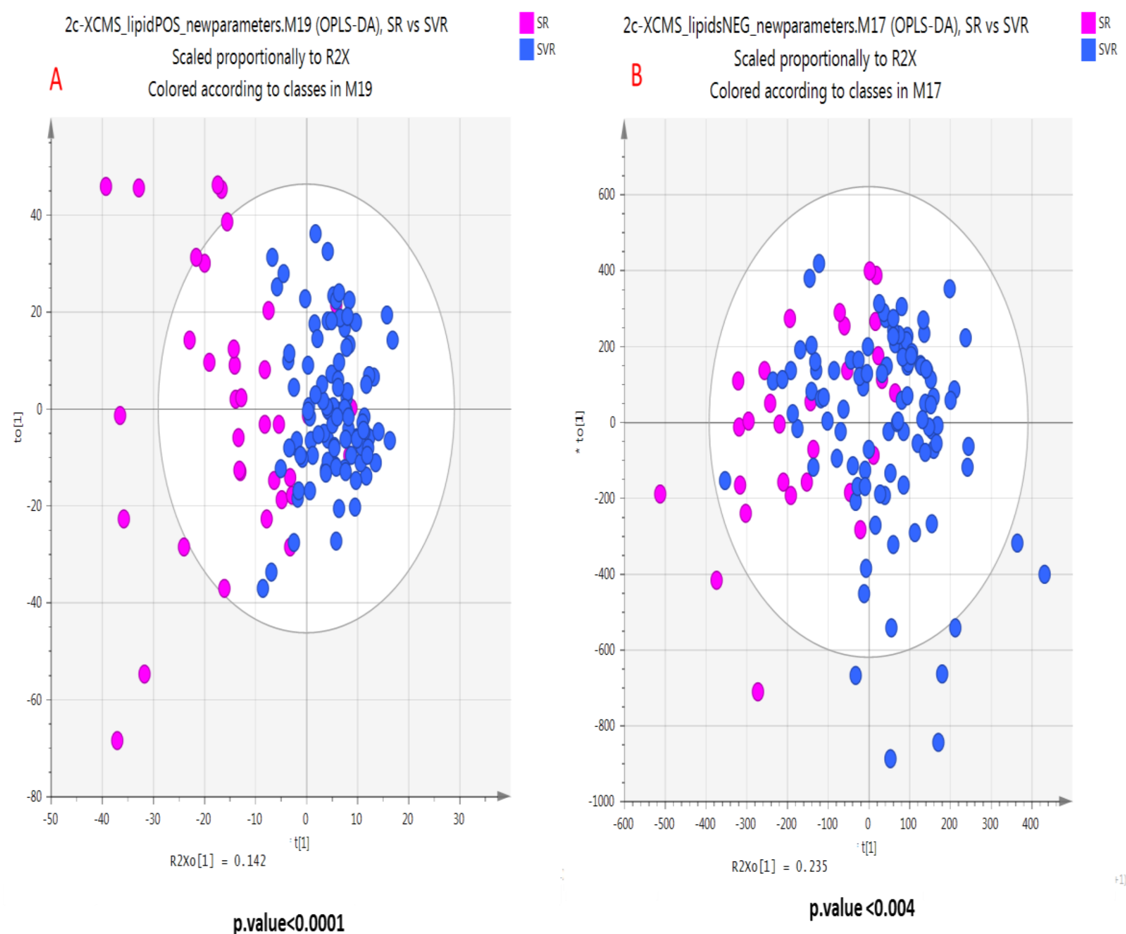


Figure 4.8 shows multivariate analysis the principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) for two groups that were both susceptible to HCV infection but cleared the infection by different mechanisms, the spontaneous resolvers (SRs) vs the sustained viral responders (SVRs).

The scores plots (OPLS-DA) models (‘A’ and ‘B’ in both ESI+ and ESI- respectively) generated from the ultra-performance liquid chromatography mass spectrometry (UPLC-MS) analysis of serum samples. A dot represents one sample, and they are coloured according to different groups. The data was subjected to multivariate statistical analysis using SIMCA-P (version 14.1, Umetrics, Umeå, Sweden). The statistical estimations utilising XCMS package within the R statistical software and SIMCA were performed using ANOVA of the cross-validated residuals (CV-ANOVA). The serum lipidomics features distinguished the EU from the SVRs in both ESI+ and ESI- (p.value <0.001, and <0.0004 respectively).

The X and Y axes are derived from the development of the PCA as orthogonal linear combinations of the factors exhibited in the dataset. The PCA are the linear combinations of the original variables. In this scores plot, the original factors were not assigned units since they were patients recognizable study numbers, therefore the PCA won't have a unit. Consequently, the units are the crude components scores. The X and Y axes in a PCA plot correspond to a mathematical transformation the X-axis (PC1 – the first principal component) and the second principal component (Y-axis) so that data can be displayed in two dimensions.

4.3.10 Host's ability to clear the HCV infection, and establishment of chronic infection.

The figure below describes an OPLS-DA model for two groups that were both susceptible to HCV infection. The SRs were HCV Ab positive but HCV RNA negative; whereas CHCV cases were both HCV Ab and RNA positive. The two groups displayed different lipid features as observed in this model. The CHCV group did not receive any treatment at the time of the study.

Figure 4.9: OPLS-DA models comparing SRs vs CHCV.

Scores plots of PCA model from UPLC-MS analyses of SR vs CHCV.

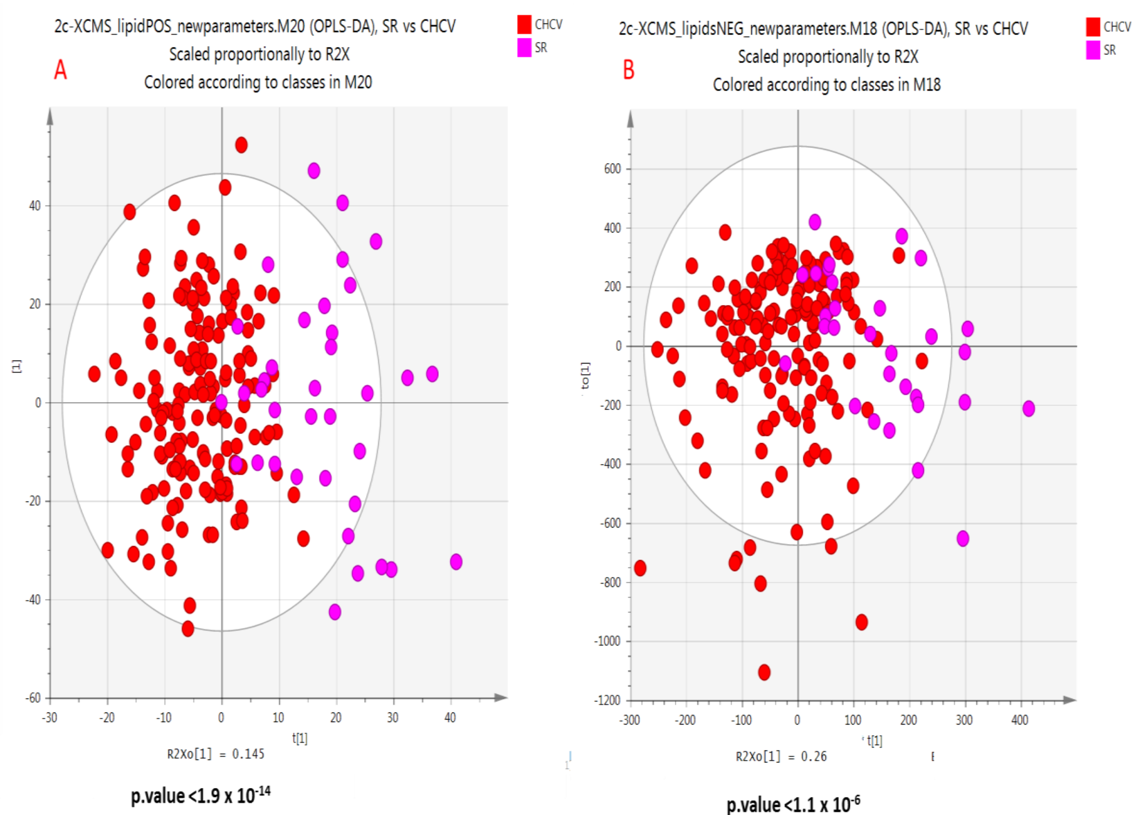


Figure 4.9 shows multivariate analysis the principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) for the spontaneous resolvers (SRs) vs the chronic HCV (CHCV) patients.

The scores plots (OPLS-DA) models ('A' and 'B' in both ESI+ and ESI- respectively) generated from the ultra-performance liquid chromatography mass spectrometry (UPLC-MS) analysis of serum samples. A dot represents one sample, and they are coloured according to different groups. The data was subjected to multivariate statistical analysis using SIMCA-P (version 14.1, Umetrics, Umeå, Sweden). The statistical estimations utilising XCMS package within the R statistical software and SIMCA were performed using ANOVA of the cross-validated residuals (CV-ANOVA). The serum lipidomics features distinguished the EU from the SVRs in both ESI+ and ESI- (p.value $<1.9 \times 10^{-14}$, and $<1.1 \times 10^{-6}$ respectively).

The X and Y axes are derived from the development of the PCA as orthogonal linear combinations of the factors exhibited in the dataset. The PCA are the linear combinations of the original variables. In this scores plot, the original factors were not assigned units since they were patients recognizable study numbers, therefore the PCA won't have a unit. Consequently, the units are the crude components scores. The X and Y axes in a PCA plot correspond to a mathematical transformation the X-axis (PC1 – the first principal component) and the second principal component (Y-axis) so that data can be displayed in two dimensions.

4.3.11 The viral effect on lipidome (CHCV vs SVR).

A serum lipidomics was analysed to assess the viral effect on the lipidome by comparing two separate HCV susceptible groups i.e. the chronic HCV (CHCV) patients and the sustained viral responders (SVRs). The 150 CHCV cases were treatment naïve and comprised 50% HCV genotype 1 and 50% HCV genotype 3 with 25% compensated cirrhosis. The SVRs achieved a sustained response following PEG-IFN and RBV based therapy. Further 100 HCV RNA negative sera post SVR was provided by the HCVRUK consisting of 50 HCV genotype 1 and 50 HCV genotype 3 SVRs also with 25% compensated cirrhosis. All samples were provided by the HCVRUK biobank in Glasgow. This was utilized to evaluate whether HCV specific variation in the lipidome persists after sustained viral response, or resolvers. The serum lipidomics showed that there are some differences in lipid features between the CHCV patients and SVR serum lipidomics post treatment.

Figure 4.10: The CHCV compared to SVRs.

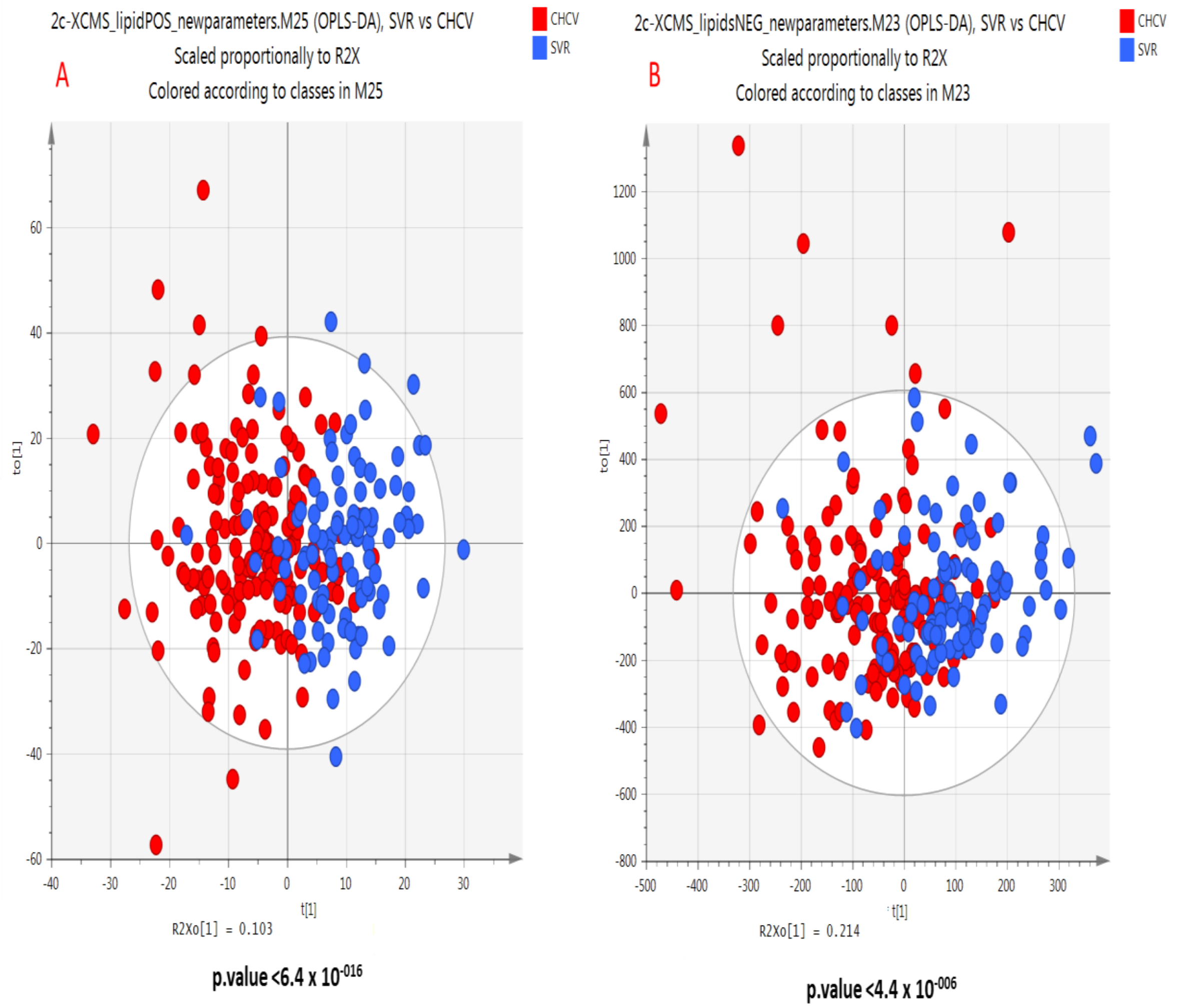


Figure 4.10 shows multivariate analysis the principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) for the CHCV patients compared to the SVRs.

The scores plots (OPLS-DA) models ('A' and 'B' in both ESI+ and ESI- respectively) generated from the ultra-performance liquid chromatography mass spectrometry (UPLC-MS) analysis of serum samples. A dot represents one sample, and they are coloured according to different groups. The data was subjected to multivariate statistical analysis using SIMCA-P (version 14.1, Umetrics, Umeå, Sweden). The statistical estimations utilising XCMS package within the R statistical software and SIMCA were performed using ANOVA of the cross-validated residuals (CV-ANOVA). The serum lipidomics features distinguished the CHCV from the SVRs in both ESI+ and ESI- (p.value $<6.4 \times 10^{-016}$, and $<4.4 \times 10^{-006}$ respectively).

The X and Y axes are derived from the development of the PCA as orthogonal linear combinations of the factors exhibited in the dataset. The PCA are the linear combinations of the original variables. In this scores plot, the original factors were not assigned units since they were patients recognizable study numbers, therefore the PCA won't have a unit. Consequently, the units are the crude components scores. The X and Y axes in a PCA plot correspond to a mathematical transformation the X-axis (PC1 – the first principal component) and the second principal component (Y-axis) so that data can be displayed in two dimensions.

4.3.12 Exposed uninfected compared to health controls.

The trends observed in the unsupervised PCA for all cohorts showed clustering of all samples that were subjected to increased freeze-thawing cycles. 22 EU and 31 health control samples were analysed separately. This OPLS-DA model shows clear separation of the EUs from the healthy controls.

Figure 4.11: The EUs compared to healthy controls.

Scores plots of PCA model from UPLC-MS analyses of EU vs HC (old samples only).

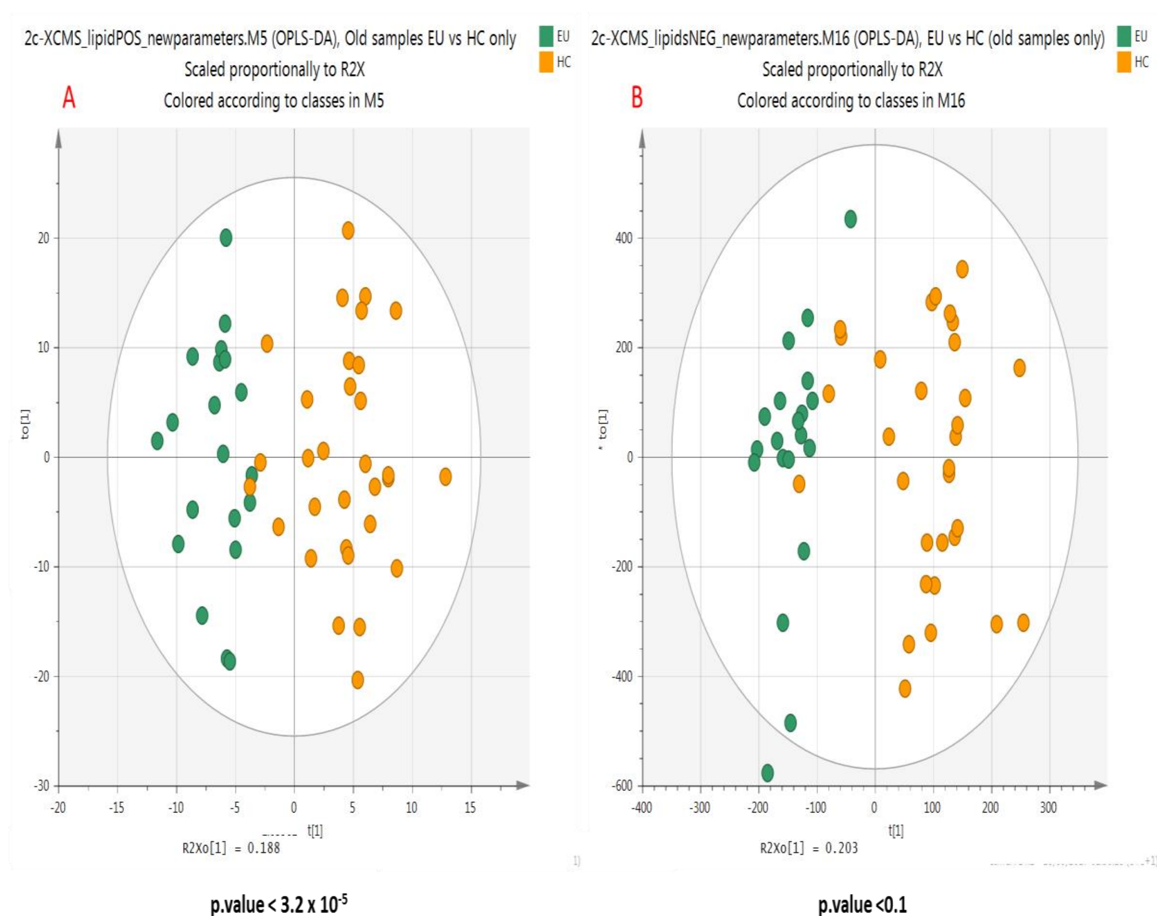


Figure 4.11 shows multivariate analysis the principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) for the EU vs healthy control (HC) group. All these samples were collected in Plymouth but underwent an increased free-thaw cycles. In this model the EUs are clearly distinctly different from the HCs.

The scores plots (OPLS-DA) models (‘A’ and ‘B’ in both ESI+ and ESI- respectively) generated from the ultra-performance liquid chromatography mass spectrometry (UPLC-MS) analysis of serum samples. A dot represents one sample, and they are coloured according to different groups. The data was subjected to multivariate statistical analysis using SIMCA-P (version 14.1, Umetrics, Umeå, Sweden). The statistical estimations utilising XCMS package within the R statistical software and SIMCA were performed using ANOVA of the cross-validated residuals (CV-ANOVA). The serum lipidomics features distinguished the EU from the SVRs in both ESI+ and ESI- (p.value $< 3.2 \times 10^{-5}$, and $< 3.2 \times 10^{-5}$ respectively).

The X and Y axes are derived from the development of the PCA as orthogonal linear combinations of the factors exhibited in the dataset. The PCA are the linear combinations of the original variables. In this scores plot, the original factors were not assigned units since they were patients recognizable study numbers, therefore the PCA won't have a unit. Consequently, the units are the crude components scores. The X and Y axes in a PCA plot correspond to a mathematical transformation the X-axis (PC1 – the first principal component) and the second principal component (Y-axis) so that data can be displayed in two dimensions.

4.3.13 ELISpot positive EUs vs ELISpot negative vs CHCV.

One potential criticism of the exposed uninfected cohort is that in the absence of serological evidence of infection, the presumed exposure and evidence of resistance to infection with HCV is determined by history alone. The presence of HCV-specific T cell responses does provide confirmation of HCV exposure but is found in only around half of the EU cases. This comparison was to see if there are any differences in lipidome between the EU cases with HCV-specific T cell responses from those without. The demonstration of HCV specific T cell responses using an IFN-gamma ELISpot is described in Chapter 5. No differences were seen between the EU with or without T cell responses. This suggests that the EU cohort as defined by injection history is uniform and that the lack of T cell responses is not likely to indicate lack of exposure as described in Chapter 5.

