“Acute HCV in HIV positive men; viral evolution and cellular immune responses”

Emma Cassandra Thomson

Thesis submitted for the degree of
Doctor of Philosophy of Imperial College London

Departments of Hepatology and Infectious Diseases
Wright-Fleming Institute
Faculty of Medicine
St Mary’s Campus
Imperial College London

Declaration of Originality

All experimental work presented in this thesis was generated by Dr Emma Thomson under the supervision of Dr Peter Karayiannis and Dr Janice Main at Imperial College London and Professor Paul Klenerman at the University of Oxford other than HLA typing which was carried out at the Anthony Nolan Trust, Royal Free Hospital London. Statistical methods were carried out by Dr Emma Thomson with advice given at STATA courses and in person by Dr Joseph Eliahoo, Department of Statistics, Imperial College London.

Acknowledgements

I am extremely grateful for the support of a large number of individuals without whom it would not have been possible to carry out this work. I would particularly like to thank my three PhD supervisors; Peter Karayiannis, Janice Main and Paul Klenerman who have provided me with continuous support, advice and encouragement throughout the last 4 years. I would especially like to thank Peter for the atmosphere of learning he creates in the lab, his open door policy and encyclopaedic knowledge of molecular biology, Janice for providing me with expertise on the clinical aspects of HCV infection, for allowing me to develop the cohort and set up a dedicated acute hepatitis C clinic and Paul for opening all the resources of his laboratory in Oxford to me and allowing me to develop my knowledge of immunology in an excellent environment. I would also like to thank Jonathan Weber for his support during this time, Peter Cherepanov who introduced me to Python programming and sequence processing in 2.3 seconds versus 6 weeks, Martin Norris who helped with Excel macros, Vicki Fleming who taught me phylogenetic analyses, Ruth Simmonds for teaching me how to do ELISpots and Ayako Kurioka for teaching me flow cytometry. I am also grateful to Joseph Eliahoo for teaching me how to analyse data using STATA.

I would like to thank my family: Caroline, Alexander and Seumas in Glasgow; Kristiina, Erika, Erkki and Jukka in Helsinki, Kate in Barcelona, Rosemary in Edinburgh, Penelope in Cambridge and my grandparents Edna and Edgar and Elaine who introduced me to the Monty Hall theorem. Finally I thank Martin and Minka who sadly did not make it to see the final version, for their encouragement, many late night meals, endless cups of tea and coffee and for waiting and waiting and waiting.

Abstract

Background: The natural history of early HCV infection has been only partially characterised previously, as primary infection is usually asymptomatic. An emerging epidemic of acute HCV virus (HCV) infection in HIV-positive men in Europe, Australia and the USA has allowed the recruitment of new cohorts of patients with early HCV. We aimed to describe the natural history of virus evolution and cellular immune responses in a rare cohort of acute HCV infected HIV-positive patients in order to identify key determinants of spontaneous clearance.

Methods: 110 patients were recruited and followed at 1-3 monthly intervals for a median period of 3 years. HCV E2 envelope, NS5B polymerase and NS5A genes were amplified at multiple time points using PCR and cloning techniques from plasma, liver and lymphocytes. A library of 2295 HCV E2 sequences were available for analysis .The functional immune response was assessed by ELISpot and flow cytometry.

Results: 14% of patients cleared HCV spontaneously while 86% progressed towards chronicity. Three patterns of infection were observed based on viral load dynamics; spontaneous clearance (SC) and 2 patterns of progression; plateau (PV) and fluctuating viraemia (FV). SC was associated with a >1.7$\log_{10}$ viral load drop within 100 days of infection (OR=2.76; p=<0.001), slow viral diversification of the envelope E2 gene and the emergence of an early multi-specific T-cell response. PV was associated with positive selection within E2 and rapid viral diversification. Superinfection with new HCV strains (40% of the cohort), delayed T-cell responses and the presence of HCV within lymphocytes were all associated with FV. CD4+ responses were particularly important in defining the final outcome of infection.

Conclusions: Spontaneous clearance of acute HCV in HIV-positive men can be predicted by rapid decline in viral load, low viral diversity and T-cell response.