Figure 4.12: The IFN-γ positive versus IFN-γ negative EUs.

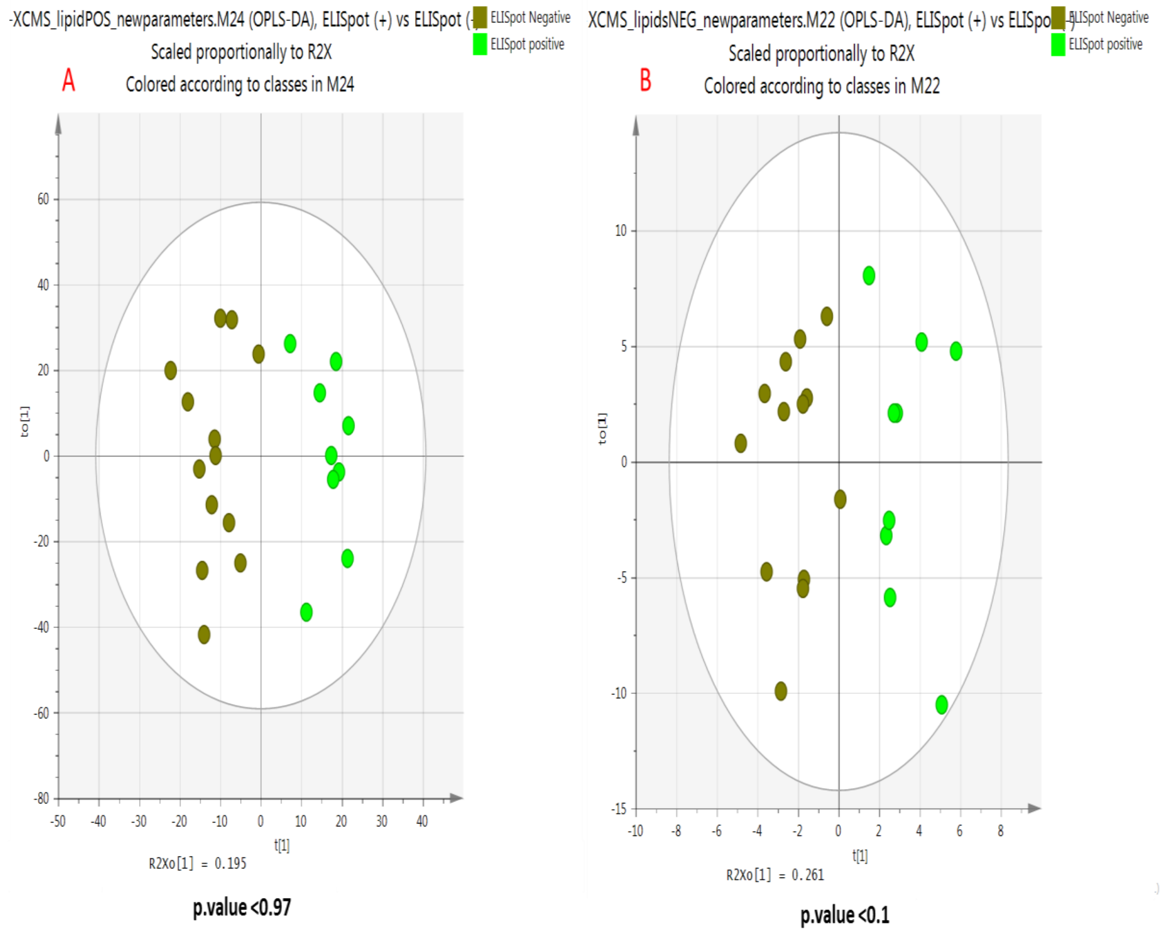


Figure 4.12 shows multivariate analysis the principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) for EU cases that were categorised as ELISpot positive or negative.

The scores plots (OPLS-DA) models (‘A’ and ‘B’ in both ESI+ and ESI- respectively) generated from the ultra-performance liquid chromatography mass spectrometry (UPLC-MS) analysis of serum samples. A dot represents one sample, and they are coloured according to different groups. The data was subjected to multivariate statistical analysis using SIMCA-P (version 14.1, Umetrics, Umeå, Sweden). The statistical estimations utilising XCMS package within the R statistical software and SIMCA were performed using ANOVA of the cross-validated residuals (CV-ANOVA). The serum lipidomics features distinguished the EU from the SVRs in both ESI+ and ESI- (p.value < 0.97, and < 0.1 respectively).

The X and Y axes are derived from the development of the PCA as orthogonal linear combinations of the factors exhibited in the dataset. The PCA are the linear combinations of the original variables. In this scores plot, the original factors were not assigned units since they were patients recognizable study numbers, therefore the PCA won't have a unit. Consequently, the units are the crude components scores. The X and Y axes in a PCA plot correspond to a mathematical transformation the X-axis (PC1 – the first principal component) and the second principal component (Y-axis) so that data can be displayed in two dimensions.

4.3.14 Sample collection centres.

Figure 4.13 shows a PCA for all samples collected at different centres. There was no difference observed in lipidomics features between samples collected in Plymouth and those obtained from the biobank.

Figure 4.13: Plymouth samples compared to HCVRUK biobank samples.

Scores plots of PCA model from UPLC-MS analyses of two separate sample collection centres.

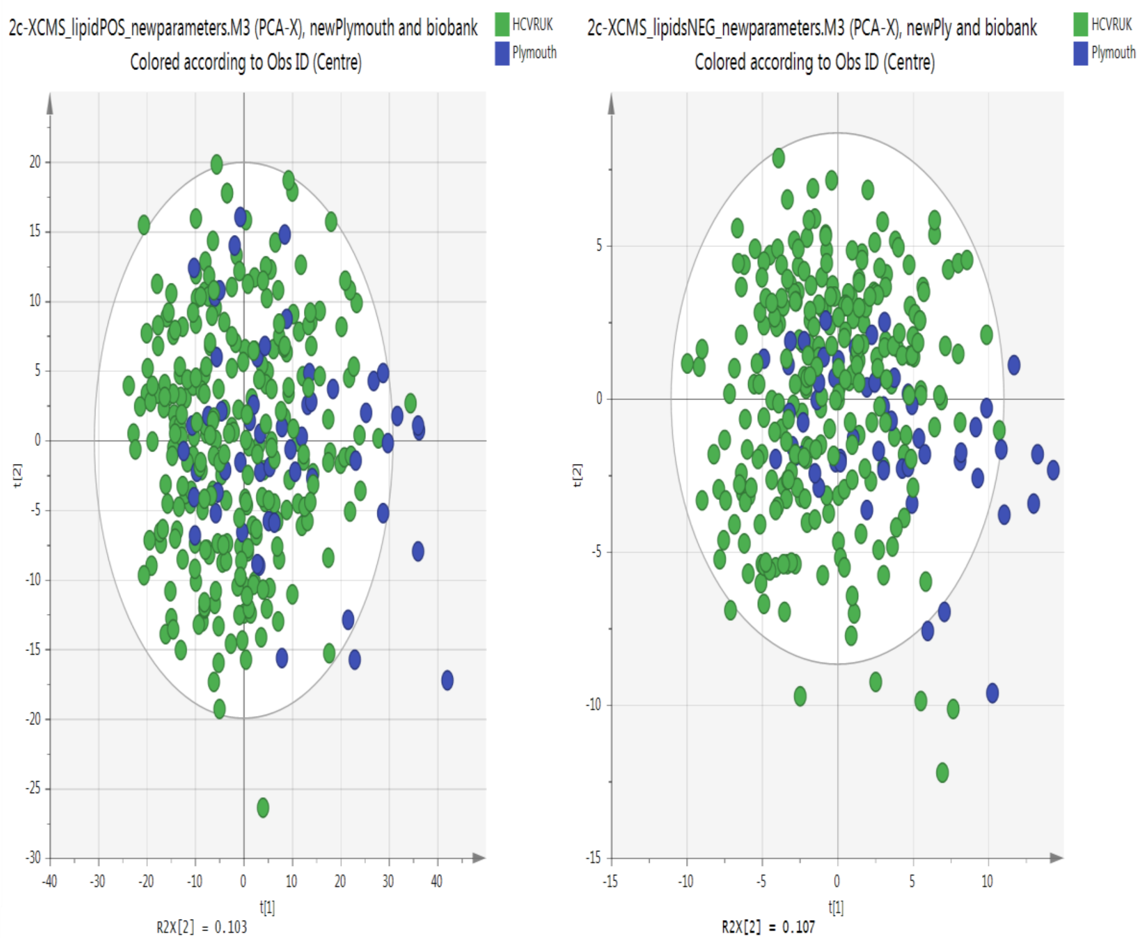


Figure 4.13 shows multivariate analysis the principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) for samples collected in Plymouth vs HCVRUK biobank samples.

The scores plots (OPLS-DA) models ('A' and 'B' in both ESI+ and ESI- respectively) generated from the ultra-performance liquid chromatography mass spectrometry (UPLC-MS) analysis of serum samples. A dot represents one sample, and they are coloured according to different groups.

The X and Y axes are derived from the development of the PCA as orthogonal linear combinations of the factors exhibited in the dataset. The PCA are the linear combinations of the original variables. In this scores plot, the original factors were not assigned units since they were patients recognizable study numbers, therefore the PCA won't have a unit. Consequently, the units are the crude components scores. The X and Y axes in a PCA plot correspond to a mathematical transformation the X-axis (PC1 – the first principal component) and the second principal component (Y-axis) so that data can be displayed in two dimensions.

4.3.15 Preliminary assignment of lipid species in lipidomics profiling.

Assignment of lipid profiling features that distinguish the EU cohort from other classes revealed phosphatidylcholines and triglycerides were significantly elevated in EUs compared to CHCV patients. The discriminant compounds (not annotated) between the EUs and the SVRs had very low retention times, eluting very early in the chromatographic domain along with the solvent front. Therefore, such polar compounds were not easily annotated using the online databases and were classified as unknowns.

Table 4.2 Assigned lipid compounds.

	Lipid species assigned	Ions	Formula	Mass	rt	ppm error
EU vs CHCV	PC(32:1)	M+H	C40H78NO8P	184.0742	5.57	5
	PC(34:1)	M+H	C42H82NO8P	759.5778	7.29	3
	PC(34:2)	M+H	C42H80NO8P	184.0742	5.90	3
	PC(36:1)	M+H	C44H86NO8P	787.6091	9.63	4
	PC(36:4)	M+H	C44H80NO8P	782.573	5.65	4
	PC(36:5)	M+H	C44H78NO8P	779.5465	4.75	5
	PC(38:4)	M+H	C46H84NO8P	809.5935	7.45	4
	PC(38:5)	M+H	C46H82NO8P	808.59	5.73	6
	PC(38:6)	M+H	C46H80NO8P	805.5622	5.26	4
	TG(52:2)	M+NH4	C55H102O6	858.7676	15.71	4
	TG(52:3)	M+NH4	C55H100O6	874.789	15.46	3
	TG(52:4)	M+NH4	C55H98O6	854.7363	15.19	5
	TG(56:5)	M+NH4	C59H104O6	908.7833	15.65	5
EU vs SR	TG(40:0)	M+NH4	C43H82O6	694.6111	14.37	2
	TG(42:1)	M+NH4	C45H84O6	720.6268	14.39	3
	TG(44:2)	M+NH4	C47H86O6	746.6424	14.44	3
	TG(42:0)	M+NH4	C45H86O6	722.6424	14.75	3
	TG(46:4)	M+NH4	C49H86O6	770.6424	14.76	4
	TG(44:1)	M+NH4	C47H88O6	748.6581	14.77	5
	TG(46:2)	M+NH4	C49H90O6	774.6737	14.80	4
	TG(46:1)	M+NH4	C49H92O6	776.6894	15.10	4
	TG(48:1)	M+NH4	C51H94O6	802.705	15.12	5
	TG(48:1)	M+NH4	C51H96O6	804.7207	15.41	5

4.4 Discussion.

Growing evidence suggests that lipids and lipid interactions are crucial to HCV infectivity. I have interrogated this in two ways; first, I have investigated the apolipoprotein levels (apoA1, apoB and apoE) in EUs compared to controls. Secondly, I have studied serum lipidomics profiling using LC-MS platform which allowed several lipid features that distinguished the EUs from the comparator groups. The use of LC-MS lipidomics approach substantially allowed assignment and annotation of lipid moieties relevant to EU phenotype.

This work has demonstrated that whilst serum concentrations of Apolipoprotein A1, B and E are similar across cohorts, the EU group are distinctly different in their lipidome from the HC, SR, and CHCV groups as discussed in Section 4.4.2.

4.4.1 Serum apolipoprotein analysis.

There were no significant differences in serum apolipoproteins A1, B and E concentrations observed among all the cohorts. This suggests that differences are not important in conferring resistance to HCV infection. Other explanations include problems related to analysis of samples that were subjected to repeated freeze-thaw cycles, or the variability from analysing non-fasting serum. It would be necessary to conduct similar investigations in fasting samples and postprandial samples since different factors would affect postprandial lipaemia.

HCV entry in hepatocytes is a complex process involving the apolipoproteins especially apoE that mediates the LVP attachment to cellular HSPG receptor thereby playing a significant role in HCV infectivity. HCV replication takes place on lipid droplets with co-localization of core protein and non-structural NS5A to the lipid droplet. Co-

dependency of HCV assembly on VLDL secretion is demonstrated by silencing of VLDL synthesis components apolipoprotein B (apoB), apoE, and microsomal triglyceride transfer protein, all of which inhibit HCV production in Huh 7 cells. Thus, HCV replication and assembly is dependent on host VLDL pathways (Shawa, Sheridan, *et al.*, 2017). Exposure to HCV alone did not show any significant changes in apolipoprotein levels as observed in EU samples compared to comparator groups.

4.4.2 Lipidomics investigations.

The lipidomics profiling demonstrated that the HCV resistant EU phenotype was clearly distinct from HCV susceptible individuals, even those who clear viremia, either spontaneously or following antiviral therapy. The HCV-lipid interaction is an essential feature of the virus lifecycle with the early formation of lipoviral particles a key step to establish infection in susceptible individuals. The demonstration of clearly distinct lipid profiles in cases apparently resistant to HCV infection provides further support that lipid pathways are crucial for early and persistent HCV infection (Shawa, Sheridan, *et al.*, 2017).

Further interrogation of the EUs compared to CHCV patients revealed marked differences between the two cohorts. The obtained OPLS-DA plot distinguished the EUs from the CHCV. The findings suggest that there are distinct lipid profiles in exposed uninfected individuals that are different from infected patients. Such lipid species could potentially confer resistance for HCV infection in EU population. Some EUs may exhibit similar lipid profiles with CHCV as observed in the OPLS-DA model where EU overlap with CHCV patients, but this could be due to small EU sample size. This observation suggests that there is presence or absence of lipid species that could be responsible for preventing the establishment of HCV infection in EU cohort. It is not clear at this stage whether the observed lipidomics differences between the EUs and the

CHCV patients could be due to the presence of the liver disease (rather than a specific EU phenotype). However, other OPLS-DA models comparing the EUs with HCV infected groups (HCV antibody positive) in this thesis also showed that the EUs are clearly distinct from the HCV infected cohorts (**Figure 4.4**).

This data shows that there is a difference between HCV susceptibility and resistance which determines the outcome of HCV exposure. This lipidomics data has revealed fundamental differences between the exposed uninfected cases and those that were susceptible to HCV infection. Additionally, the virus – lipid interaction as expected is important and that the different lipid profiles are associated with very different outcomes. Identification of the precise lipid moieties that distinguish the susceptible from resistant phenotypes is the next step in finding out an explanation of this key interaction.

Likewise, an OPLS-DA model was created to explore the host's ability to clear the infection (i.e. EUs vs SRs). The aim was to investigate how the host's lipid-viral interaction affects host's susceptibility to HCV infection. A number of research groups have investigated the ~ 20% that are able to clear HCV spontaneously (Baden, Rockstroh and Buti, 2014). Despite both cohorts being aviremic, the SRs were susceptible to HCV infection but they spontaneously cleared the infection. The EUs were observed to be clearly different from the SRs. This represents variations in lipid profiles among HCV RNA negative populations. Several lines of evidence indicate that the host's cellular immune response to multiple HCV epitopes is vital in controlling early HCV infection. Successful early immune responses may lead to spontaneous resolution. Conversely, ineffective early cell-mediated response may lead to development of chronic HCV infection (Bowen and Walker, 2005). These lipidomics findings open another chapter that may provide useful insights into the existence of the

EU phenotype which is distinct from the SRs. Our immunological investigations also support the existence of the EUs as described in the subsequent paragraphs of this chapter. These findings strongly indicate that the EUs are a distinct group different from all the comparator groups; and the differences are very statistically significant. Therefore this data has helped to provide further insights into the existence of EUs, and rest any debate whether the EUs is a genuine group of PWIDs or are similar to SRs.

Another OPLS-DA model was created in order to observe if the HCV resistance is associated with a different lipidome than in those susceptible to HCV but resolved the infection following IFN-based treatment (i.e. EUs vs SVRs). The clear lipid lipidomics separation observed in this model suggests that there are lipid pathways that are involved in offering protection for HCV infection. The HCV sustained viral response is defined as aviremia 24 weeks following completion of antiviral therapy (Lindsay, 2002; Ghany *et al.*, 2009). In this case, SVR was achieved after interferon-based treatment, and the subjects tested HCV RNA negative but anti-HCV antibody positive. Discriminant compounds (not annotated) between the EUs and the SVRs had very low retention times, eluting very early in the chromatographic domain along with the solvent front. Therefore such polar compounds were not easily annotated using the online databases and were classified as unknowns. The effects of therapy on perturbations of lipid metabolites have not been investigated yet, but it is clear that antiviral therapy changes the host lipidome.

The host's ability to resolve HCV infection either spontaneously or following antiviral therapy is dependent on a complex set of interactions between the virus and the host that ultimately changes the lipidome. In addition, two different groups who were both susceptible to HCV infection were also investigated in this study. These two cohorts achieved viral resolution by different mechanisms. The OPLS-DA plot distinguished

the SRs from the SVR group. The separation between the two HCV susceptible groups indicated presence of different lipid profiles even after viral clearance. The lipidomics separation confirms that there are different mechanisms involved in host's ability to resolve infection spontaneously and after interferon-based therapy. Variables that affect virus/host lipid interactions that predict clinical outcomes are different between the two cohorts. I suggest further investigation of lipid profiling between spontaneous resolvers and CHCV patients that failed to respond to treatment in order to establish the differences in their lipidomic classes.

The OPLS-DA model for SR vs CHCV was explored and it indicated the host's ability to clear HCV infection. This model was aimed at investigating the lipid factors that enable the host to resolve HCV infection spontaneously compared to absence of similar lipidomics compounds that lead to development of chronic infection. Again the SRs were observed to be different from the CHCV patients.

The OPLS-DA model for CHCV vs SVR was also explored. There were some lipid profile differences observed between the CHCV and SVR groups. This suggests that variations in serum lipidomics features that specifically related to HCV, change after HCV clearance following treatment. It is unclear if similar differences would be observed if the SVRs achieved sustained response post DAAs therapy.

I have analysed the lipidomics to assess the HCV exposure between the EUs that secreted IFN-γ and those that did not. One of the traditional criticisms of the EU group is that you cannot confirm exposure. The surrogate for exposure has been the presence of T cell responses as detected by ELISpot assay. The similar lipidomics profiles seen in this study in both ELISpot positive and negative EU cases suggests that the EU population is similar and that the presence or absence of T cell responses does not confirm or exclude evidence of HCV exposure.

The LC-MS technique using PCA has a high degree of reliability and reproducibility in both ionisation modes.

The effect of storage time and freeze-thaw cycles was observed when the PCA model was created for all the samples. This suggests that increased freeze-thaw cycles affect lipid stability and potential viability in MS analysis. Therefore lipidomics studies utilising samples of increased freezing cycles must be interpreted with great care; where possible such samples should be avoided. Freeze thaw cycles clearly showed great impact on lipid profiles. Previous studies showed that some lipid metabolites were reduced by freeze-thawing (Ishikawa *et al.*, 2014). Interestingly, when all old samples were analysed separately they still clearly showed the EU lipidome to be different from healthy volunteers.

Furthermore, there were no variations observed in samples collected in Plymouth and those obtained for the HCVRUK biobank based on OPLS-DA model in Section 4.3.14 . This confirmed that the Plymouth samples met the required standard and were comparable to the biobank samples. Therefore the findings in this study were not influenced by differences between centres.

4.4.3 Annotation of lipid species that distinguish the EUs from the other groups.

Lipidomics profiling has shown clear difference between the EUs and the control groups. It is essential to identify where these differences lie in the spectrum. I have assigned lipid species focussing on the highest ranked species (top 10) that discriminate the EUs from the other cohorts; prioritized based on their VIP values. Assignment of lipid species is a complicated process; but candidate lipidomics moieties were carefully annotated utilising their *m/z* values as described is Section 2.9.2.7.

ESI-MS is a robust sensitive tool that has reliably been used in clinical and biomedical research to study small (femto-molar) quantities of lipids of various polarities that are hardly detectable by other conventional methods. The preliminary assignment of lipid species in this lipidomics study identified phosphatidylcholine (PC) and triglycerides (TG) as the main lipidomics classes among the top 10 compounds highly expressed among EUs.

A classic OPLS-DA comparison of EUs versus CHCV group, the EUs showed significant increase in PCs and TGs as opposed to the CHCV patients. Similarly, TGs were also the main lipid class identified that distinguished the EUs from the SRs. The TGs were increased in SRs which supports the evidence that lipid metabolism plays a crucial role in HCV infectivity.

The findings of elevated PC and TGs in EUs versus CHCV OPLS-DA model reinforces the evidence that HCV infection relies on host-viral lipids interactions at all stages of its life cycle (i.e lipid metabolism affects host susceptibility to HCV infection or vice versa). The elevated levels of lipoproteins in the circulation could suggest a defect in their clearance mechanisms through the liver which is consistent with our data because of the expression of high PCs in EUs. Our data further shows that HCV utilises lipid metabolism at each stage of its life cycle in that we observed different lipid compounds that separated the EUs from those that were susceptible to HCV.

Furthermore, the following TGs: TG(52:4), TG(52:3), TG(52:2), and TG(56:5) were assigned in EUs as opposed to CHCV patients but their acyl side chains were not fully characterised. All TG compounds were detected in positive ionisation mode and the dominant ions were $[M+NH_4]^+$ adducts, their related dimers and isotopes were observed as well. Due to the lack of hydroxyl groups in the fatty acid chain, TGs cannot ionise properly and are not detected in negative mode. The following TGs: TG(40:0),

TG(42:0), TG(42:1), TG(44:1), TG(44:2), TG(46:1), TG(46:2), TG(46:4) and TG(48:1) were also high in SRs as opposed to EUs. In separate studies, HCV infection was reported to be associated with decreased plasma TG levels (Marzouk *et al.*, 2007) and this supports the evidence that HCV infection is associated with lipid metabolism. Previous studies have reported low TGs levels in HCV chronic cases as opposed to SRs (Dai *et al.*, 2008) which is consistent with our data in that TG levels were low in CHCV group as opposed to SRs.

The TGs are neutral lipids secreted by the liver and intestines in association with lipoproteins such as VLDL and chylomicrons. The TGs secreted as VLDL and chylomicrons are derived from lipid droplets (Wiggins and Gibbons, 1992). To link TGs and PCs, the PC biosynthesis was reported to be associated with TG secretion, therefore any interference in PCs secretory pathway directly influences changes in TGs synthesis (Moessinger *et al.*, 2014). Inhibition of PCs secretory pathway leads to potential increase in TGs formation (Jackowski, Wang and Baburina, 2000). Therefore, the role of TGs in confirming resistance to HCV infection is of interest.

4.4.4 Phosphatidylcholine synthesis.

The PC is the major phospholipid component of lipoproteins (Skipski *et al.*, 1967) responsible for lipoprotein assembly and excretion. Three pathways have been described for PC synthesis namely: 1. The CDP-choline pathway, 2. The phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway and 3. The Land Cycle.

The PC de-novo synthesis via CDP-choline also known as Kennedy pathway is the major pathway occurring in all nucleated cells (Kennedy and Weiss, 1956). Three enzymatic steps are involved in catalysis of choline to PC. Firstly, choline obtained

from a dietary source is phosphorylated by choline kinase using adenosine triphosphate (ATP) to produce phosphocholine. Secondly, this follows a reaction between phosphocholine and CTP catalysed by CTP:phosphocholine cytidylyltransferase (CT) for production of CDP-choline. The third enzyme in this pathway is CDP-choline:1,2-diacylglycerol cholinephosphotransferase that converts CDP-choline to form PC (Vance and Vance, 2004; Cole, Vance and Vance, 2012; Vance, 2015). This reaction occurs at the surface of the endoplasmic reticulum (ER).

The second pathway for PC synthesis is PEMT pathway. PC is endogenously synthesised primarily in the liver through this second pathway where 20 – 40% of total hepatic PC is synthesised (Sundler and Akesson, 1975; Noga, Zhao and Vance, 2002; Da Costa *et al.*, 2011) and consists of ~70% of total phospholipids of VLDL (Ågren, Kurvinen and Kuksis, 2005). PEMT is active in the ER where three repeated methylation reactions that convert phosphatidylethanolamine (PE) to PC occur. The PE is methylated by PEMT for the production of PC through phosphatidylmonomethylethanolamine (PMME) and phosphatidyldimethylethanolamine (PDME) intermediates (Shields *et al.*, 2003; Hartz and Schalinske, 2006; Jacobs *et al.*, 2010). PC(38:6) and PC(40:6) fragments are products of PEMT hepatic pathway that may influence the biosynthesis of PC (DeLong *et al.*, 1999). In this study, PC(38:6), PC(38:5), PC(36:4), PC(36:5), PC(34:2),and PC(34:1) were highly expressed in EU subjects which may indicate a possible perturbation linking the viral-lipid interaction in the liver; resulting in protection from establishment of HCV infection. The PC (40:6) was not annotated in this study.

Thirdly, PC is also synthesised through the Lands cycle (Jacobs *et al.*, 2010). In the Lands cycle PC is synthesised through the activity of phospholipase A2 (PLA2), an enzyme that removes fatty acids at the *sn-2* position for PC resulting in the production

of lysophosphatidylcholine (LPC) (Moessinger *et al.*, 2014). When fatty acids are added (re-acylation) on PC's *sn-2* position, resulting in formation of PC (Moessinger *et al.*, 2014) that is catalysed by lysophosphatidylcholine acyltransferases (LPCATs) (Lands, 1958) present in the ER. Two important subgroups of LPCATs (LPCAT1 and LPCAT2) were found to be expressed on the surface of lipid droplets (LDs); and were essential for the production of PC. The LDs are metabolic cellular organelles that regulate storage and trafficking of neutral lipids (Liu *et al.*, 2004). The LDs consist of neutral lipids core of triglycerides, and PC as a lipid monolayer (Tauchi-Sato *et al.*, 2002; Walther and Farese, 2009). HCV replication takes place on double membrane vesicles of LDs with co-localization of structural core protein and non-structural NS5A (Masaki *et al.*, 2008).

Studies have reported that decreased PC concentration was reported to impair hepatic secretion of VLDL (Rusiñol, Verkade and Vance, 1993). It was also reported that PC expression on the surface of VLDL is involved in viral-host cell receptor interaction that consequently affects the rate of lipoprotein removal from the circulation (Fielding and Fielding, 2008). Therefore, one additional possible explanation for the increased PC expression in EUs is the altered interation between the LVPs and the host cellular receptors. No previous lipidomics studies were performed in the EU cohort.

4.4.5 PC regulation.

When there is adequate supply of choline through the diet, approximately 30% of PC is synthesised in the liver through the first and second pathways described above. Choline supply is essential for regulation of PC synthesis through the CDP-choline pathway. When choline supply is limited to ensure PC synthesis, PEMT pathway maintains PC

supply in the liver. It is clear that sustainability of CDP-choline pathway is dependent on choline and CTP that are the rate limiting factors. On the contrary, PC production via PEMT pathway increases when choline supply is limited for PC production via CDP-choline pathway. Other factors that regulate PC production are the Lands cycle enzymes (LPCAT1 and LPCAT2) that affect the PC content in LDs (Moessinger *et al.*, 2014).

4.4.6 Possible pathway involved in EUs resistance.

The above explored evidence suggests that the increased PCs production in EUs may occur via the PEMT pathway or the Lands cycle pathway. Increased PCs levels in the circulation suggest perturbations in PCs clearance from the circulation through mechanisms that remain unclear. It is clear that regulation of PEMT pathway maintains normal lipid metabolism. Another possible explanation could be the interruption of the viral-host interaction at the host cellular receptors that prevents successful initiation of the viral life cycle. Again, I speculate that high serum PC concentration could be due to blocked clearance through the hepatocytes. PC is highly expressed in the lipoproteins whose clearance from the circulation is affected by the integrity of host cellular receptors. This is in agreement with the earlier possibility about perturbations in host cellular receptors. The PC is highly expressed in lipoproteins whose clearance from the circulation is affected by integrity of host cellular receptors. It is necessary to determine the lipoprotein class that expresses high PC level. It is not clear which host receptors that could be involved; but HSPG seems a possible candidate based on its reported interaction with apoE during attachment stage.

On the whole, I explored the lipidomic alterations that could be association with HCV resistance. The serum lipidomics profiling findings reveal that the EUs have unique profiles that are different from SR, SVR and CHCV groups. Serum PCs and TGs were found to be significantly elevated in EUs compared to CHCV patients. Assignment of potential lipid classes provided biological evidence that discriminated the EUs from other comparator groups. The non-targeted LC-MS 'omics' approach employed in this study has limitations such as lack of direct biomarker identification. Despite the limitations, this work demonstrates the utility of serum lipidomics profiling for identification of lipid moieties that are associated with physiological processes and disease. However, lipidomics profiling and not targeted lipid identification was the major objective of this study, justifying this approach. This data also refines the available knowledge about the HCV life cycle involving host lipid metabolism as well as re-affirming the existence of the EU phenotype among PWIDs. Therefore understanding mechanisms in which HCV disrupts metabolic pathways for viral replication provides an essential area for development of vaccine and therapeutic agents.

4.5 Conclusion.

Investigation of serum lipidomics utilising UPLC-MS system has identified perturbed lipid pathways that may be associated with resistance for HCV infection. The study cohorts were matched by demographics and clinical characteristics in order to control confounding factors that may influence the data interpretation.

The primary aim of this study was to identify lipid profiles that may be highly associated with HCV resistance. This thesis has clearly shown the difference between the EUs and the comparator groups. The preliminary efforts to identify the discriminant lipids point toward PC and TG. The next efforts should focus specifically on these compounds and others to look for biologically plausible mechanisms that can be associated with resistance to HCV infection.

The results point very strongly to the profound influence on HCV susceptibility by the lipidome. This reinforces how important the host lipid-virus interaction is; therefore, this observation calls for further study. I have preliminary identified PC and TG as being important but precisely how these perturb host lipid-virus interaction remains to be determined.

5 CHAPTER FIVE

5.1 HCV-specific T cell responses in exposed uninfected cohort compared to healthy volunteers and spontaneous resolvers.

5.2 Background.

There is published evidence including our group's previous work (Thurairajah *et al.*, 2008), suggesting that the HCV exposed uninfected cases demonstrate specific immune responses without detectable anti-HCV antibodies and HCV-RNA (Kamal *et al.*, 2004); and such cellular immune responses may result in the clearance of infection (Zeremski *et al.*, 2009). Our previous studies reported 50% of HCV-specific T helper cell responses in exposed but uninfected individuals (Thurairajah *et al.*, 2008). The frequency of HCV virus-specific T cell responses are usually low that makes it difficult to control viraemia and to design in vitro detection experiments.

The purpose of this chapter was to verify the proportion of HCV exposure among exposed uninfected IDUs as previously reported by our group. There is an on-going debate as to whether the EU phenotype exists or they are the same as spontaneous resolvers. The lipidomics investigations identified unique lipid moieties that suggest that the EU cohort is distinctly different from the comparator groups.

The exposure to HCV was assessed for production of IFN-γ utilising ELISpot assay. Detection of ELISpot positivity among the EU cases will confirm that the lipidomics investigations were performed on a truly exposed cohort; and consequently confirm that a good proportion of EU cases demonstrate T cell immune responses. The principal technique employed to identify immune responses was ELISpot assay in order to

determine the frequency of cytokine secreting T cells; and details of this assay has been described in Chapter 2.

5.3 Results.

5.3.1 Demographics of EU and SR subjects.

Due to limited number of cryopreserved peripheral blood mononuclear cells (PBMCs), twenty-two EU subjects were studied for T cell immune response to determine the exposure to HCV. All the EU cases had persistently tested negative for anti-HCV antibodies and HCV RNA using standard diagnostic methods reviewed in Section 1.7.1 and described in Section 2.2.2. All the EU cases reported current drug injection and sharing either needles or other paraphernalia. The 22 EU cases, 16/22 (72.7%) were males, whereas 6/22 (22.3%) females. Eight cases that spontaneously cleared HCV infection were analysed for the purpose of this study; of these five subjects were males (62.5%). Demographics of all cases recruited for the purpose of this thesis are detailed in Table **3.1**. The mean duration of injection drug use was 11.7 years but ranged from 1 to 34 years. All the participants had reported a minimum of 1 – 3 injecting episodes per day and they had admitted currently sharing needles or other injecting equipment.

5.3.2 High HCV-specific T cell response to HCV peptides in EUs compared to control group.

The frequency of IFN-γ T cell immune responses determined by ELISpot assay indicated that 41% of EU cases showed sufficient HCV specific T cell responses to at least one HCV peptide as shown in Figure **5.1** below. ELISpot positivity was determined by calculating the mean plus 2 standard deviation of IFN-γ response produced by the healthy volunteers. The figure below summarises the number spot forming units per million cells of EU cases that gave a positive IFN-γ response to at least one pool of HCV peptides.

Figure 5.1: Proportion of IFN-γ ELISpot positives.

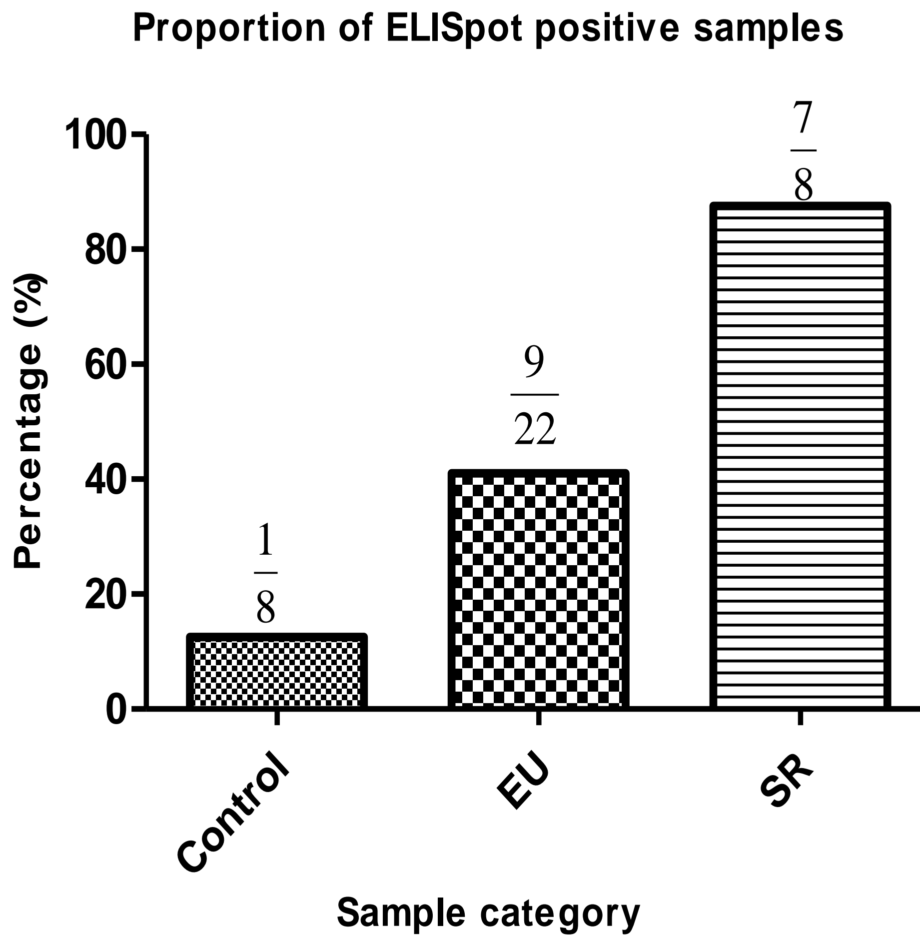


Figure 5.1 shows the percentage and the fraction of subjects studied that gave the IFN-γ ELISpot response. Only one healthy volunteer was ELISpot positive to HCV NS5B peptides. The EUs exhibited high IFN-γ response as opposed to the health volunteers and seven out of eight SR subjects were ELISpot positive to different HCV peptides. Key: EU = exposed uninfected; SR = spontaneous resolvers.

5.3.3 IFN-γ responses for specific HCV peptides for EUs compared to healthy volunteers.

Individual HCV peptides were considered to ascertain the production of IFN-γ in EU cohort compared to the healthy volunteers. The spot forming units (SFU) per million cells were counted in response to HCV Core, NS3, NS5A, and NS5B peptides. The EU showed high IFN-γ response as compared to healthy volunteers but the differences did not reach the desired statistical difference (p.value >0.05) for all the HCV peptides between the two cohorts. The p.values were as follows: Core =0.4070; NS3 =0.5638; NS5A =0.3223; NS5B =0.6877 using Mann Whitney test to compare the two groups. Wilcoxon signed rank test was also used to compare the two groups but there was no statistical difference observed with p.value >0.05 for all comparisons.

Figure 5.2: Cross sectional IFN-γ response to specific HCV peptides.

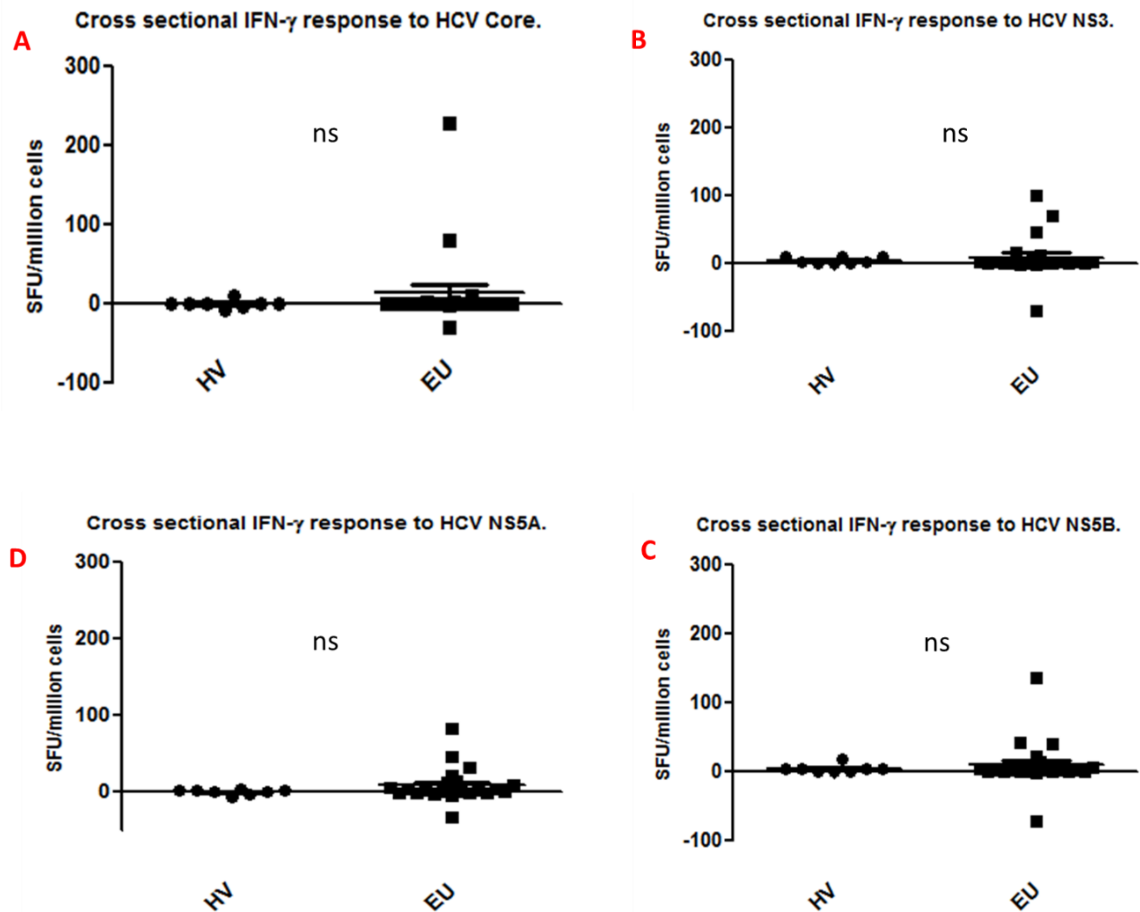


Figure 5.2 shows a cross sectional IFN-γ response for HCV peptides for EUs compared to control group. The production of IFN-γ by PBMC stimulated with HCV Core, NS3, NS5A, and NS5B peptides was measured in healthy volunteers (HV; n =11) and the exposed uninfected cases (EU; n = 22). The IFN-γ production was expressed as the number of spot forming units (SFU) per million cells. Each dot represents the mean SFU per well for one subject. All statistical estimations were evaluated by the Mann-Whitney U test but the differences were insignificant ($p>0.05$). Key: HV = healthy volunteers; EU = exposed uninfected; ns = not significant.