Key words: HIV, HCV, HVR-1

Publications arising from this thesis

1. Thomson, E. C., Fleming, V. M., Main, J., Klenerman, P., Weber, J., Eliahoo, J., Smith, J., McClure, M. O. & Karayiannis, P. (2011) Predicting spontaneous clearance of acute hepatitis C virus in a large cohort of HIV-1-infected men. *Gut, in press.*
2. Thomson, E. C., Nastouli, E., Main, J., Karayiannis, P., Eliahoo, J., Muir, D. & McClure, M. O. (2009) Delayed anti-HCV antibody response in HIV-positive men acutely infected with HCV. *AIDS (London, England),* 23 (1), 89-93.
3. Huang, K. H., Bonsall, D., Katzourakis, A., Thomson, E. C., Fidler, S. J., Main, J., Muir, D., Weber, J. N., Frater, A. J., Phillips, R. E., Pybus, O. G., Goulder, P. J., McClure, M. O., Cooke, G. S. & Klenerman, P. (2010) B-cell depletion reveals a role for antibodies in the control of chronic HIV-1 infection. *Nature Communications,* 1 (7), 102.
4. Nastouli, E., Thomson, E. C., Karayiannis, P., Main, J., McClure, M. & Muir, D. (2009) Diagnosing acute hepatitis C in HIV-infected patients: nucleic acid testing compared with antibody and antigen-antibody detecting methods. *Journal of Clinical Virology : The Official Publication of the Pan American Society for Clinical Virology,* 44 (1), 78-80.
5. Smith, J. A., Aberle, J. H., Fleming, V. M., Ferenci, P., Thomson, E. C., Karayiannis, P., McLean, A. R., Holzmann, H. & Klenerman, P. (2010) Dynamic coinfection with multiple viral subtypes in acute hepatitis C. *The Journal of Infectious Diseases,* 202 (12), 1770-1779.
6. Thomson, E. C. & Main, J. (2008) Epidemiology of hepatitis C virus infection in HIV-infected individuals. *Journal of Viral Hepatitis,* 15 (11), 773-781.
7. Winston, A., Garvey, L., Scotney, E., Yerrakalva, D., Allsop, J. M., Thomson, E. C., Grover, V. P., Main, J., Cox, J. I., Wylezinska, M. & Taylor-Robinson, S. D. (2010) Does acute hepatitis C infection affect the central nervous system in HIV-1 infected individuals? *Journal of Viral Hepatitis,* 17 (6), 419-426.
8. Garvey, L., Thomson, E. C., Main, J. & Winston, A. (2007) Response to "Persistence of Neuropsychologic Deficits Despite Long-Term Highly Active Antiretroviral Therapy in Patients With HIV-Related Neurocognitive Impairment". *Journal of Acquired Immune Deficiency Syndromes,* 46 (5), 7-author reply 657.
9. Fox J, Nastouli E, Thomson E, Muir D, McClure M, Weber J, Fidler S. (2008) Increasing incidence of acute HCV in individuals diagnosed with primary HIV in the United Kingdom. *AIDS(London, England),* 12, 22(5), 666-8.

Table of Contents

Declaration of Originality 2

Acknowledgements..... 3

Abstract..... 4

Publications arising from this thesis..... 5

Table of contents..... 6

List of tables..... 11

List of figures..... 13

Abbreviations..... 15

Chapter 1: General introduction

1.1 The epidemiology of HCV in HIV-positive individuals..... 20
1.1.1 Blood transfusion and unsafe injections..... 20
1.1.2 Intravenous drug use..... 22
1.1.3 Sexual transmission..... 23
1.1.4 Control of infection..... 24
1.2 HCV discovery..... 25
1.3 HCV structure and function..... 26
1.3.1 HCV models of infection..... 28
1.3.2 HCV life cycle..... 29
1.3.2.1 Attachment and entry..... 29
1.3.2.2 Replication within the cell..... 30
1.4 HIV-1 and HIV-2 discovery, structure and function..... 34
1.5 The clinical course of HCV in HIV-positive patients..... 37
1.6 The clinical course of HIV in HCV-positive patients..... 39
1.7 Treatment of HCV in HIV-positive patients..... 40
1.7.1 Current treatments for HCV..... 40
1.7.2 The treatment of chronic HCV in HIV-positive patients..... 40
1.7.3 The treatment of acute HCV in HIV-positive patients..... 43
1.7.3.1 Interferon monotherapy..... 45
1.7.3.2 Interferon and ribavirin combination therapy..... 45
1.7.3.3 Treatment limitations..... 46
1.7.4 Prescribing antiretroviral therapy in HCV-infected individuals..... 47
1.8 The natural history of acute HCV infection in HIV-negative and positive populations..... 49
1.9 Aims and hypotheses..... 54

Chapter 2: Predicting outcome in HIV-positive MSM with acute HCV

2.1 Background..... 55
2.1.1 The phyloepidemiology of acute HCV in HIV-positive men..... 55
2.1.2 Methods used to assess viral diversity..... 56
2.1.3 Predicting spontaneous clearance of acute HCV using measures of

viral evolution..... 58
2.1.4 Response to treatment..... 59
2.2 Hypotheses..... 63
2.3 Aims..... 63
2.4 Methods 64
2.4.1 Patient cohort..... 64
2.4.2 Case definition and end-points..... 64
2.4.3 Storage of blood samples..... 65
2.4.4 Anti-HCV Enzyme Linked Immunosorbent Assays (ELISAs)..... 65
2.4.5 Extraction of viral RNA from plasma samples..... 66
2.4.6 Quantitative RT-PCR assays..... 66
2.4.7 Amplification of the HCV 5'Untranslated Region (5'UTR)..... 67
2.4.8 Amplification of the HCV Hypervariable region-1 (HVR-1) 68
2.4.9 Amplification of NS5A by RT-PCR..... 69
2.4.10 Amplification of NS5B by nested PCR..... 70
2.4.11 Gel Electrophoresis and DNA extraction..... 70
2.4.12 Cloning..... 71
2.4.13 Preparation of DNA following clonal amplification..... 71
2.4.14 Sequencing..... 72
2.4.15 Estimated PCR error rate..... 73
2.4.16 Sequence analyses and construction of phylogenetic trees..... 74
2.4.17 Statistical analyses..... 75
2.5 Results..... 76
2.5.1 Patient cohort diagnosis, treatment and follow-up..... 76
2.5.2 Diagnosis of acute HCV..... 76
2.5.2.1 Seroconversion time..... 78
2.5.2.2 Diagnostic testing..... 79
2.5.3 Viral load dynamics during early HCV infection..... 80
2.5.4 Cohort characteristics..... 83
2.5.5 HVR-1 sequence analysis..... 86
2.5.5.1 Cross-sectional phylogenetic analysis of the HIV-positive MSM population with acute HCV..... 86
2.5.5.2 E2 HVR-1 sequence evolution within patients and prediction of spontaneous clearance..... 87
2.5.5.3 E2 HVR-1 sequence evolution and prediction of SVR..... 93
2.5.6 NS5B sequence analysis..... 95
2.5.7 NS5A sequence analysis..... 97
2.5.8 PCR and cloning error rate..... 100
2.5.8.1 Sensitivity of PCR assays..... 100
2.5.8.2 Estimated PCR error rate..... 100
2.5.8.3 Actual PCR/cloning error rate..... 101
2.6 Discussion..... 103
2.6.1 Diagnosis of acute HCV in HIV-positive patients..... 103
2.6.2 Predicting spontaneous clearance of acute HCV in HIV-positive men based on viral dynamics, liver function and other clinical measures 105
2.6.3 Predicting spontaneous clearance of acute HCV in HIV-positive men based on sequence analysis..... 107
2.6.3.1 Superinfection with new strains of HCV..... 107
2.6.3.2 Viral diversification within the E2 HVR-1 is predictive of spontaneous clearance..... 109