5.3.4 EUs exhibit high IFN-γ response to HCV NS5A peptides.

The strength of IFN-γ response for each HCV peptides was assessed for the EU cohort. High IFN-γ response was elicited by NS5A (31.8%), followed by NS5B (18.2%), NS3 (18.2%), and Core (13.6%) respectively.

Figure 5.3: The strength of IFN-γ response for each HCV peptide for EU cohort.

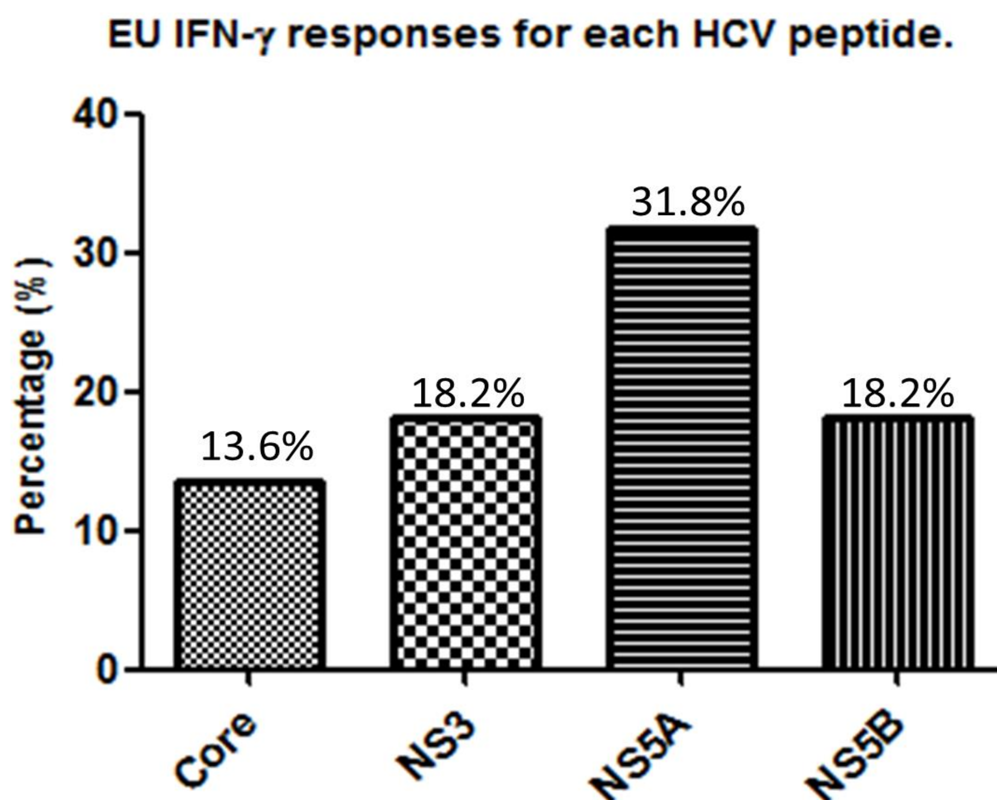


Figure 5.3 shows the IFN-γ response for EU cohort to each HCV peptide. The production of IFN-γ by PBMC stimulated with HCV Core, NS3, NS5A, and NS5B peptides was measured in healthy volunteers (HV; n =11) and the exposed uninfected cases (EU; n = 22). The EUs showed high IFN-γ responses to the HCV NS5A, NS3, NS5B and Core peptides respectively. No significant statistical differences were observed.

5.3.5 EUs demonstrated multi-specific IFN-γ responses to HCV peptides compared to healthy volunteers.

The EU subjects demonstrated multi-specific IFN-γ responses to HCV peptides. All EU ELISpot positive subjects showed IFN-γ to at least a single pool of HCV peptides (Figure 5.4A) whereas, the healthy volunteers, only one case (HV22) gave an IFN-γ response to HCV NS5B peptides.

Figure 5.4: IFN-γ response for individual EU subjects to HCV peptides.

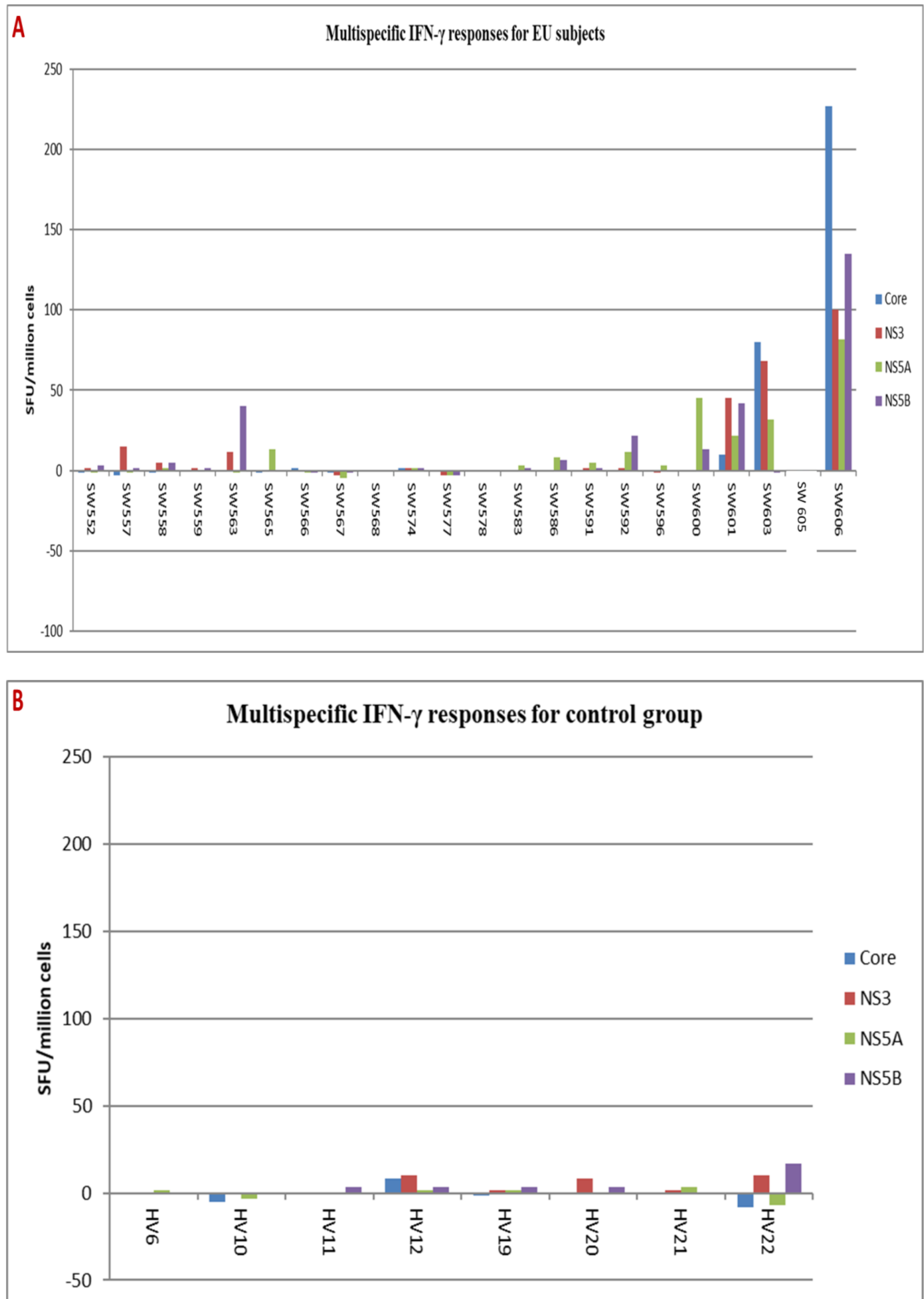


Figure 5.4 shows multi-specific responses for IFN-γ production for EUs vs HVs. The magnitude of the HCV-specific IFN-γ response to different HCV peptides (Core, NS3, NS5A, and NS5B) in exposed uninfected subjects compared to healthy control group. The exposed uninfected subjects demonstrated multi-specific IFN-γ responses to HCV peptides as compared to healthy volunteers. Each bar on the graph represent the IFN-γ response for an individual sample expressed as the spot forming unit (SFU). Key: SW = South West; HV = healthy volunteer.

5.3.6 SRs elicit stronger IFN-γ production compared to EU subjects.

In order to validate the CEFT peptide pool as an effective recall antigen (positive control) in ELISpot assay, eight samples (62.5% males) from spontaneous resolvers were analysed. Out of the eight cases, seven were ELISpot positive for at least one HCV peptide as shown in Figure 5.5 below. The strength of IFN- γ production for EU cohort was also compared to the spontaneous resolvers. The SRs elicited strong IFN- γ response to all the HCV peptides. Only one subject (SW560) was ELISpot negative.

Figure 5.5: ELISpot results for SR cases.

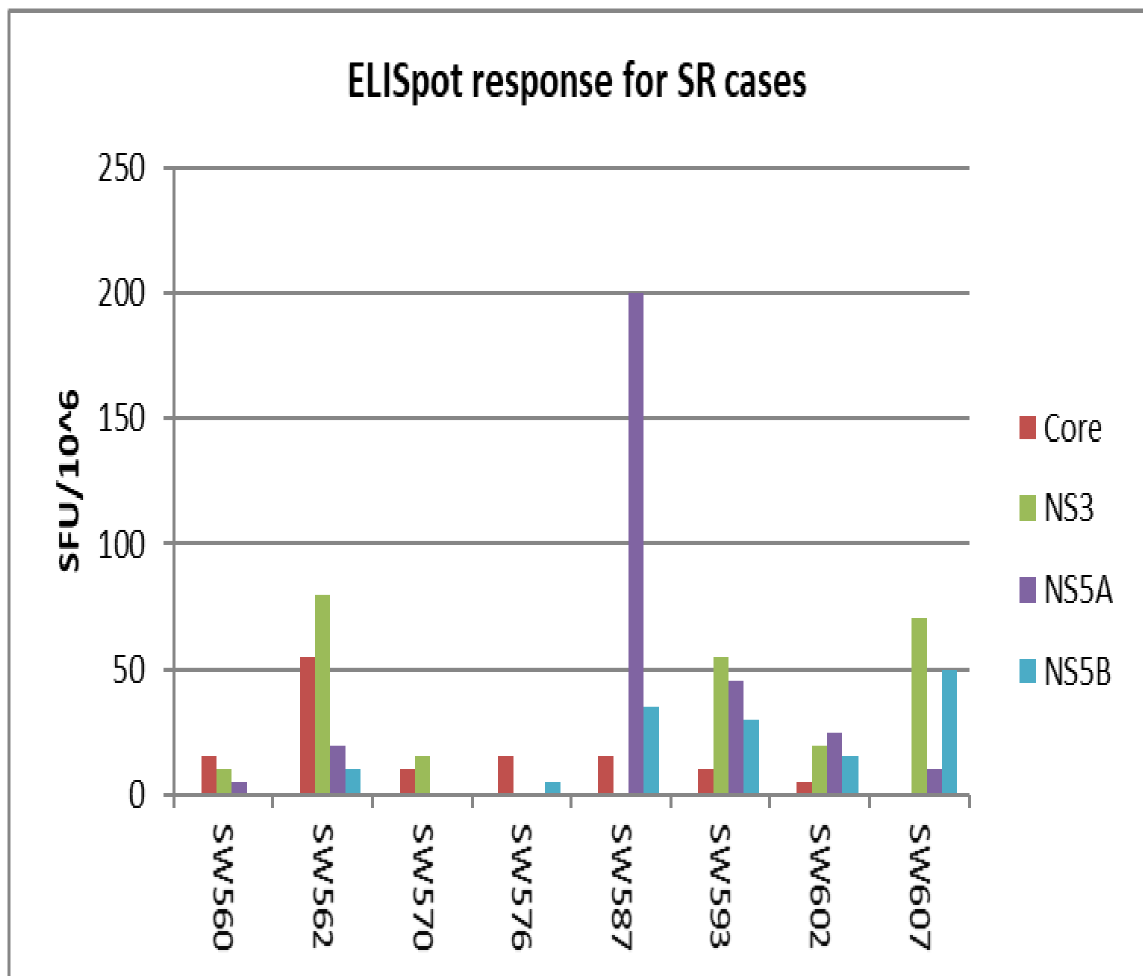


Figure 5.5 shows a proportion of positive the IFN-γ ELISpot responses to the HCV-specific peptides (Core, NS3, NS5A, and NS5B) for the spontaneous resolvers (SRs). ELISpot responses expressed as spot forming units (SFU) per million cells (peripheral blood mononuclear cells). All samples gave the IFN-γ response to atleast a single pool of HCV peptides. Each bar on the graph represent the IFN-γ response for an individual sample expressed as the spot forming unit (SFU). Key: SW = South West; SR = Spontaneous resolver; SFU = Spot forming unit.

5.3.7 The strength of IFN-γ response in EU cohort is different from comparator groups.

The strength of all IFN-γ responses was assessed by calculating the sum of SFU for all 4 HCV peptides in each subject and compared between study groups. Overall, the EUs elicited strong IFN-γ responses followed by the SR, and healthy volunteers (median 186.7; 149.2; 14.17 SFU) respectively with a significant p.value <0.0231estimated by Kruskal-Wallis test.

Figure 5.6: Strength of pooled IFN-γ responses.

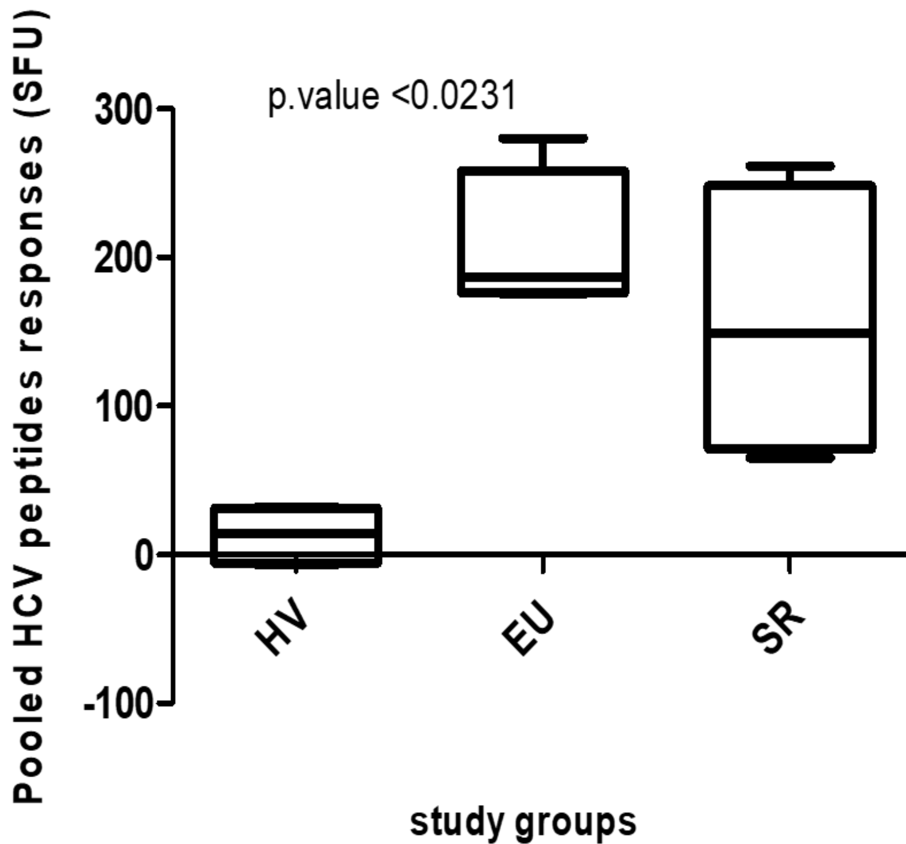
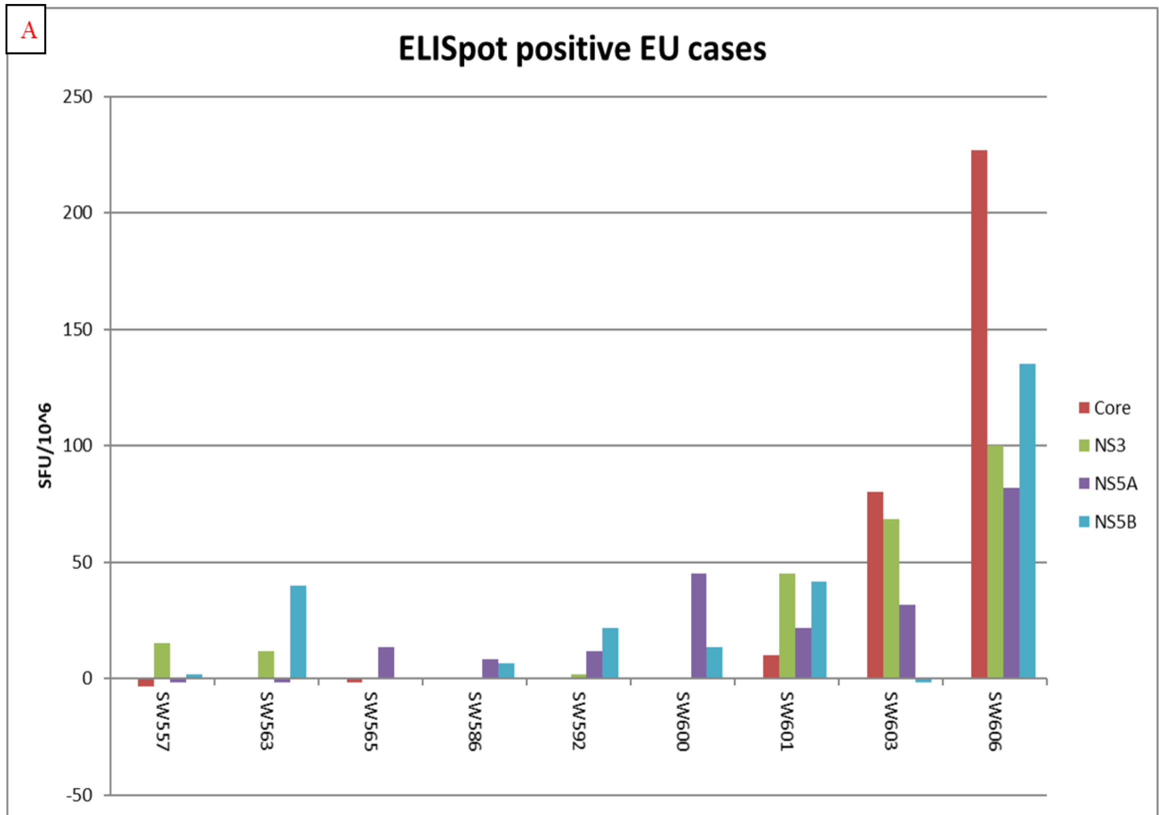


Figure 5.6 shows the strength of the sum of IFN-γ responses for 4 HCV peptides (Core, NS3, NS5A, and NS5B) for EUs compared to the SRs and healthy volunteers (p.value <0.0231, Kruskal-Wallis test (ANOVA). The EUs elicited significant IFN-γ responses when compared to the spontaneous respolvers (p.value >0.23 estimated by Kruskal-Wallis test). There was no difference in IFN-γ responses when compared to the healthy volunteers. Box and whisker plots show the median, upper and lower quartiles, as well as the ranges of the IFN-γ responses. Key: HV = healthy volunteer; EU = exposed uninfected; SR = spontaneous resolver.

5.3.8 Two separate phenotypes of EU the population exist.

The existence of the EU phenotype was confirmed by the production of HCV-specific T cell responses. The nine subjects out of the 22 were ELISpot positive whose demographics are summarised in Table 3.1.

Figure 5.7: ELISpot positives vs ELISpot negatives.