2.6.3.3 Fluctuating Viraemia; superinfection versus recrudescence..... 109
2.6.4 Predicting SVR in HIV-positive men with acute HCV..... 110
2.6.5 Artefactual error during PCR and clonal sequence analysis..... 112
2.7 Conclusions..... 113

Chapter 3: The viral evolution of acute HCV

3.1 Background..... 115
3.1.1 Evolutionary theory 115
3.1.2 The evolution of HCV 117
3.1.2.1 Transmission diversity..... 118
3.1.2.2 Superinfection or recrudescence 119
3.1.2.3 The adaptive immune response..... 120
3.2 Hypotheses..... 121
3.3 Aims..... 121
3.4 Methods used to analyse viral sequence diversification; theory.... 122
3.4.1 Measuring nucleotide diversity (π)..... 123
3.4.2 Inferring phylogeny..... 123
3.4.2.1 Nucleotide substitution models..... 123
3.4.2.2 Methods for the construction of phylogenetic trees..... 124
3.4.2.3 Stepwise methods..... 125
3.4.2.4 Exhaustive methods 126
3.4.2.5 Bayesian analysis..... 126
3.4.2.6 Application of the molecular clock hypothesis to the evolution of HCV..... 128
3.4.2.7 Estimating effective population size..... 129
3.5 Laboratory methods 130
3.5.1 Extraction of viral RNA from formalin-fixed, paraffin-embedded (FFPE) liver biopsy specimens..... 130
3.5.2 Extraction and concentration of RNA from PBMCs..... 130
3.5.3 Strain-specific PCR 131
3.6 Computer-based methods for viral sequence analysis..... 131
3.6.1 Program for cropping sequence files using Python..... 131
3.6.2 Sequence alignment..... 132
3.6.3 Construction of neighbour-joined phylogenetic trees..... 132
3.6.4 Construction of maximum likelihood trees..... 132
3.6.5 Path-O-Gen v1.2..... 132
3.6.6 Estimating tree topologies using a molecular clock model 133
3.6.7 Detecting evidence of purifying or positive section by pairwise comparison of sequences using the codon-based Z test..... 135
3.6.8 Testing for neutrality using Tajima and Fu and Li's D test in DNAsp v5..... 136
3.6.9 Codon-specific models of positive, neutral or purifying selection... 316
3.6.9.1 Detecting codon-specific selection using HyPhy..... 136
3.6.9.2 Detecting codon-specific selection using CODEML..... 137
3.7 Results..... 137
3.7.1 The impact of transmission diversity on outcome..... 137
3.7.2 The impact of superinfection and recrudescence of minority strains on viral diversity over time..... 144
3.7.3 The impact of compartmentalisation on recrudescence of minority strains over time..... 146
3.7.3.1 Compartmentalisation within PBMCs..... 146

3.7.3.2 Compartmentalisation within the liver..... 147
3.7.4 The impact of positive or negative selection pressure on viral diversification..... 149
3.7.4.1 Codon based z test..... 150
3.7.4.2 Neutrality testing..... 152
3.7.4.3 Codon-specific selection 153
3.8 Discussion..... 154
3.8.1 Transmission diversity..... 154
3.8.2 Compartmentalisation..... 156
3.8.3 Superinfection..... 157
3.8.4 Adaptive immunity..... 158
3.9 Conclusions..... 159