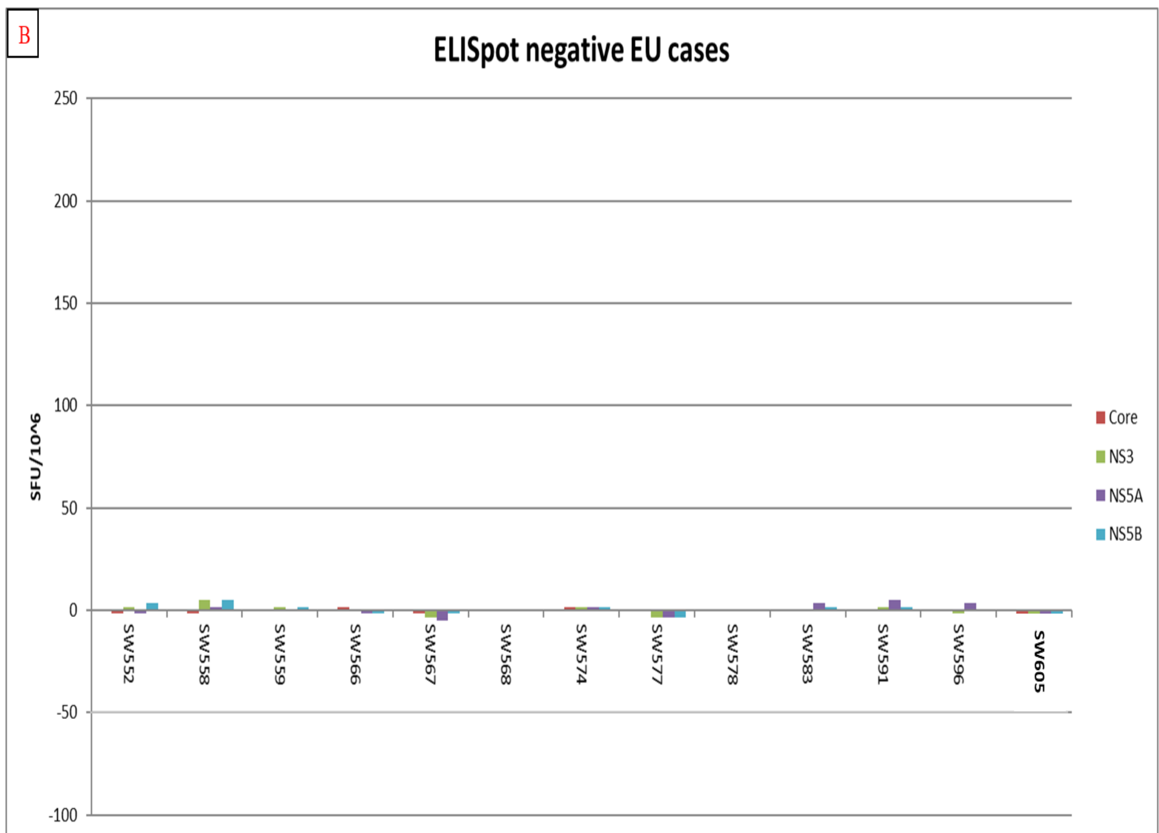


Figure 5.7 shows IFN-γ responses for individual subjects that separated the EU cohort between the IFN-γ ELISpot positive and the IFN-γ ELISpot negative phenotypes. The positive IFN-γ responses were determined based on the mean + 2SD of the healthy volunteers. The positive IFN-γ ELISpot responses were detected in nine EU cases (Figure 5.7 A), whereas thirteen EU cases (Figure 5.7 B) were IFN-γ ELISpot negative. The bars in the graphs represent an individual IFN-γ ELISpot responses for each sample expressed as spot forming units (SFU) per million cells.

5.4 Discussion.

The HCV-specific T cell responses were analysed in this study comparing the exposed uninfected cohort to the healthy volunteers in order to establish the previous exposure to HCV.

A total of thirty-eight samples from exposed uninfected cases were recruited, but only twenty-two EU subjects were studied for T cell immune response to determine the exposure to HCV since the extracted PBMCs were also used for other investigations. The findings in this chapter help to redefine the exposed uninfected cohort. Nearly half of the studied EU subjects demonstrate HCV-specific T cell responses secreting IFN-γ to at least one pool of HCV peptides. Our group (Thurairajah *et al.*, 2008) and others (Mizukoshi *et al.*, 2008) previously reported the presence of HCV-specific adaptive immune responses detected by IFN-γ ELISpot assay, among individuals who remained uninfected despite repeated exposure to HCV risk factors. Consistent with these findings, this data showed that IFN-γ production was prominent in EU subjects as opposed to the healthy volunteers; however, the differences were not significant enough between the two groups. IFN-γ responses for EUs were significantly different when compared to subjects who achieved spontaneous resolution to HCV infection.

In addition, all the 4 HCV peptide pools of Core, NS3, NS5A, and NS5B elicited IFN-γ ELISpot response where NS5A showed dominant immune responses among EU subjects. However, there was no significant difference observed in IFN-γ cross-sectional to the 4 HCV peptides. This data shows that the studied EU subjects were indeed exposed to HCV; further supporting evidence obtained through risk assessment questionnaire during case identification and recruitment process.

IFN-γ production was further assessed among SRs after PBMC stimulation with the HCV peptides utilised in similar experiment for the EU cohort. The SRs elicited strong IFN- γ response to all the HCV peptide pools; whereas the EU subjects exhibited a weaker response comparatively. This data suggests that the EU phenotype is distinct from the spontaneous resolvers. All recruited subjects had high frequency rate of injecting and sharing needles which represents a high risk group that had multiple exposures to HCV for a mean duration of 11.7 years (refer to Table **3.1**). The highest incidence of HCV infection was reported to occur during the first 2 years following initial injection drug use; and the possibility of getting infected rises with increased duration (Lorvick *et al.*, 2001). Therefore, this data supports the notion that HVC-specific T cell immune response could be used to describe HCV exposure in EU cohort. One SR subject (SW560) was a low CEFT responder; and did not give IFN-γ response to HCV peptides as well.

Evidence suggests that Th-1 cell responses are essential in spontaneous or treatment-related resolution of HCV infection. Some researchers suggested that T cell mediated immune response may offer protection in an event of re-exposure (Osburn *et al.*, 2010). Furthermore, cross-sectional studies have previously reported that HCV-specific T cell responses are primed and maintained in individuals upon repeated exposure to low viral doses; which may offer protection against development of HCV infection (Freeman *et al.*, 2004; Zeremski *et al.*, 2009). In this data, eligible study participants were identified and adequate information was obtained in relation to their risks of exposure that included duration. For this reason, and consistent with these findings, the studied participants were indeed exposed despite remaining uninfected. The IFN-γ responses dispute the possibility of the development of humoral responses as suggested by other researchers (Cramp *et al.*, 2000; Takaki *et al.*, 2000) who reported that the EU

phenotype may have resolved an acute infection in the past and had lost HCV-specific antibodies.

Equally important, IFN-γ responses have categorised the EU phenotype into two separate groups of individuals who are either ELISpot positive or negative. This data suggests that within the exposed but uninfected cohort, there are some individuals who do not elicit IFN-γ T cell responses. It will be interesting to further interrogate the immunological and genetic characteristics of these two categories of the EU population.

5.5 Conclusion.

I have demonstrated that HCV-specific T cell immune responses can reliably be utilised as an immunological marker of low dose HCV exposure. Determination of the high probability of HCV exposure based on T cell responses coupled by robust risk assessment questionnaire, and laboratory assays for detection of HCV antibodies and viraemia may aid description of the EU cohort.

The HCV-specific T cell responses were observed among EU subjects. Such T cell responses were associated with injecting habits that are high risk factors for HCV exposure. The findings suggest that the study subjects were truly exposed to HCV; further confirming the existence of the EU phenotype among some PWIDs. Though the EU cohort exhibited IFN-γ responses, but the strength of the T cell responses were weaker as compared to the spontaneous resolvers. The findings in this Chapter are consistent with our previous work; where more than half of the EU cases were expected to give demonstrable HCV specific ELISpot responses as previously reported by our group (Thurairajah *et al.*, 2008).

It is still unclear whether these T cell immune responses represent protective immunity or the involvement of the early innate immune responses. The role of the innate immunity will be addressed in the next chapter of this thesis (Chapter 6).

6 CHAPTER SIX

6.1 Upregulated innate immune responses in an HCV exposed uninfected cohort.

6.2 Background.

The findings presented in Chapter 5 of this thesis showed that nearly half of the studied EU subjects demonstrate HCV-specific T cell responses secreting IFN-γ to at least one pool of HCV peptides. Such T cell responses were not strong enough and I propose that the innate immune responses may be important.

The type I IFNs is a key mediator of the innate immune response to viral infection by directly inhibiting viral replication, preventing viral attachment to host cellular receptors, as well as indirectly stimulating innate and adaptive responses (Stetson and Medzhitov, 2006). The direct antiviral activity for type I IFNs is exerted by preventing viral transcription, translation, and cleavage of RNA (Haller, Stertz and Kochs, 2007) and at the same time promoting an anti-viral state in surrounding uninfected cells (Yokota, Okabayashi and Fujii, 2010).

The type I IFNs is a family of cytokines secreted by virally infected cells, and have diverse effects on both the innate and adaptive immune responses that promote host defence against viral infections. All the type I IFNs bind to specific IFN-α/β cellular receptor (IFNAR) complex that consists of IFNAR1 and IFNAR2. IFN-α, IFN-β, -IFN-ε, IFN-kappa (IFN-κ) and IFN-omega (IFN-ω), have been described as members of human type I IFNs (Sun, Rajsbaum and Yi, 2015). Both the type I IFNs (IFN-α/β) play an important role in activation of NK cell cytotoxicity, and upregulation of MHC-I on host cells and costimulatory molecules on APC (Biron *et al.*, 1999; Vivier *et al.*,

2008) to facilitate antigen specific T cell responses (Curtsinger *et al.*, 2005; Kolumam *et al.*, 2005). Different cell types have the potential to secrete type I IFNs.

The type I and II IFN subsets have different signal transduction receptor complexes to type III IFNs, but they activate similar intracellular signalling pathway that induce an antiviral immune response. The type III IFNs (IFN-λ) and their subsets IL-29, IL-28A, and IL-28B induce their immune response through a receptor complex distinct from the type I IFNs (Kotenko *et al.*, 2003; Siren *et al.*, 2005). The antiviral activity of IFN-λs in vivo is poorly described, but has been demonstrated in-vitro to be induced by ssRNA viruses.

All the IFNs are produced in response to viral recognition by cellular receptors and modulate the host immune responses. The virus infected cells have the potential to release viral particles that can infect the neighbouring cells. Therefore, the infected cells send IFN warning signals to the nearby cells to help them prepare against a potential virus infection. In response to IFNs, the cells secrete protein kinase RNA-activated (PKR) to facilitate shut-off of general translation, induction of apoptosis and inhibition of virus replication (Park and Rehermann, 2014).

In this study, I investigated the expression of a panel of cytokines secreted by PBMCs in response to overnight stimulation with low infective doses of Influenza A virus H3N2 strain or with synthetic model ligands of RNA viruses such as Polyinosinic-polycytidylic acid (PolyIC):Lyovec complex (RIG-I/MDA-5 ligand), and R848 (Resiquimod, TLR7/8 ligand). The data presented in Chapter 5 of this thesis, and previous work of our research group has reported low level T cell and humoral responses to HCV, but it remains unclear if these can prevent infection and recent evidence points to an important role for innate immune responses in determining the outcome of HCV exposure (Shawa, Felmlee, *et al.*, 2017).

In this chapter I have looked for differences in innate immune response to low level RNA viral exposure (influenza) and to stimulation with RIG-I and TLR7/8 ligands in exposed uninfected cases compared to healthy controls and some spontaneous resolvers of HCV infection. 38 EU, 8 SRs, and 11 healthy controls cases all collected from Plymouth were analysed for IFN-α ELISA, and the multiplex cytokine bead array. The EU and healthy control cases only were analysed for a panel of cytokines in response to the lower dose of Influenza A virus used to mimic the likely low level exposure of EU cases to HCV. For details refer to the methodology section (Chapter 2).

6.3 Results.

The data of this chapter will be presented in two categories. Firstly, the IFN-α ELISA, and secondly the luminex multiplex bead array. There were no commercial microbeads for IFN-α available to be included on the multiplex panel, hence the utilisation of ELISA technique. The Luminex multiplex assay was preferred as it provides a more comprehensive representation of the immune responses unlike measuring individual cytokines which is prone to inter-assay variabilities.

6.3.1 EUs demonstrate stronger IFN-α responses to TLR, RLR ligands and Influenza A virus than healthy controls and spontaneous resolvers.

This data shows that the EUs demonstrate a stronger IFN-α responses to TLR, RLR ligands and Influenza A virus than healthy controls and spontaneous resolvers.

Both R848 and Influenza virus induced a strong IFN-α response as opposed to PolyIC:Lyovec complex. There were IFN-α signals detected in unstimulated cells for some EU subjects as compared to healthy volunteers (p.value < 0.0013). There was insignificant difference in IFN-α response between healthy volunteers and EUs following PolyIC:Lyovec complex and R848 stimulations (p.value < 0.23, and < 0.56 respectively). Little differences were also observed in SR group that were stimulated with PolyIC:Lyovec complex and R848. The SRs were stimulated with the Influenza virus low dose only and they showed good response, whereas the EU and healthy controls were stimulated with the lower dose of Influenza virus. I observed no significant difference for IFN-α production in EU subjects in response to PolyIC:Lyovec complex stimulation, and the unstimulated cells. I observed an increase in IFN-α production in SRs in response to cell stimulation with R848, and low dose

influenza exposure. No IFN-α production was observed in response to PolyIC:Lyovec complex.

Figure 6.1: IFN-α responses for EUs, healthy controls and the SRs.

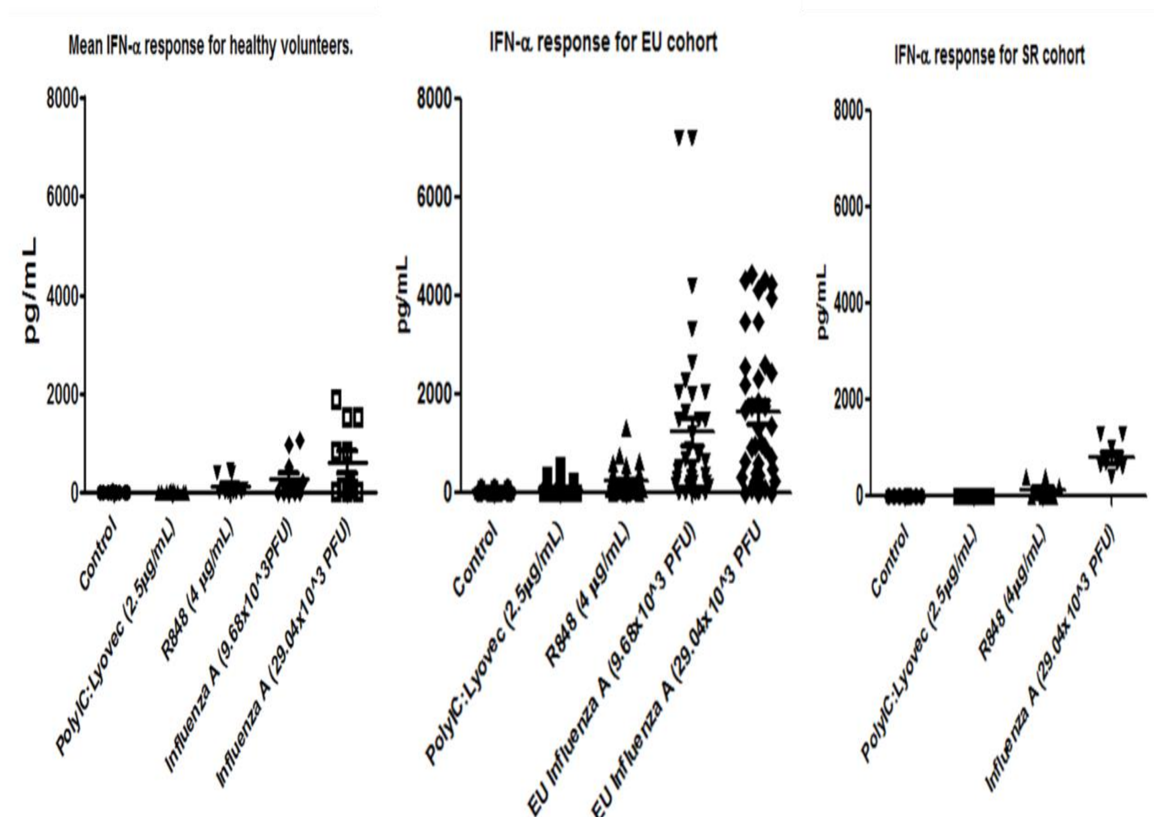


Figure **6.1** shows IFN-α production for the healthy volunteers (n=11), the EUs (n=38), and the spontaneous resolvers (n=8) in response to PolyIC:Lyovec complex, R848, and low doses of Influenza A virus measured by ELISA assay. Due to limited number of peripheral blood mononuclear cells (PBMCs), only one dose of Influenza A virus was used to assess IFN-α production in SRs. The dot plots and the error bars represent the mean and the standard error of the mean. The EUs demonstrated a strong IFN-α production in response to the lower dose of Influenza A virus but no difference was observed other stimulants.

6.3.2 EUs demonstrate stronger IFN-α responses to low doses of Influenza A virus compared to healthy volunteers.

A significant high level of IFN-α was observed in EUs compared to healthy controls following stimulation with two different low doses of Influenza A virus (p.value <0.0005). The increased IFN-α response was sustained in EUs as a result of PBMC stimulation with a low dose of Influenza A virus (p.value <0.002). Some high IFN-α secretors with low Influenza doses are coloured blue in both graphs. The blue coloured dots were samples SW565 SW568, SW552, SW559, and SW557.

Figure 6.2: IFN-α response for EUs vs healthy controls stimulated with Influenza virus.

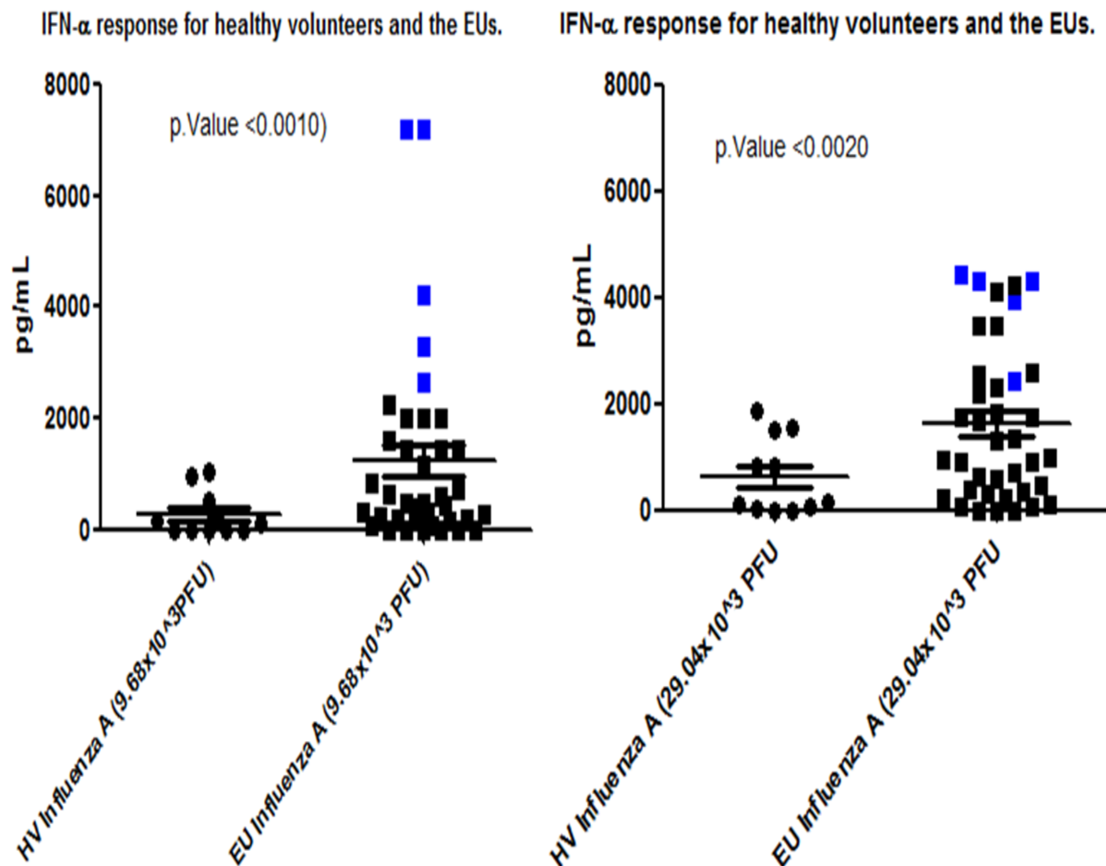


Figure 6.2 shows a separate presentation for IFN-α production in response to two doses of Influenza A virus (9.68 x 10^3 PFU – lower dose, and 29.04 x 10^3 PFU – low dose). The IFN-α production in the EUs (n=37) measured by ELISA assay was compared to the healthy volunteers (n=11). The EUs showed a significant increased IFN-α production in response to the low doses of Influenza A virus as compared to the healthy volunteers ($p<0.001$, and $p<0.002$ for lower and low doses respectively estimated by Wilcoxon signed rank test). The plots and the error bars represent the mean and the standard error of the mean. The blue coloured dots represent the high IFN-α secretors (same individuals) in both cohorts. Key: HV = healthy volunteer. EU = exposed uninfected.

6.3.3 EUs demonstrate stronger IFN-α responses to Influenza A virus than SRs.

As described above, again the EUs show significant stronger IFN-α production than the SRs when stimulated with a low dose of Influenza virus. This data is presented separately for a good graphical presentation of the separation between EUs and SRs.

Figure 6.3: The EUs secrete high IFN-α than SRs.