Chapter 4: Cellular immune responses to HCV in HIV-positive individuals

4.1 Introduction: 160
4.1.1 T cells and their role in the adaptive immune response..... 160
4.1.2 The adaptive immune response to HCV..... 163
4.1.3 The adaptive immune response to HIV-1 and 2..... 171
4.1.4 Cellular immune responses during co-infection with HCV and HIV... 173
4.2 Hypotheses..... 175
4.3 Aims..... 175
4.4 Methods..... 176
4.4.1 Separation of peripheral blood mononuclear cells (PBMCs)..... 176
4.4.2 Thawing of PBMCs..... 176
4.4.3 HLA typing..... 177
4.4.4 Ex-vivo IFNγ ELISpot assay 177
4.4.5 Flow cytometry..... 181
4.5 Results..... 186
4.5.1 HLA typing..... 186
4.5.2 ELISpots..... 186
4.5.3 Flow cytometry..... 191
4.5.3.1 Flow cytometry results by outcome..... 191
4.5.3.1.1 CD4+ responses by outcome group..... 191
4.5.3.1.2 CD8+ responses by outcome group..... 193
4.5.3.1.3 Fluctuating viraemia..... 194
4.5.3.2 Flow cytometry results by route of infection..... 195
4.5.3.2.1 CD4+ responses in IDUs versus non-IDUs..... 195
4.5.3.2.2 CD8+ responses in IDUs versus non-IDUs..... 198
4.6 Discussion..... 201
4.6.1 The evolution of cellular immune responses in spontaneous clearance and progression to chronicity..... 201
4.6.2 Fluctuating viraemia..... 204
4.6.3 Cellular immune responses in intravenous methamphetamine users..... 205
4.7 Conclusions..... 206

Chapter 5: Final Discussion

The natural history of acute HCV in HIV-positive MSM

5.1 Spontaneous clearance..... 207
5.2 Plateau viraemia..... 208

5.3 Fluctuating viraemia..... 208
5.4 Route of transmission and final outcome..... 211
5.5 Treatment outcome and viral evolution..... 212
5.6 Conclusions..... 213

Chapter 6: Future work..... 215

Bibliography..... 216

Appendices..... 241

List of Tables

Table 1.1: Treatment of acute HCV in HIV-infected patients

Table 1.2: Predictors of spontaneous clearance of HCV in experimentally infected chimpanzees

Table 1.3: Predictors of spontaneous clearance of acute HCV in humans

Table 2.1: Primers used for RT-PCR amplification of the 5'UTR

Table 2.2: Primers used in RT-PCR for amplification of the E2 HVR-1

Table 2.3: Primers used for amplification of NS5A

Table 2.4: Primers used for amplification of NS5B

Table 2.5: Sequencing primers

Table 2.6: Sequential viral load measurements in untreated spontaneous clearers and progressors

Table 2.7: Clinical measures and outcome (spontaneous clearance versus progression)

Table 2.8: Viral sequence analyses and predictors of spontaneous clearance

Table 2.9: Viral sequence analyses and predictors of SVR

Table 2.10: Amino acid differences within the ISDR compared with the HCV-1 reference strain

Table 3.1: Clinical and virological measures and route of exposure

Table 3.2: HCV detection within PBMCs and outcome

Table 3.3: IFNγ expression in HCV+ and HCV- PBMCs following stimulation with pooled HCV peptides

Table 3.4 Number of codons under positive and negative pressure by group

Table 4.1: Protein expression during T cell maturation in naive, effector and memory T cells

Table 4.2: Peptide pools

Table 4.3: Peptide pools used for flow cytometry

Table 4.4: Antibodies used for flow cytometry

Table 4.5: Total CD4+ cell responses: all peptide pools

Table 4.6: CD4+ cell responses following stimulation with peptide pool 3

Table 4.7 Dual cytokine expression in CD4+ cells to all peptides

Table 4.8: Dual cytokine expression in CD4+ positive cells stimulated with peptide pool 3

Table 4.9: Dual expression in CD8+ cells to all peptides

Table 4.10: Total responses in CD4+ cells to all pools

Table 4.11: CD4+ cell responses by peptide pool

Table 4.12: Dual cytokine expression in CD4+ cells to all peptides

Table 4.13 Dual cytokine expression in CD4+ positive cells by peptide pool

Table 4.14 Total CD8+ responses to all peptide pools

Table 4.15: CD8+ cell responses by peptide pool

Table 4.16: Dual cytokine expression in CD8+ positive cells by peptide pool

List of Figures

Figure 1.1: Structure of the HCV genome (H77 reference strain)

Figure 1.2: HIV-1 genome structure (HXB2 reference strain)

Figure 2.1: Patient diagnosis, treatment and follow-up

Figure 2.2: Time to seroconversion

Figure 2.3: HCV viral load, bilirubin and ALT levels over time

Figure 2.4: Multiple strains of HCV are circulating in the HIV-positive MSM population

Figure 2.5: Genetic diversity within HVR-1 (uncorrected) over time

Figure 2.6: Patterns of acute HCV based on phylogenetic analysis

Figure 2.7: Genetic variation within E2 HVR-1 over time by group

Figure 2.8: Phylogenetic tree using HVR-1 and NS5B sequences derived from the same time points in a) a patient with evidence of 3 circulating strains at baseline and b) a patient with similar strains at 2 time points

Figure 2.9: Phylogenetic tree using NS5A sequences derived from 20 patients at 2 time points

Figure 2.10: Alignment of NS5A sequences in a) Patients with SVR and b) Non-responders/relapsers

Figure 2.11: End-point dilution of the 5'UTR

Figure 3.1: Models of evolution

Figure 3.2: Bayes theorem

Figure 3.3: Tracer file output

Figure 3.4: Neighbour-joined phylogenetic tree of IDU and non-IDUs

Figure 3.5: Bayesian phylogenetic trees a) IDU exposure b) Mucosal exposure

Figure 3.6: Bayesian phylogenetic analysis of transmitting couples

Figure 3.7: Strain-specific PCR

Figure 3.8: Neighbour-joined circular phylogenetic tree of subject 1 and reference sequences.