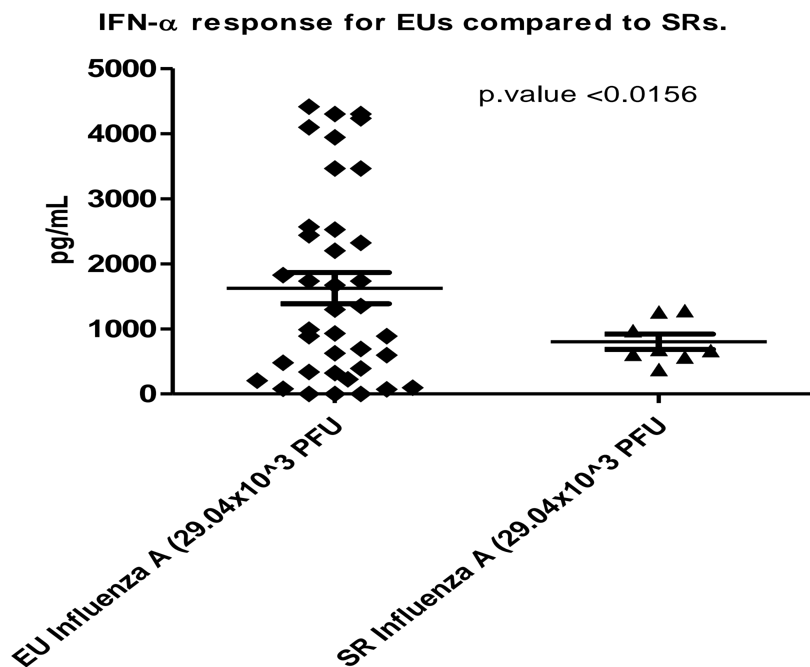


Figure 6.3 shows IFN-α production in response to the low dose (29.04 x 10^3 PFU) of Influenza A virus. The IFN-α production in the EUs (n=37) measured by ELISA assay was compared to the SRs (n=8). The EUs showed a significant increased IFN-α production in response to the low dose of Influenza A virus as compared to the spontaneous resolvers ($p<0.0156$ estimated by Wilcoxon signed rank test). The plots and the error bars represent the mean and the standard error of the mean. Key: EU = exposed uninfected, SR = spontaneous resolver.

6.3.4 EU cohort produce high IFN-α with lower doses of Influenza A virus.

In order to establish the strength of IFN-α production following exposure to lower doses of the virus, 3 EU samples (SW601, SW603, and SW606) were stimulated with different doses of Influenza A virus. 0.9×10^3 PFU was the lowest dose tested; and there was no significant difference observed in IFN-α level secreted by the other seven doses as indicated in Figure 6.4 below.

Figure 6.4: Dose dependent IFN-α secretion for EU cohort.

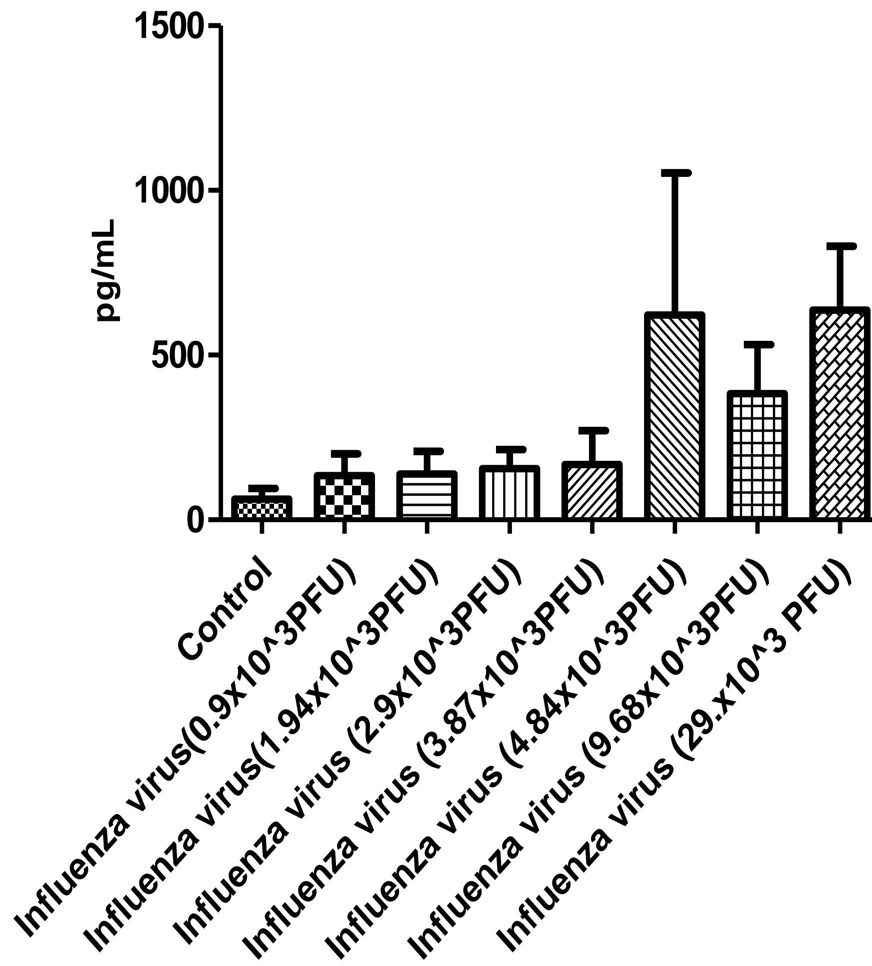


Figure 6.4 shows IFN-α production for 3 exposed uninfected samples (SW601, SW603, and SW606) only that had more PBMCs, stimulated with 7 different possible low doses of Influenza A virus ($0.9x10^3$PFU, $1.94x10^3$PFU, $2.9x10^3$PFU, $3.87x10^3$PFU, $4.84x10^3$PFU, $9.68x10^3$PFU, and $29x10^3$PFU). The ANOVA (Kruskal-Wallis test) or Wilcoxon signed rank test analysis did not show significant differences between doses. Key: PFU = Plaque forming unit.

6.3.5 EUs secrete higher levels of cytokines than the healthy volunteers in response to influenza virus.

In this next part of the study I analysed secretion of six different cytokines in response to Influenza A virus using a Luminex 6-plex magnetic bead-based assay in supernatants of stimulated PBMCs. I selected a Luminex quantitative assay that allowed detection of IFN-γ, IL-6, IL-10, IL-27, IL-28A, and TNF-α. These cytokines were carefully selected to demonstrate the main type of the immune system that is activated following cell stimulation with ligands for ssRNA virus. I studied the capacity of stimulated PBMCs to secrete different cytokines in response to exposure to low doses of Influenza A virus. IFN-γ, IL-10, and IL-27 production was significantly higher in EUs than healthy controls whereas IL-6, IL-28A, and TNF-α production was similar. The Wilcoxon signed rank test was performed on each comparison. The EU subjects that were identified as high IFN-α secretors with low Influenza dose above are coloured blue in this graph. Those values that clustered together on x-axis close to zero are hardly visible.

Figure 6.5: Cytokine production for EUs compared to healthy controls.

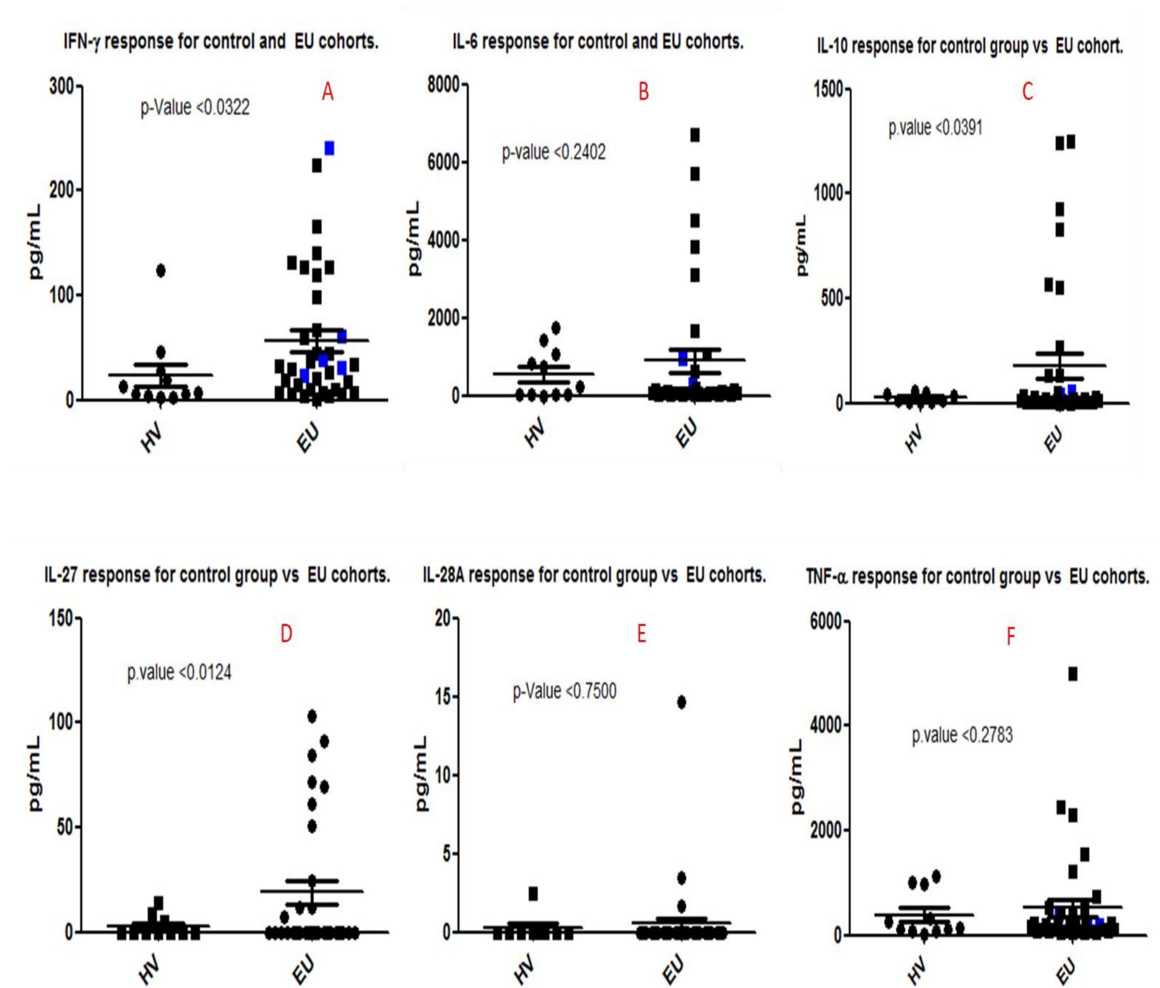


Figure 6.5 shows scatter plots for cytokine levels for EU cohort in comparison to healthy volunteers using Luminex magnetic bead-based assay. The EUs showed significant increased production of IFN-γ (fig. 6.5A p.value <0.0322), IL-10 (fig. 6.5C p.value <0.0391), and IL-27 (fig. 6.5D p.value <0.0124) compared to the healthy volunteers whereas IL-6 (fig. 6.5B), IL-28A (fig. 6.5E) and TNF-α (fig. 6.5F) production was similar estimated by Wilcoxon signed rank test. The plots and the error bars represent the mean and the standard error of the mean. Key: EU = exposed uninfected, HV = healthy volunteer.

6.3.6 ELISpot positive vs ELISpot negative characteristics.

IFN-γ ELISpot assay used for the screening of immune responses to Influenza A virus were grouped into ELISpot positive and negative subsets (Chapter 5). To further characterise the EU cohort using the multiplex Luminex assay, the ELISpot positive subgroup secreted slightly high levels of IFN-γ, IL-6, IL-10, IL-27, and TNF-α cytokines than the ELISpot negative subgroup. The IL-6, IL-10, IL-27, and TNF-α cytokines expression was significantly different, and increased in ELISpot positive subgroup than in the ELISpot negative subgroup (p.values <0.03; <0.03; 0.06; and <0.08 respectively). The ELISA assay showed slightly elevated IFN-α levels in the ELISpot negative subgroup, but the difference was not significant enough (p.value $=1.0$).

Figure 6.6: Cytokine secretion between ELISpot positive and ELISpot negative EU groups.

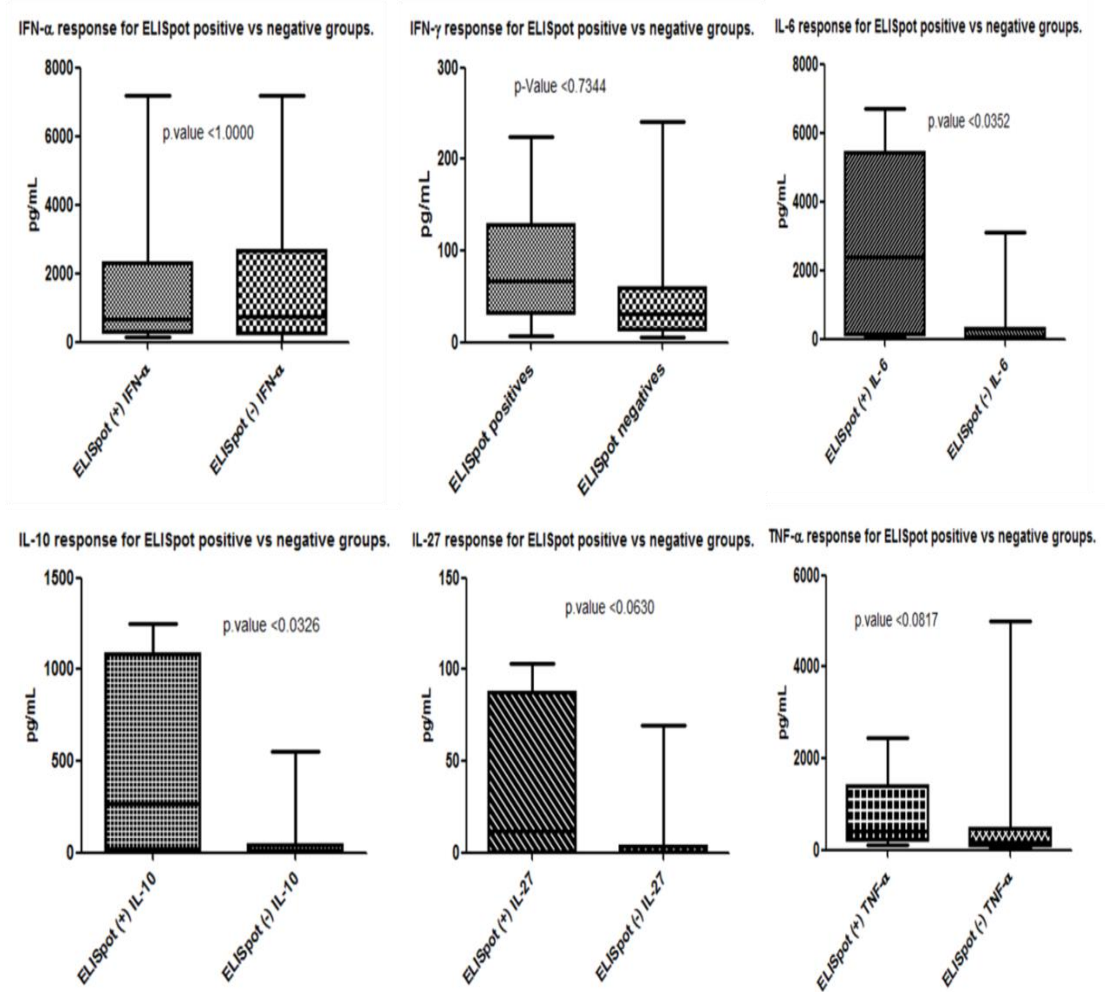


Figure 6.6 shows the comparison of cytokine secretion between ELISpot positive and negative groups. The ELISpot positives showed significant increased IL-6, IL-10, IL-27, and TNF-α production as compared to the ELISpot negative group. The box and whisker plots show the median, upper and lower quartiles, as well as the ranges of the cytokine productions.

6.4 Discussion.

The cytokines play an essential role in the mediation and regulation of immune and inflammatory responses to viral infection.

I have demonstrated significantly increased secretion of IFN-α, IFN-γ, IL-10, and IL-27 in EUs compared to healthy controls in response to Influenza A virus RNA. The differences in IFN-α production are especially striking and indicates a strong innate immune response. The EUs showed high IFN-α secretion in unstimulated cells which is consistent with the role of type I IFNs in mediating an early innate immune response that is able to prevent establishment of HCV infection.

In this study, the cytokine panel was carefully selected to demonstrate the main type of the immune system that is activated following cell stimulation with ligands for ssRNA virus. These results show that both IFN-α and IFN-γ are secreted by human PBMCs after stimulation with the lower dose of Influenza A virus. The IFN-α is reported to be produced by pDCs consistent with reports suggesting the role of pDCs in secretion of IFN-α and other cytokines (Hao, Kim and Braciale, 2008) whereas IFN-γ is produced by T cells, monocytes, and NK cells. Therefore, it is necessary to determine the cell type that secretes increased IFN-α in the EUs. The IFN-α was the principal cytokines that was strongly produced in EUs compared to the comparator groups. The high IFN-α production in the EUs is indicative of an initiation of a primary cell defence against establishment of HCV infection or others.

A complex set of interactions between the host's ability to induce an effective antiviral immune response and the viral mechanisms to oppose them, determines the outcome of exposure to HCV. The low levels of IFN-α secretion were also observed following cell stimulation with PolyIC:Lyovec complex (a RIG-I/MDA-5 ligand) in both the EU

cohort and healthy volunteers, suggesting that cytosolic PRRs are not involved in mediating the upregulated immune response found in the EU cohort. In contrast, R848 which activates cells via TLR7/8 induced some IFN-α production suggesting activation of an innate immune response.

The type 1 IFNs were discovered to have an antiviral activity against Influenza virus (Lindenmann, 1982). Th1 cells are characterised by the production of IFN-γ, IL-2, TNF-α and other cytokines; whereas Th2 cells secrete IL-5, IL-10, IL-13 and other cytokines (Lauer, 2013). Therefore, if early HCV-specific T helper cells do not produce sufficient cytokines, the possibility of development of CHCV is high. Loss of CD4 T helper cells function directly affects the proliferation of HCV-specific CD8 T cells (Wedemeyer *et al.*, 2002). Th2 cytokines are important in the inhibition of cytokines derived from Th1cell that consequently downregulates the Th1 immune responses by inhibiting APC capacity as well as promoting B cell proliferation.

I would like to speculate that individuals with more coordinated protective factors such as an upregulated innate immune response, perturbed lipid metabolism, and early recruitment of T cell response have a greater possibility of remaining uninfected despite repeated exposure to HCV.

It is interesting to note that IFN-α production was upregulated in the SR individuals than in healthy controls, but not with the same magnitude as the EUs. This data confirms that the SRs are different to the healthy control group, as well as the exposed uninfected cases. Therefore, this data shows that the SRs may not have enough, a strong innate immune response to prevent establishment of HCV infection, hence the need for an adaptive response.

I attempted to explore the dose response to lower levels of viral RNA to mirror what happens in vivo with IDUs following exposure to low levels of viral particles but the study was hampered by the limited number of cells. Therefore, due to the limited number of cryopreserved cells, $9.68x10^3$ PFU of Influenza A virus was the lower number of viral particles tested to determine cytokines secretion for EU cohort in this study. Three EU subjects (SW601, SW603, and SW606) who had more cryopreserved cells were stimulated with additional lowest viral doses. These three cases studied were selected for convenience since they had more cells than others. None of these three cases were high IFN-α secretors. The high IFN-α levels produced by the three samples suggest induction of the innate immune response due to exposure to low HCV viral particles since PWIDs are thought to be exposed to varying amounts of viral particles. It is not clear if there was any cross-reactivity as a result of exposure to other viral proteins. I used Influenza A virus in this work to mimic HCV since they are both ssRNA viruses.

One of the cytokines that was highly secreted by the EUs was IL-6 which is a pleotropic cytokine released by a number of human cells such as monocytes, DCs, and epithelial cells. The IL-6 has multiple roles that include activation of the innate immune response during an acute phase of HCV exposure (Bode *et al.*, 2012). The IL-6 level was increased in EU cohort compared to healthy volunteers. The increased IL-6 levels may be a result of activation of the innate immune cells including DCs and macrophages.

IL-10 was also investigated in order to determine the production of type II cytokines. The IL-10 is an anti-inflammatory cytokine that activates B cells for antibody production and also downregulates the development of Th1 response (Couper, Blount and Riley, 2008; Darrah *et al.*, 2010). The IL-10 was high in EUs compared to the healthy controls but not as high as IFN-α. The IL-10 is essential in offering a good

balance between pro- and anti-inflammatory responses. I therefore speculate that the high production of IL-10 in EU cohort may suggest that the host immune system had recognised the viral particles resulting in pro-inflammatory stimulus, hence the increased pro-inflammatory cytokines. These IL-10 levels would be considered low levels when interpreted in relation to high IFN-α production. Other researchers reported that IL-27 and IL-6 induced T helper cells to secrete IL-10 (Stumhofer *et al.*, 2007).

Another member of the IL6/IL-12 family of cytokines (IL-27) was also tested in this project. The EU cohort showed significant higher IL-27 secretion in response to Influenza virus stimulation than the healthy volunteers. The IL-27 is secreted by APCs and is important in activating antiviral Th1 cells (Siebler *et al.*, 2008) (Hunter and Kastelein, 2012). Similarly, increased IL-27 levels in EU subjects suggest the presence of an early induction of pro-inflammatory cytokines as discussed above in this section. Consistent with this data, the IL-27 possibly induces T cell proliferation through activation of the IL-10 which is another anti-inflammatory cytokine. Available evidence suggests that IL-27 induces T cell secretion of the IL-10 (Stumhofer *et al.*, 2007). The IL-27 has both pro- and anti-inflammatory properties that aid in bridging both innate and adaptive immune responses (Hunter and Kastelein, 2012). In addition to the immune regulatory functions, IL-27 was reported to inhibit HCV replication (Frank *et al.*, 2010) signifying its role in mediating a pro-inflammatory response. Genome wide association studies described the role of SNPs near IL-28B gene are associated with response to chronic HCV genotype 1 response. IL-28A was not identified in the cases studied.

On the other hand, those individuals who exhibit immunological evidence of exposure employ multifaceted approach including the involvement of the innate and adaptive responses to prevent HCV infection. While ELISpot responses suggesting HCV

exposure was detected, there was a weak correlation of high IFN-γ secreting subjects and IFN-α secretion offering protection from establishment of the infection. The fact that the presence of an adaptive immune T cell response has demonstrated that ELISpot did not correlate with the production of IFN-α, gives further credibility that the innate immune response is a key part in protective immunity against HCV infection.

This data suggests that low viral dose elicits an early innate immune response but such a response is not strong enough. It is not known whether early T cell activation observed in this study was as a result of Influenza viral particles or presence of an immune response already primed by exposure to HCV particles. This data concurs with previous studies that have shown clearance of low levels of HCV viraemia in the absence of seroconversion can occur without demonstrable adaptive immune responses (Post *et al.*, 2004; Meyer *et al.*, 2007). Increased injection episodes, and long duration were the main factors that delineated the EU cases into ELISpot positive and negative categories.

6.5 Conclusion.

The EUs demonstrate stronger IFN-α responses to Influenza A virus than healthy controls or spontaneous resolvers. In this study, I have demonstrated that the exposed uninfected cohort elicit HCV-specific immune responses through induction of type I IFNs, and recruitment of adaptive responses. The increased IFN-α production in response to low dose of Influenza virus indicates an enhanced induction of early anti-viral innate cytokine responses in EU subjects. I suggest that following exposure to low doses of HCV an enhanced innate immune response may contribute to the resistance to clinical HCV disease seen in our EU cohort. Since this study was performed using Influenza RNA, it would be ideal to conduct similar experiments using HCV RNA.

The possible immunological mechanisms that protect the EUs are yet to be fully described but it is possible that these responses are a consequence of exposure to low doses of HCV. This thesis confirms that these virus specific immune responses represent a footprint of exposure to HCV. I therefore hypothesis that the upregulated innate immune responses are a possible mechanism for HCV resistance in concert with the adaptive T cell and B cell responses. Further studies involving multiple cytokines and increasing the samples sizes for both the EU cohort and the comparator groups should be considered.

In conclusion, this data has shown that the EUs produce high levels of cytokines in response to Influenza virus, but we would like to know if they can produce similar levels with HCV.

7 CHAPTER SEVEN

7.1 General discussion.

This study has provided a number of new insights into potential mechanisms of resistance to HCV infection in PWIDs. I have studied urine metabolic biomarkers to seek profiles able to distinguish individuals who inject drugs who develop chronic HCV infection from those who resolve it spontaneously and those who appear to be resistant to HCV and remain uninfected despite their long history of injection drug use. The findings of this investigation revealed altered urinary metabolic profiles in EU subjects weakly indicative of altered gut microbial metabolism. However, I observed that detection of urinary metabolic signatures was prone to variability from dietary and possibly lifestyle factors resulting in the exclusion of outliers from the analysis. These findings that potentially link the microbiome to viral resistance are novel, but require confirmation in a larger cohort where careful account is taken of dietary and lifestyle influences.

The serum metabolome (complete set of metabolites) contains diverse classes of compounds including lipids among others. The lipidome is a general term used to refer to complete set of lipid classes and their subclasses, including small lipid signalling molecules. The lipidomics spectra were acquired from a large data set of 386 serum samples comprising 60 EU, 36 SR, 100 SVR, 31 HC, and 159 CHCV. Analysis was performed using an ACQUITY UPLC system in both positive and negative electrospray ionisation modes (ESI+ and ESI-). A composite quality control sample prepared by combination of equal aliquots of all samples and injected at regular intervals (i.e. one QC injection after every 9 samples) throughout the run determined the system's stability and offered reliable results. In this study, UPLC-MS lipidomics profiling identified

very highly significant differences in lipid profiles between EU and all other groups with a number of lipid moieties found to be highly expressed in EU cohort compared to chronic HCV patients. Of note, the serum lipidomics analysis identified phosphatidylcholine (PC- the building block of the family of lipids) as one of the discriminant lipid class that was highly expressed among the EUs compared to the CHCV patients.

I also measured the HCV-specific T cell responses whose magnitude was determined by IFN-γ secretion upon recognition of HCV-peptides using ELISpot assay. As expected from previous work by our research group (Thurairajah *et al.*, 2008), approximately 50% of the EU cases had demonstrable T cell responses to HCV peptides using ELISpot. However, the ELISpot positive and negative cases could not be distinguished on the basis of their self-reported injection behaviour and importantly did not differ in their lipidomics profile or in their innate immune response to stimulation with a low dose of single stranded RNA virus (Influenza). The fact that all EU cases, regardless of HCV-specific T cell reactivity, have similar lipid profiling and innate immune responses provides further evidence to support the fact that we have identified a true cohort of HCV exposed but uninfected cases and that the HCV-specific T cell response on its own is unlikely to confer protection. In this thesis I have demonstrated that exposure to low doses of ssRNA virus induces a much greater IFN- α response in EU cohort than in healthy controls and spontaneous resolvers. We know that the outcome of HCV infection is determined by series of complex host – viral interactions and that effective innate and adaptive immune responses are crucial in controlling HCV infection (Sun, Rajsbaum and Yi, 2015).

This thesis has also shown increased production of pro-inflammatory innate cytokines such as IFN-α, IL-6, and TNF- α in EU cohort as opposed to healthy volunteers

suggesting a potential role of the innate immune response in offering protection to PWIDs exposed to HCV but remain uninfected. The TLR dependent pathway (Iwasaki and Medzhitov, 2004; Kawai and Akira, 2009, 2011; Yokota, Okabayashi and Fujii, 2010; Kumar, Kawai and Akira, 2011), and the cytosolic pathway (Yoneyama *et al.*, 2004; Kato *et al.*, 2006; Park and Rehermann, 2014) are the two well described pathways that detect the HCV viral genome to limit development of CHCV infection. It would be informative to measure additional cytokines such as IL-12 which is an important pro-inflammatory cytokine that is secreted as a result of IFN-γ stimulation (Vignali and Kuchroo, 2012) to support the IFN-α, IL-6, IL-10, IL-27 and other cytokines reported in this thesis. Other researchers already reported the polymorphism of IL-12 and KIR:HLA genes as important factors associated with resistance. This thesis provides further evidence for the role played by the innate immune response in providing some protection from HCV infection following low dose exposure.

The hypotheses of this thesis were: firstly, that there are host metabolic determinants involved in interrupting the HCV life cycle; and secondly, that there is involvement of host innate immunological responses that could potentially confer resistance to HCV exposed uninfected cohort. Based on the cases studied in this thesis, both hypotheses were upheld. Therefore, I speculate that there are concerted host mechanisms involving the innate immune response, which consequently recruit an adaptive response, and perturbed host lipid – viral interactions together effectively offering protection for HCV infection. The exact putative mechanisms involved in this complex interplay that offer potential resistance for the HCV exposed uninfected cohort remain unclear and require further interrogation. The disruption of formation of LVPs in the vascular compartment, interrupted LVP attachment to host cellular receptors, the activated innate and weaker adaptive responses, all work in concert to offer protection to HCV infection. In general,

this work has provided a benchmark for further studies to describe how the innate responses and lipid – viral interactions are related.

7.2 Limitations of the study.

There were some inevitable limitations in this thesis as described below:

The major limitation of this work was the sample size for EU subjects and the comparator groups which resulted in reduced power for detection of significant differences that define the EU phenotype in comparison to the CHCV, SR, and control population. The PWIDs represent a transient subgroup within the HCV high risk population who are difficult to reach; therefore, restricted recruitment of study participant that include imposing dietary restrictions to enable sufficient recording of demographic data becomes difficult.

The multivariate analysis results from urinary NMR were less impressive than UPLC-MS. Lack of dietary and BMI data, 19 cases that were excluded from the analysis, made it difficult for the urinary NMR study to be other than observational. Larger studies are needed to tease out any significant urinary metabolic fingerprint differences between the EUs and other comparator groups.

The UPLC-MS protocols were labour intensive that involved many steps. Assignment of candidate lipid biomarkers involves complex steps that require adequate time to complete all the extracted features. Furthermore, identification of final biomarkers was difficult owing to the need for further fragmentation experiments, technical training, and external validation.

7.3 Recommendations for future work.

The research that has been undertaken for this thesis has generated very useful information and opens new important avenues on which further research would be greatly beneficial. Several gaps were identified and highlighted in the Introduction Chapter. Whilst this thesis has addressed some of them, others still remain to be considered. There are a number of potential areas for further research that have been highlighted by findings of this thesis. These include further recruitment of the EU study participants, healthy controls and other comparator groups in order to increase the sample size, and extrapolate the findings of this thesis to a larger population.

The identification and recruitment of the EU individuals is more challenging which makes longitudinal follow up even more challenging. Therefore, I propose engaging the nurses who work in needle exchange centres to assist in future case identification and recruitment process, they can combine this with their routine duties since they already work with PWIDs. This will obviously be an additional responsibility on top of their already busy schedule, therefore such decisions have to be made in liaison with the relevant authorities. More healthy volunteers have to be recruited to offer meaningful comparison and improve the statistical power of the research findings. Despite the challenges of recruiting the EUs, if possible, a longitudinal follow up on the EU cases is important to assess their serostatus changes and confirm that their resistance to HCV continues and they remain exposed but uninfected, as opposed to exposed and infected.

The existence of the ELISpot positive and negative subsets within the EU phenotype is of great interest. Future studies might, for example, look at the specific phenotypic, immunological, and genetic characteristics that describe these two subsets of PWIDs. This would help to confirm, and possibly to quantify the magnitude of any changes in

relation to cytokines productions and demographics characteristics of EUs that can emerge during case identification process.

There are also several areas for further development, such as developing an in vitro HCV replicon system to be used to stimulate PBMCs and monitor secretion of wide range of cytokines. This would give a better impression of the HCV doses that stimulate an effective innate immune response, and allow comparison of findings from our approach using low dose Influenza.

The discriminant features identified using lipidomics that separate the EUs from the other cohorts reported in this thesis need to be confirmed, and could then potentially be validated by performing the knock-in or knockdown experiments such as siRNA or CRISPR Cas 9 in HCV model systems. Further insights into alterations in PC and TG metabolism could be gained by detailed lipoprotein fractionation and interrogation of lipoprotein compositions, to assess whether this disrupts HCV-lipoprotein interactions.

Other as yet unidentified factors may also play a role in resistance to HCV infection following low dose exposure. Additional numbers of EU cases will be needed for any meaningful genome wide association study, but with collaboration with other groups this may be possible.

7.4 Conclusions.

The study of a unique subgroup of PWIDs who remain uninfected despite long and repeated exposure to HCV, has unravelled novel findings that suggest a perturbation of host lipid-viral interactions, and activated innate immune response that works in concert with adaptive responses to protect from HCV infection. This phenomenon has never been described before.

This thesis has demonstrated the role of the activated innate immune response as a potential key factor for HCV resistance. The human innate immune response plays a crucial role in providing immediate defence against invading pathogens. Putative mechanisms of HCV resistance in HCV-lipid interactions are postulated that demand further investigations to enhance understanding of resistance and immunity to HCV infection. Resistance to HCV infection depends on a complex balance between upregulation of pro-inflammatory immune response and host – viral lipid interactions.

This work further suggests that there is no single mechanism that monopolises the putative mechanisms of resistance. There is a combination of multifaceted factors involved in the HCV resistance. However, the exact nature and sequence of how the innate and adaptive responses, and lipid metabolism work to affect the HCV resistance phenotype remains unclear and requires further elucidation.

Therefore, elucidation of the mechanisms that confer resistance for HCV infection remains important in understanding why some individuals who are subjected to high risk behaviours for HCV transmission get infected whereas others do not.

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9 Appendices

9.1 Appendix A

9.1.1 Consent form.

Plymouth Hospitals NHS
NHS Trust

PATIENT CONSENT FORM

SOUTHWEST STUDY OF PATIENTS WITH HCV INFECTION

Why are some people susceptible to hepatitis C and not others?

A study of innate and immunological mechanisms of protection

	The patient should complete the whole of this sheet himself/herself	(Please circle one)
1.	Have you read the patient information sheet? (Please take a copy home with you to keep)	YES/NO
2.	Have you had an opportunity to discuss this study and ask any questions?	YES/NO
3.	Have you had satisfactory answers to all of your questions?	YES/NO
4.	Have you received enough information about the study?	YES/NO
5.	Who has given you an explanation about the study? Dr /Mr/Ms …………………………………………………………	
6.	Do you understand that you are free to withdraw from the study: • At any time? • Without having to give reason? • Without affecting your future medical care?	YES/NO
7.	Do you agree to your GP being informed?	YES/NO
8.	Have you had sufficient time to come to your decision?	YES/NO
9.	Do you agree to have some of your blood and/or urine kept and used to study genetic/immune factors; changes in the lipidome during HCV infection	YES/NO
11	Do you agree to take part in this study?	YES/NO

Participant

Name (BLOCK
LETTERS) …………………………………………………………………………………..

Address: ……………………………………………………………………………………..

D.O.B: ………………………………………………………………………………………….

Hospital/NHS Number.…………………………………………………………………...

Signed……………………………………………………………………………………….

Date…………………………………………………………………………………………

INVESTIGATOR

I have explained the study to the above participant and he/she has indicated his/her willingness to take part.

Signed………………………………………………………………………………………

Date…………………………………………………………………………………………

Name (BLOCK LETTERS) ……………………………………………………………….

9.1.2 Questionnaire (version 4/2015).

Why are some people susceptible to hepatitis C and not others?

A study of innate and immunological mechanisms of protection.

Questionnaire

Date: Trial No:

Initials: Date of Birth: Age:

1. How old were you when you first used drugs IV?..

2. Did anyone else teach/ help you with your first injection? YES/NO

3. Roughly how old was the person who injected for you?...............................

4. How long were you being injected before you could to do it yourself?.........

5. How many years have you been/were you injecting drugs? ……………….

6. At the most, how often were you injecting during that time?

Less than once a month	□	About once a month	□
2 – 3 times a month	□	About once a week	□
2 – 3 times a week	□	Most days	□
1 – 3 times a day	□	4 – 6 times a day	□
More than 6 times a day	□		

7. Which drug(s) did you mainly inject?

Heroin	□
Crack	□
Cocaine	□
Amphetamines (speed)	□

Other…………………………………………………………………………..

8. Do you still use drugs IV? YES/NO

If not, when did you stop? ………………………………………………….

If yes, how often do you inject at the moment?

Less than once a month	□	About once a month	□
2 – 3 times a month	□	About once a week	□
2 – 3 times a week	□	Most days	□
1 – 3 times a day	□	4 – 6 times a day	□
More than 6 times a day	□		

9. Are you on a maintenance script? YES/NO

10. Which drugs have you injected in the last 3 months?

Heroin □

Crack □

Cocaine □

Amphetamines (speed) □

Other……………………………………………………………………

11. Do/have you ever injected with others around? YES/NO

If yes, roughly how often do you inject around others?

Every time □

About half the time □

Most times □

Rarely □

12. Where is the most common place you inject with others?

Home □

Friend's house □

Shooting gallery/ Sorter house □

Outside/ Public areas □

Other place □

Please specify……………………………………………………………

13. Have you ever shared a needle (pin) with anyone else (using it either before OR after them)? YES/NO

If yes, roughly how many times?

Once	□	Rarely	□
About half the time	□	Most times	□
Every time	□		

If yes, when was the last time? …………………………………………..

14. Have you ever shared a syringe (barrel) with anyone else (using it either before OR after them)? YES/NO

If yes, roughly how many times?

Once	□	Rarely	□
About half the time	□	Most times	□
Every time	□		

If yes, when was the last time? …………………………………………..

15. Have you ever shared a spoon/ water container/ filter with anyone else (using it either before OR after them)? YES/NO

If yes, roughly how many times?

Once	□	Rarely	□
About half the time	□	Most times	□
Every time	□		

If yes, when was the last time? ……………………………………………

16. Have you ever shared ANY injecting items with someone you KNOW has hepatitis C (using it before OR after them)? YES/NO

If yes, what did you share? ………………………………………………….

If yes, roughly how many times have you shared with someone you know has hepatitis C?

Once	□	2 – 10 times	□
More than 10 times	□	Every time	□

When was the last time?..

17. Have you ever snorted drugs? YES/NO
If yes, did you share the straw/banknote etc with anyone else (using it either before OR after them)? YES/NO

Have you ever shared a crack pipe with anyone else (using it before OR after them)? YES/NO

18. Have you ever shared a crack pipe with anyone else (using it before OR after them)? YES/NO

19. Have you ever received a blood transfusion or blood products? YES/NO/DON'T KNOW
If yes, in which year/years? …………………………………………………

20. Do you have tattoos? YES/NO
If yes, how many? ………………………………………………………..

If yes, where did you get these done?

Professional parlour □ Friend did it □

Did it yourself □ In Prison □

Other (please state) …...

21. Have you ever had any part of your body pierced? YES/NO
If yes, how many? ……………………………………………………….

If yes, where did you get this done?

Professional parlour □ Friend did it □

Did it yourself □ In Prison □

Other (please state) …...

22. Have you ever had acupuncture? YES/NO
If yes, where? ………………………………………………………………

23. Do you know if anyone you've ever had sex with has/had hepatitis C? YES/NO/DON'T KNOW

If yes, did you use a condom? YES/NO

24. Does anyone else that you live with inject drugs? YES/NO
25. Have you ever been in Prison? YES/NO

Thank you.

9.1.3 Patient information sheet.

Study Title

<u>Why are some people susceptible to hepatitis C and not others.</u>

What is the purpose of the study?

Hepatitis C virus (HCV) currently infects 200,000 to 400,000 people in the United Kingdom. Many people have become infected through injection drug use and up to 75% of people who have injected drugs for 6 months or more will have hepatitis C virus infection. However, some people who have injected drugs for years or shared injecting equipment with people known to have hepatitis C do not become infected and test negative for both antibody and virus. It is these people we are interested in for this study. We wish to understand what can make some people resistant to infection with hepatitis C virus.

The aim of this project is to identify immunological and / or inherited factors responsible for protection from HCV infection. There is still a large gap in our understanding of how this may happen, but information gained from this study has the potential to be of great importance in the development of new treatments and possibly design of a vaccine.

Why have I been chosen?

You have been potentially exposed to hepatitis C through injection drug use, however your results show no sign of hepatitis C virus infection. It may be that your immune system has protected you against hepatitis C virus, or it may be that you have inherited some factor that makes you resistant to this infection.

We wish to study your blood and urine to analyse immune, metabolic and genetic factors that may have protected you.

Who is organising the study?

Professor Matthew Cramp, Consultant Hepatologist and Honorary Senior Lecturer in Medicine is running this research project with the help of NHS Research and Development money at Derriford Hospital, Plymouth. The research will take place at Derriford Hospital, Plymouth University Peninsula Schools of Medicine and Dentistry in collaboration with other research units in London and Glasgow.

What will happen to me if I take part?

If you agree to take part in our study

- We will ask you to fill in a questionnaire detailing your injection drug usage to assess the duration and degree of your exposure to hepatitis C virus infection.
- We will ask your permission to take about 40 mls of your blood (two tablespoonfulls) and a small volume of your urine. We may ask you to provide blood samples in the morning before your breakfast (fasting) another specimen after we have provided you with some food.
- Your blood will be tested for hepatitis C once again and will be used to study immune responses and genetic factors and lipid profile that may have protected you from infection.
- With your permission, we will store some of your blood and urine samples for testing in the future when additional genetic factors likely to influence susceptibility to hepatitis C have been identified.
- We may ask you to return at specific time intervals in the future for further blood tests.

Are there any disadvantages in taking part in this study?

There may be some soreness and bruising after having the blood sample taken.

We will be testing you on several occasions for any evidence of hepatitis C virus infection and it is possible that you will be found to have hepatitis C virus infection by highly sensitive modern tests. If this is the case then you will be informed of the result and you will be referred to our hepatitis clinic for further information and assessment.

What are the possible benefits of taking part?

There are no direct benefits to you from participating in this study. However, information learnt from you may help towards our understanding of this disease and will be a step closer towards developing a vaccine for hepatitis C.

Is my doctor being paid for including me in the study?

No.

Are there any restrictions on what I might eat or do?

No.

What if something goes wrong?