Figure 3.9: Evidence of selection using the codon-based Z test

Figure 3.10: Fu and Li's D test in a) clearers and b) PV/FV patients

Figure 3.11: Frequency of positively selected codons within the E2 HVR-1 region

Figure 4.1: Flow cytometry analysis

Figure 4.2: *Ex vivo* IFNγ ELISpots by group over time

Figure 4.3: ELISpot responses in representative patients from each group

Figure 4.4: IFNγ and IL-17 expression in CD4+ cells

Figure 4.5: CD4+IFNγ and IL-17 expression in an FV patient

Figure 4.6: Dual secretion of IL-2 and IFNγ from CD4+cells in HIV-positive IDUs versus non-IDUs

Figure 5.1: Model of viral evolution in FV patients

Figure 5.2 Transmission diversity and outcome; route of transmission hypothesis

Abbreviations

µg	Microgram
µl	Microlitre
µmol	Micromole
3'UTR	3' Untranslated Region
5'UTR	5'Untranslated Region
A	Absorbance
AIDS	Acquired Immunodeficiency Syndrome
ALT	Alanine transaminase
APRICOT	AIDS Pegasys Ribavirin International Coinfection Trial
bp	Base pairs
CD	Cluster of differentiation
cDNA	Complementary DNA
CI	Confidence intervals
Da	Dalton
DC SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non- integrin
dH20	Distilled water
DMSO	Dimethyl sulfoxide
dN	Number of non- synonymous substitutions per site
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
dS	Number of synonymous substitutions per site
E1	Envelope protein 1
E2	Envelope protein 2
EDTA	Ethylenediaminetetra-acetic acid
elf3	Eukaryotic initiation factor 3
ELISA	Enzyme Linked Immunosorbent Assay

ELISpot	Enzyme Linked Immunosorbent Spot
ESS	Effective sample size
EVR	Early virological response
FasL	Fas ligand
FFPE	Formalin-Fixed, Paraffin-Embedded
FV	Fluctuating Viraemia
g	Gram
GAG	Glycosaminoglycan
GBV-B	GB Virus B
HAART	Highly Active Anti-Retroviral Therapy
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HCV E2	HCV Virus Envelope Protein 2
HCVcc	HCV infectious in cell culture
HCVpp	HCV pseudoparticles
HDL	High density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus-1
HIV-2	Human Immunodeficiency Virus-2
HLA	Human Leukocyte Antigen
HR	Hazard Ratio
HVR-1	Hypervariable Region 1
HVR-2	Hypervariable Region 2
IDU	Intravenous Drug User
IFN	Interferon
IFNy	Interferon gamma

IFNα	Interferon alpha
IFN-λ	Interferon lambda
IL	Interleukin
INDU	Intranasal Drug Use
IQR	Interquartile range
IRES	Internal Ribosomal Binding Site
ISDR	Interferon Sensitivity Determining Region
IU	International Units
JFH-1	Japanese Fulminant Hepatitis-1
kb	Kilobase
kDa	KiloDalton
KIR	Killer- cell Immunoglobulin-like Receptor
l	Litre
LB	Luria-Bertani broth
LDL	Low-density lipoprotein
L-SIGN	Liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin
LTR	Long Terminal Repeat
MCMC	Markov Chain Monte Carlo
mg	Milligram
MHC	Major Histocompatibility Complex
min	minutes
MIP1β	macrophage inflammatory protein-1ß
miR-122	microRNA-122
ml	Millilitre
mm3	Cubic millimetre
mmol	Millimole
MSM	Men who have sex with men

MU	Million Units
Ne	Effective population size
ng	Nanogram
NJ	Neighbour Joining
NPV	Negative Predictive Value
NRTIs	Nucleoside analogue Reverse Transcriptase Inhibitors
NS	Non-Structural Protein
OR	Odds ratio
ORF	Open reading frame
OTU	Operational Taxonomic Unit
PAMPs	Pathogen-Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PD-1	Programmed Death 1
pegIFNα	Pegylated interferon alpha
Pfu	*Pyrococcus furiosus* DNA polymerase
pg	Picogram
PILAR	Proliferation-induced lymphocyte associated receptor
PKR	Protein kinase
PPV	Positive Predictive Value
PRESCO	Pegasys Plus Ribavirin for HCV Treatment in HIV/HCV Coinfection
PV	Plateau viraemia
r_N	Non-synonymous substitutions
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
r_S	Synonymous substitutions

RT-PCR	Reverse Transcription PCR
SC	Spontaneous Clearance
SFU	Spot forming unit
siRNA	Small interfering RNA
SIV	Simian immunodeficiency Virus
SVR	Sustained Virological Response
Taq polymerase	*Thermus acquaticus* polymerase
TAR	Trans Activation Response
TC	Transmitting couple
Tc	T cytotoxic
Tgo	*Thermococcus gorgonarius* DNA polymerase
Th	T helper
TLRs	Toll-Like Receptors
Tm	Melting temperature
TNFα	Tumour necrosis factor
TS	Transition
TV	Transversion
UPGMA	Unweighted Pair Group Method with Arithmetic means
UTR	Untranslated region
Vif	Viral infectivity protein
VL	Viral Load
VLDL	Very low density lipoprotein
WHO	World Health Organisation
π	Nucleotide frequency

Chapter 1: General introduction

1.1 The epidemiology of HCV in HIV-positive individuals

Around the world, silent infection with hepatitis C virus (HCV) is emerging as a major cause of future morbidity and mortality in individuals infected with human immunodeficiency viruses 1 (HIV-1). Liver disease has recently overtaken AIDS-defining illnesses as a cause of death in many patient populations following the successful introduction of highly active antiretroviral therapy (HAART) for the treatment of HIV (Bica et al 2001; Arnold et al 2006).

In 2006, a total of 130 million people were estimated to be infected with HCV and 40 million with HIV. Shared routes of transmission mean that co-infection is common, affecting at least 4–5 million individuals (Alter et al, 2006). In the developing world, infection with HCV is frequently associated with unsafe injection practices and unscreened blood transfusions. In westernized countries it is most commonly transmitted as a result of recreational intravenous drug use (IDU). Patients who have received untested blood products prior to the introduction of screening blood for HCV are also at risk. Sexual transmission of HCV occurs infrequently in HIV-negative couples (Hallam et al, 1993; Scott et al, 2010) but is commoner in HIV-positive patients, particularly in men-who-have-sex-with-men (MSM) (Danta et al, 2007).

1.1.1 Blood transfusion and unsafe injections

Following the identification of HIV in 1983 (Barré-Sinoussi et al, 1983) and HCV in 1989 (Choo et al, 1989), diagnostic tests were rapidly developed and screening of blood and blood products became routine in resource-rich countries. Prior to this, patients receiving

blood products were at risk of acquiring both HCV and HIV; haemophiliac patients, for example, were frequently infected with both HIV and HCV from contaminated Factor VIII. Between 1982 and 2003, 96.5% of Canadian HIV-infected haemophiliac patients also had HCV (Arnold et al, 2006). In countries with established effective screening programmes transmission of HIV and/or HCV is now very rare (Pillonel, 1998).

The risk of HIV and HCV acquisition in resource-poor countries unfortunately remains high. Between 2001 and 2002, 47.5% of blood transfusions in developing countries were unscreened for HCV infection (WHO Global Database on Blood Safety). Blood transfusions in the developing world are most often given to women as a result of peripartum haemorrhage (the commonest cause of death in women of child-bearing age) and to children with anaemia secondary to severe falciparum malaria (70% of blood transfusions in Africa). Between 8 and 12 billion injections are administered yearly around the world and 50% of these are thought to be unsafe through non-properly sterilised needles and syringes (given mainly in sub-Saharan Africa and Asia) (Kane et al, 1999). This estimate does not include medical injections given by non-traditional practitioners and exchange of blood during ceremonial activities such as circumcision, tattooing and body piercing. HIV positive patients from countries in sub-Saharan Africa have an HCV antibody prevalence of 3.7–8.2%, the majority of which are likely to have been the result of nosocomial transmission (Ayele et al, 2002; Agwale et al, 2004; Karuru et al, 2005). These figures may be somewhat artificially elevated by the inclusion of highly selected populations of patients. The above studies are surveys of hospital attendees and may therefore be biased as iatrogenic transmission of HCV is known to be common in this setting. The largest documented outbreak of HCV infection associated with unsafe injection practice was in Egypt following mass treatment

for schistosomiasis with intravenous tartar emetic resulting in an estimated seroprevalence of 25% in at-risk populations (Frank et al, 2000). Health care workers have often been implicated in transmission of HIV and HCV to patients. Unsafe injection practice at the Al-Fateh hospital, Benghazi, Libya is likely to have resulted in transmission of HCV and HIV to 418 children (111 of whom were infected with both viruses) and until recently the health care workers thought to be responsible were threatened with the death penalty (de Oliveira et al, 2006).

1.1.2 Intravenous drug use

HCV is highly prevalent in HIV-infected IDUs. A cross-sectional analysis of the US adult AIDS Clinical Trials Group revealed an overall HCV seroprevalence of 16.1% with a prevalence of 72.7% in the high risk subgroups – those with haemophilia or a history of IVDU (Sherman et al, 2002). Similarly, a study of HIV-infected current and ex-IDUs in New York City demonstrated an HCV seroprevalence of 75% (Strasfeld et al, 2003). European HIV-infected individuals have an extremely high prevalence of HCV (33%) which is related to high levels of IDU (Mendel et al, 1995). The distribution of HCV in the HIV positive population in Europe varies from country to country with highest prevalence levels in urban areas of eastern and southern countries. European HIV-infected patients who practice IDU have an HCV prevalence of 78–97% (Quaranta et al, 1994; Rauch et al, 2005; Quaglio et al, 2003), and 90% of injecting drug users who have injected drugs for more than 10 years have HCV (Quaglio et al, 2003).

1.1.3 Sexual transmission

HIV positive MSM have an estimated prevalence of HCV infection of 4–11.5% with a higher risk in men who use condoms inconsistently (Cohen et al, 2006). An epidemic of acute HCV infection in HIV positive MSM has recently been reported in urban centres in the UK, Germany, France, the Netherlands and the USA (Cohen et al, 2006; Gilleece et al, 2005; Vogel et al, 2005; Dominguez et al, 2006; van de Laar et al 2007). The majority of these patients have been asymptomatic and identified by deranged liver function tests at routine HIV follow-up clinics. Risk factors for acquisition of HCV in such patients include multiple sexual partners (median number of partners 30 versus 10 in the preceding 12 months), unprotected anal intercourse, sex under the influence of drugs, internet-arranged sex, and sexual practices with a high risk of mucosal trauma such as fisting and use of sex toys. Patients were noted in one study to be seven times more likely to use the internet as first point of contact (Danta et al, 2007). It is likely that high risk (mucosally traumatic) sex is significantly associated with transmission of HCV. Limited data are as yet available on HIV-negative MSM and incidence of HCV, but early reports would suggest that it is uncommon (Scott et al, 2010). Heterosexual spread of HCV in HIV-infected patients may also be higher than in HIV negative couples (Eyster et al, 1991; Vandelli et al, 2004; D'Oliveira et al, 2005). Again it has been suggested that this relates to the higher levels of HCV RNA in genital secretions of those with HIV infection. A French study investigating 1000 patients who acquired HIV sexually found a prevalence of HCV infection of 5.7% in MSM compared with 12.9% in patients who acquired HIV through heterosexual sex (D'Oliveira et al, 2005). The female partners of haemophiliac men are more likely to have become HCV infected if their partner also has HIV infection (Eyster et al, 1991).