If taking part in this study harms you, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action. Regardless of this, if you have any cause to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you.

Confidentiality – who will know I am taking part in the study?

The information will be shared with doctors and nurses involved in the study only. The questionnaire you fill in will be identified by a number only and will be kept securely.

GP Notification

With your permission your GP will be informed that you are taking part in this study.

What will happen to the results of the study?

The results of this study will be presented during national and international specialist meetings. Results will be published in national and international peer review journals. No information identifying you as an individual will be published or presented.

Contact for further information.

If you have any problems, concerns, complaints or other questions about this study you should contact Professor. Matthew Cramp on 01752 432722. Alternatively, you may contact the Patients Services, Derriford Hospital on 01752 439884.

Thank you for taking time to consider entering this study.

9.1.4 HCV study flier/poster.

RESEARCH

WITH PLYMOUTH UNIVERSITY PENINSULA
SCHOOLS OF MEDICINE & DENTISTRY

Have you tested negative for

Hepatitis C?

Perhaps you would be interested in being involved in a study?

If you are actively injecting intravenous drugs and have shared pins, barrels or other works we need your help.

The Hepatology Research group, based at the Medical School in Derriford Hospital, has been carrying out research on people who are at risk from getting hepatitis C, but have not become infected. We are trying to determine if these people are protected from Hepatitis C which may help future work on a vaccine.

Participation simply involves filling in a questionnaire on your drug use habits and having some blood and urine samples taken.

There is a payment of £10 for participation

Please call or text the **Hepatology Research Group** on **07980 143385** for further information.

Approved by National Research Ethics Service REC 1703

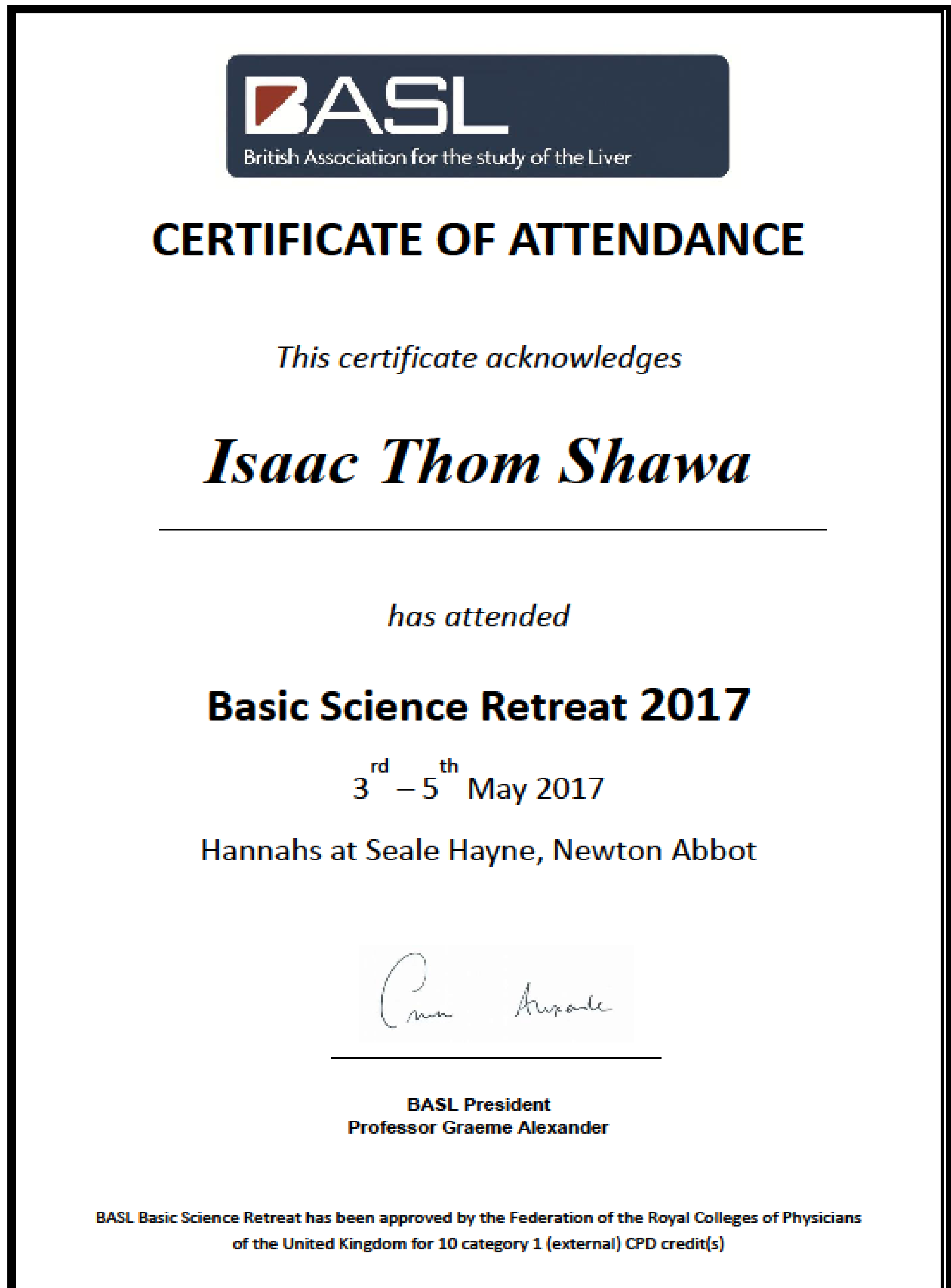
9.2 Appendix B: Urinary NMR confounding metabolites.

IoH code	NMR date	UCL code	SW	Status	Gender	Mannitol	Ethanol	Paracetamol	Taurine	Others	Dietary sheet
1	01/03/2016	AR-1	SW552	EU	F			↑			
2	01/03/2016	AR-2	SW553	EU	M		↑↑	↑			
3	01/03/2016	AR-3	SW554	Chronic	M			↑			
4	01/03/2016	AR-4	SW555	EU	M			↑↑			
5	01/03/2016	AR-5	SW556	Chronic	M		↑↑				
6	01/03/2016	AR-6	SW557	EU	M			↑		to be assigned	
7	01/03/2016	AR-7	SW558	EU	F		↑	↑			
8	01/03/2016	AR-8	SW559	EU	M			↑			
9	01/03/2016	AR-9	SW560	SR	F			↑			
10	01/03/2016	AR-10	SW561	Chronic	F			↑			
11	01/03/2016	AR-11	SW562	SR	F			↑		d 1.15ppm	
12	01/03/2016	AR-12	SW563	EU	M	↑↑		↑			
13	01/03/2016	AR-13	SW564	EU	F						
14	01/03/2016	AR-14	SW565	EU	M			↑			
15	01/03/2016	AR-15	SW566	EU	M			↑		to be assigned	
16	01/03/2016	AR-16	SW567	EU	F				↑↑		
17	01/03/2016	AR-17	SW568	EU	M					2-hydroxybutryate	
18	01/03/2016	AR-18	SW569	Chronic	M			↑			
19	01/03/2016	AR-19	SW570	SR	M			↑			
20	01/03/2016	AR-20	SW571	EU	M				↑		
21	01/03/2016	AR-21	SW572	Chronic	M		↑↑	↑			
22	01/03/2016	AR-22	SW573	EU	M		↑↑	↑		low citrate	
23	01/03/2016	AR-23	SW574	EU	F			↑			
24	01/03/2016	AR-24	SW576	SR	F			↑			
25	01/03/2016	AR-25	SW577	EU	M						

26	01/03/2016	AR-26	SW578	EU	M			↑↑			
27	01/03/2016	AR-27	SW580	Chronic	M					2-hydroxybutryate	
28	01/03/2016	AR-28	SW581	EU	M	↑					
29	01/03/2016	AR-29	SW582	Chronic	M			↑			
1	02/03/2016	AR-30	SW583	EU	M	↑		↑↑		no citrate, d 1.15ppm	
2	02/03/2016	AR-31	SW585	EU	M		↑↑			scyllo-inositol	
3	02/03/2016	AR-32	SW586	EU	M		↑	↑↑	↑↑		
4	02/03/2016	AR-33	SW587	SR	M	↑		↑			
5	02/03/2016	AR-34	SW588	EU	F						
6	02/03/2016	AR-35	SW589	EU	F			↑	↑		
7	02/03/2016	AR-36	SW590	EU	M			↑			
8	02/03/2016	AR-37	SW591	EU	F			↑		?s 3.15ppm, s 3.30ppm	
9	02/03/2016	AR-38	SW592	EU	M		↑↑	↑		scyllo-inositol	
10	02/03/2016	AR-39	SW593	SR	M	↑↑	↑	↑		low citrate	
11	02/03/2016	AR-40	SW594	Chronic	M			↑			
12	02/03/2016	AR-41	SW595	EU	M			↑			
13	02/03/2016	AR-42	SW596	EU	M	↑↑	↑	↑		low citrate	
14	02/03/2016	AR-43	SW597	EU	M			↑			BMI 21.4, Alc 1.5u/w
15	02/03/2016	AR-44	SW598	EU	M			↑			BMI n/a, Alc 8u/w & 2u 24h
16	02/03/2016	AR-45	SW599	EU	M			↑			BMI 30.0, Alc n/a, meat
17	02/03/2016	AR-46	SW600	EU	M			↑			BMI 20.7, Alc n/a
18	02/03/2016	AR-47	SW601	EU	F		↑↑	↑		scyllo-inositol	BMI 20.4, Alc 50u/w
19	02/03/2016	AR-48	SW602	SR	M		↑↑	↑		scyllo-inositol	BMI 21.8, Alc cider
20	02/03/2016	AR-49	SW603	EU	M			↑		low citrate	BMI 24.7, Alc 18u/w
21	02/03/2016	AR-50	SW604	Chronic	M			↑			BMI 33.0, Alc 9u 24h
22	02/03/2016	AR-51	SW605	EU	M			↑	↑↑		BMI n/a, Alc 2 bottles 24h
23	02/03/2016	AR-52	SW606	EU	M			↑			BMI 19.4, Alc n/a
24	02/03/2016	AR-53	SW607	SR	M			↑	↑		BMI 24.9, Alc 2u/w, methadone

9.3 Appendix C: Trainings and courses related to this thesis.

9.3.1 BASL Basic Science Retreat 2017.



9.3.2 Research Governance Training: Good Clinical Practice.

Certificate of Attendance

Isaac Shawa

PhD Research Fellow

Has attended

Research Governance – Good Clinical Practice (GCP)

On 10th May 2017

Areas covered:

- Introduction to clinical research, PPI
- Historical perspective - Nuremburg code, Declaration of Helsinki, Tuskgee Study
- Unethical research, fraud
- Organisation involved in research
- Ethical & regulatory frameworks
- Sponsor, Investigator & Researcher responsibilities
- CT & Device regulations
- Data quality
- Safety & Pharmacovigilance
- WHO GCP, ICH GCP & MRC GCP
- Informed consent
- Additional Regulation, MCA, HTA, HEFA & Data Protection
- MHRA GCP Inspection findings
- R&D Contacts

"This ICH E6 GCP Investigator Site Training meets the Minimum Criteria for ICH GCP Investigator Site Personnel Training identified by TransCelerate BioPharma as necessary to enable mutual recognition of GCP training among trial sponsors."

Dr Chris Rollinson

Research Governance Manager, PHNT

Leading with excellence, caring with compassion

9.3.3 BASL Annual Meeting 2016.

Certificate of Attendance

BASL Annual Meeting 2016

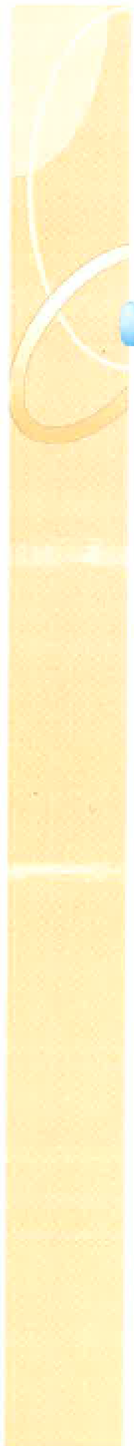
7th– 9th September 2016
Manchester Central Convention Complex
Manchester, UK

Isaac Thom Shawa

BASL President
Professor Graeme Alexander

'BASL Annual Meeting 2016'
has been approved by the
Federation of the Royal Colleges of Physicians of the United Kingdom
for up to 20 category 1 (external) CPD credit(s)

9.3.4 Human Tissues Training.



Certificate of Training

RESEARCH WITH PLYMOUTH UNIVERSITY

Human Tissues Training Session

– The Human Tissue Act 2004 - Working with Human Tissues at PU

(Please sign two copies. One copy to be completed and signed for participant and one copy to be retained by DI)

This is to certify that *Isaac Shawa* attended the HTA training session held at Plymouth University on 30th June 2015.

Dr Garry Farnham
Human Tissue Authority (HTA)
Designated Individual for University of Plymouth, License number 12103

The session covered:

- The Human Tissue Act
- Human Tissue Authority licensing, guidelines and compliance
- Relevant material and its use for the scheduled purpose of Research under the PU HTA licence:12103
- Consent, Disposal and Training : legal requirements and obligations
- Plymouth University HTA Standard Operating Procedures
- Traceability: Transfers and use, record keeping and monitoring

I understand my obligations while working with human relevant material at Plymouth University as outlined above and in the training session.

Signed attendee: Date 30/06/15

9.3.5 General Teaching Associates (GTA) course.

University of Plymouth

Certificate of Professional Development

Learning and Teaching for General Teaching Associates

A course run by Educational Development:
see www.gtacourse.co.uk for further details

This is to certify that

Isaac Shawa

has attended the General Teaching Associates course, which included taught sessions and online activities as detailed below

Sessions Attended

Theories of Learning and Teaching
Planning Sessions; Delivering Presentations
Learning in Groups; Equality and Diversity
Assessment
Evaluating your Teaching; Giving Feedback

Online Activities

Dealing with Difficult Situations
Assessment Criteria and Marking

Signed: ....

Date of issue: 8th July 2015

Dr Sharon Gedye – Educational Developer and GTA Lead

9.3.6 An Introduction of Immunology Certificate.

Certificate of attendance

This is to certify that

Isaac Shawa

attended the course

An Introduction to Immunology

held at Warwick University over the period 6 - 7 July 2015

and completed 11 hours (all) of the programme.

Signed ....[signature]............ (Course organiser). Date: 7 July 2015

School of Life Sciences

[illegible]
[illegible]
[illegible]
[illegible]
[illegible]
[illegible]

[illegible]

9.3.7 Metabolic Phenotyping training at Imperial College London.

Imperial College London

It is hereby certified that

Mr Isaac Thom Shawa

completed the

Metabolic Phenotyping in Disease Diagnosis & Personalised Health Care

organised by the Department of Surgery and Cancer

on

23/06/14 - 26/06/14

Held at South Kensington Campus

Vice Provost (Education)

Wednesday, 25 June 2014

Date

Academic Registrar

IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY AND MEDICINE

9.4 Appendix D: Peer-reviewed publications and conference abstracts.

Basic Science

Serum lipid profiling using Ultra-performance liquid chromatography mass spectrometry (UPLC/MS) discriminates HCV exposed uninfected injection drug users from those susceptible to infection.

Isaac Thom Shawa [1] Maria Gomez Romero [2] Alexandros Pechlivanis [2] Daniel J Felmlee [1] Mary Crossey [2] Elaine Holmes [2] Maggie Bassendine [2] Simon Taylor Robinson [2] David A Sheridan [1] Matthew E Cramp [3]

[1] *Hepatology Research Group, Institute of Translational & Stratified Medicine, Plymouth University Peninsula Schools of Medicine & Dentistry, Plymouth, United Kingdom*

[2] *Imperial College London*

[3] *Hepatology Research Group, Institute of Translational & Stratified Medicine, Plymouth University Peninsula Schools of Medicine & Dentistry, Plymouth, United Kingdom*

Introduction

We have previously defined a cohort of injection drug users who remain uninfected with HCV (HCV Ab and RNA negative) despite long term drug use who appear resistant to HCV infection and have termed them exposed uninfected (EU). Metabolomics and lipidomics techniques are powerful tools for detection of unique fingerprints of molecular species and metabolites in serum samples.

Aim

Our aim was to determine metabolic alterations associated with HCV resistance to gain mechanistic insight into HCV protection. In this study, serum lipidomics analysis was performed to characterise the lipidome of EU cases compared to cases with chronic HCV (CHCV – HCV Ab and RNA positive), those with spontaneous resolution of HCV (SR – HCV Ab positive, RNA negative) and healthy controls.

Method

The lipidome of 286 serum samples were analysed comprising 60 EU, 36 SR, 159 CHCV patients and 31 healthy controls , utilising EU / SR cases collected locally and additional CHCV samples from the HCV Research UK biobank. An ACQUITY UPLC/MS system (Waters) in both positive and negative electrospray ionisation modes (ESI+ and ESI-) was used following established protocols. A composite QC sample defined the system suitability, analytical stability, and sample repeatability. Raw data was extracted and converted to NetCDF files using Databridge module within MassLynx software 4.1 (Waters); and pre-processed using XCMS package within the R (3.2.5) statistical software and in-house developed scripts. The data was subjected to multivariate statistical analysis using SIMCA-P 14.1 (Umetrics). Principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) were performed on all data after pareto scaling and log transformation for detection of patterns, trends and outliers; and construction of discriminant models for classification and the discovery of potential biomarkers.

Results

Multivariate analysis of the lipidomics data using PCA and OPLS-DA showed that the HCV exposed uninfected cohort had distinctly different lipidomics features from the SR and CHCV cohorts in both positive and negative ionisation modes. The EU lipid profiling features were clearly distinguished from other groups, with strong discriminating features observed in negative ionisation mode.

Conclusion

UPLC/MS lipidomics discriminate EU from SR, and CHCV. Our preliminary findings suggest lipid perturbations may be associated with the resistance to HCV infection seen in EU intravenous drug users. Assignment and identification of lipid profiling features that distinguish the EU cohort from other classes is ongoing to gain mechanistic insights into HCV resistance.

Abstract Submitted: 06/06/2016

Basic Science

URINE METABOLIC PROFILING DISTINGUISHES HCV EXPOSED UNINFECTED INJECTION DRUG USERS FROM THOSE WITH CHRONIC OR RESOLVED HCV INFECTION

Isaac Thom Shawa [1] I.Jane Cox [2] Antonio Riva [2] James N Fullerton [3] David A Sheridan [1] Daniel J Felmlee [1] Shilpa Chokshi [2] Matthew E Cramp [1]

[1] *Hepatology Research Group, Institute of Translational and Stratified Medicine, Plymouth University Peninsula Schools of Medicine and Dentistry, UK*

[2] *Institute of Hepatology, Foundation for Liver Research, London*

[3] *Rayne Institute, University College London*

Introduction

Host resistance to HCV infection in a small proportion of individuals that are highly exposed but remain uninfected represents a unique outcome in the natural history following HCV exposure.

Aim

Determination of systemic metabolic alterations associated with HCV exposed but uninfected (EU) cases may provide information on factors that confer resistance to HCV infection. Urine metabolic profiling studies in EU cases may provide such insight.

Method

Urine samples were obtained from 10 healthy controls (HC) who had no history of HCV infection and 53 injection drug users exposed to HCV, 36 of whom remained uninfected (EU, HCV RNA and antibody negative), eight spontaneously resolved (SR) HCV infection (HCV RNA negative, antibody positive) and nine were chronically infected (CHCV, HCV RNA and antibody positive). Samples were stored at -20 ºC until NMR study. NMR spectra were obtained at 600MHz and spectral regions were integrated, normalised to the sum of the total spectral integral and mean-centred prior to multivariate analysis performed by KnowItAll® Informatics v9.0. Data were also pre-processed and subjected to principal component analysis (PCA) and orthogonal projection on latent structures-discriminant analysis (OPLS-DA) using Simca-P (Umetrics, v14).

Results

Urinary NMR data from all subjects showed good spectral resolution (linewidths of ≤1Hz) and quality. However, 19 of 53 HCV data sets and one control were excluded for various confounding factors (dominant signals from ethanol (5 EU, 1 SR, 3 CHCV); comparatively strong signals from taurine (3 EU); multiple peaks from mannitol (3 EU, 1 SR); resonances from 2-hydroxybutyrate (1 CHCV); high levels of paracetamol metabolites (1EU); and unassigned peaks (1EU, 1 SR). The final cohort for multivariate NMR analysis therefore comprised 25 EU, 4 SR, 5 CHCV, 9 controls. The EU cohort was distinguishable from SR, CHCV and controls using multivariate analysis techniques with class separation. Differences in hippurate, creatinine and TMAO accounted for the class separation.

Conclusion

Urinary metabolic profiles can distinguish EU from SR, CHCV and HC. The association between hippurate and TMAO is of interest as both are derived from gut microbial activity. Hippurate is endogenously synthesized through metabolism of gut derived polyphenols and detoxification of their bi-products in the liver and TMAO is derived from activities of gut microbiota on choline and absorption of TMAO containing diet in the liver. The potential link between the gut microbiome and resistance to HCV infection is of interest and warrants further study.

Abstract Submitted: 06/06/2016