It is clear that the high rate of HCV infection in HIV-positive MSM is at least in part related to increased exposure to infection (Danta et al, 2007). What remains unclear is if the immune response to HCV in HIV-infected individuals results in a higher risk of sexual transmission of the virus. HCV viral loads in plasma tend to be higher in HIV-positive individuals (Cribier et al, 1995) and HCV is also more commonly detected in semen and genital secretions (Leruez-Ville et al, 2000). In MSM, a reduction in CD4+ cells in the rectal mucosa associated with HIV infection (Bishop et al, 1987) could also theoretically facilitate sexual transmission of HCV.

1.1.4 Control of infection

It is estimated that 70% of injections given by primary health care providers in developing and transitional countries are unnecessary or could have been given in an oral formulation (WHO Global Database on Blood Safety 2001–2002). About 40% of these may have been administered with no sterilization procedure. Blood transfusions in developing countries are often donated by first-degree relatives without screening for HIV or HCV. In an attempt to reduce nosocomial transmission events, the World Health Organization co-ordinates the Safe Injection Global Network which provides advice on safe injection practice and mechanisms by which health care providers in developing countries can access equipment, and promote the training of health staff in order to change health care worker behaviour patterns, ensure the availability of adequate equipment and advise on appropriate waste disposal. Screening of blood used for transfusion is also a priority but there is still limited availability in resource-poor settings.

Intravenous drug users have been shown to develop multiple infections over time suggesting multiple and ongoing transmission events (Herring et al, 2004). We have observed the same phenomenon in HIV positive men who have contracted HCV sexually. Patients who have access to treatment should be given advice on reducing reinfection risk either by risk avoidance or modification strategies such as attending needle exchange facilities, using clean needles, syringes and paraphernalia for injection drug use and using condoms.

In summary, HIV/HCV infection is common and has a variable distribution around the world reflecting differing routes of transmission. Reduction of transmission of HCV in coinfected patients is a priority and will only be achieved by a combination of public health campaigns targeting affected groups and health care workers in resource-poor settings.

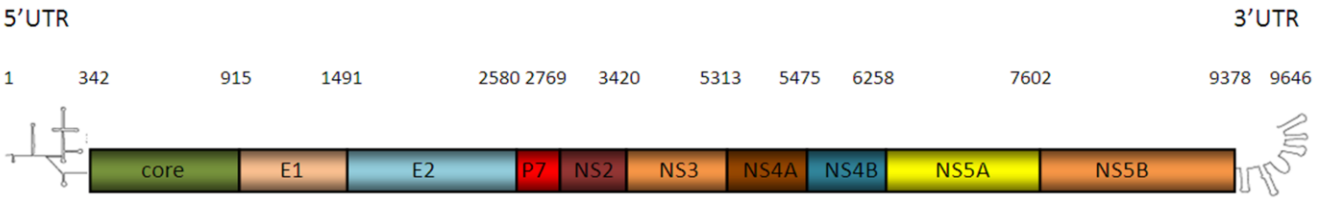
1.2 HCV discovery

The 9600kb in length RNA genome of HCV was first characterised in 1989 using a shotgun sequencing approach. The genetic sequence places the virus in the *Hepacivirus* genus within the Flaviviridae family (Simmonds 1994; Lindenbach & Rice, 2001). The identification of multiple genotypes and strains of HCV have subsequently provided evidence that it originated between 500 and 2000 years ago (Simmonds, 2001). Infection with HCV, the infecting pathogen in the majority of cases of “nonA-nonB” hepatitis is currently estimated to account for about 130 million carriers around the world, 4-5 million of whom have HIV co-infection (Alter et al, 2006).

1.3 HCV structure and function

The HCV genome organisation as shown in Figure 1.1 is so arranged that the structural genes (blue) are located at the 5' end of the genome and the non-structural ones at the 3' end. It is flanked by 5' and 3' untranslated regions (UTRs) on either side of an open reading frame (ORF) that encodes a polyprotein which is post-translationally cleaved into ten proteins divided by function into structural and non-structural ones. A small protein translated from an alternative short open reading frame of unknown function is also formed.

Figure 1.1: Structure of the HCV genome (H77 reference strain)



Adapted from the Los Alamos HCV database http://hcv.lanl.gov/content/sequence/HCV/MAP/) Nucleotide positions are labelled above. Regions of interest include the hypervariable regions 1 and 2 (HVR-1 and 2) located at positions 384-410 and 473-480 respectively. The interferon sensitivity determining region (ISDR) is located at positions 2209-2248 and the interferon inducible RNA activated protein kinase PKR binding site at positions 2209-2274

1.3.1 HCV models of infection

While many aspects of the early stages of the HCV life cycle remain unknown, recent advances in *in vitro* cell culture systems have made the prospect of a stronger understanding of these events more likely. A number of systems are now available, including replicon-based culture, pseudovirus culture, chimeric cell culture and culture of the genotype 2a JFH-1 strain. *In vivo*, chimpanzees can be infected with HCV and have been used to characterise host-virus dynamics during early infection (reviewed by Bukh, 2004). Other models include tamarins infected with GBV-B (a closely related Flavivirus, Karayiannis et al, 1995) and transgenic mice with human liver xenografts.

The development of viral sub-genomic replicons (RNA molecules capable of replicating autonomously but not capable of forming infectious viral particles) was the first successful in vitro culture strategy. Subgenomic replicon systems (in which the structural genes core, E1 and E2 are absent) have allowed study of the non-structural protein functions in an artificial setting, for example, by site-directed mutagenesis. Bicistronic replicons may include selection genes e.g. neomycin or reporter genes such as luciferase (Lohmann et al, 2003). Retroviral pseudoparticles (HCVpp) (Bartenschlager et al 2003) consisting of a retroviral backbone attached to HCV envelope glycoproteins have allowed studies into attachment and entry of HCV and the development of neutralisation assays. A further advance in the *in vitro* culture of HCV was the successful introduction of chimeric virus particles infectious in cell culture (HCVcc) consisting of the structural proteins of genotype 2a (Lindenbach et al 2005), 1b, 1a or 3a HCV (Pietschmann et al, 2002) fused to the replicase (non-structural proteins) of the genotype 2a strain JFH-1. In 2005, cDNA prepared from RNA extracted from

a genotype 2a virus from a **J**apanese patient with **F**ulminant **H**epatitis (designated JFH-1) was successfully cloned and transcribed RNA shown to replicate in the Huh-7 hepatoma cell line. Moreover, such cultures were able to release mature infectious virions as shown by infection of other cells *in vitro* as well as chimpanzees (Wakita et al, 2005) and transgenic mice with human liver xenografts (Lindenbach et al, 2005). Other genotypes, however, have not yet been cultured successfully *in vitro*. Use of these culture systems have allowed investigation into the attachment and entry stages of infection, as well as replication of HCV *in vitro*.

1.3.2 HCV life cycle

1.3.2.1 Attachment and entry

The search for the attachment receptor on hepatocytes has focussed on a number of proteins. It is clear that the HCV envelope protein E2 binds with high affinity to the ubiquitous tetraspanin protein CD81 (Pileri *et al* 1998), and this has been found to be required as a co-receptor during the cell-entry process using both HCVpp and JFH-1 culture systems (Bartosch et al, 2003; Hsu et al, 2003; Cormier et al, 2004; Zhang et al, 2004; Wakita et al, 2005; Morikawa et al, 2007). Inhibition of entry in Huh-7 hepatoma cells with anti-CD81 monoclonal antibodies or siRNA prevents infection, while expression of CD81 in previously non-permissive HepG2 cells renders them susceptible to infection.

Various attempts have been made to identify the area of E2 required for binding to CD81 using each of the *in vitro* models available and a variety of techniques including blocking monoclonal antibodies, deletion and mutagenesis studies. Using the Con1 chimera in

culture (genotype 1b structural proteins attached to the JFH-1 replicase) and blocking monoclonal antibodies, amino acids 412-447 of the HCV E2 protein appear to be required for attachment to CD81 (Hsu et al, 2003). Mutagenesis studies have revealed that the HVR-1 (Callens et al 2005) and residues 420, 527, 529, 530 and 535 may also be required for CD81 binding (Patel et al, 2005). However, HCV cannot penetrate into all CD81 positive cells and HCV lacking the HVR1 sequence resulted in attenuated infection in chimpanzees. It is likely, therefore, that another receptor present on hepatocytes facilitates binding to the cell surface. There is evidence that the low-density lipoprotein (LDL) receptor (Agnello *et al* 1999; Monazahian *et al* 1999), scavenger receptor class B type 1 (Scarselli *et al* 2002), glycosaminoglycans (GAGs) (Germi *et al* 2002; Barth *et al* 2003; Morikawa *et al* 2007), Claudin 1 also appears to facilitate entry into hepatocytes.

Other cells may also be involved in binding HCV. DC-SIGN which is expressed on dendritic cells and L-SIGN expressed on endothelial cells in liver sinusoids (Gardner *et al* 2003; Lozach *et al* 2003; Pohlmann *et al* 2003) may capture circulating HCV particles and facilitate virus infection of proximal hepatocytes and lymphocytes (Cormier et al, 2004). HCV can also enter and replicate within other cells such as peripheral blood mononuclear cells (PBMCs), especially in HIV-infected individuals (Blackard *et al* 2005; Laskus *et al* 2007; Blackard *et al* 2007), intestinal cells (Deforges *et al* 2004) and the central nervous system (Forton *et al* 2004). The presence of negative strand RNA within such cells is highly suggestive of active replication.

1.3.2.2 Replication within the cell

After entry into a hepatocyte, the positive strand RNA acts as mRNA and binds directly to the 40S ribosomal subunit via a highly conserved 4-domain region of the 5'UTR of the viral

genome called the internal ribosome binding site (IRES), which then recruits the 60s subunit. This direct binding of the IRES to the ribosome occurs without requirement for pre-initiation factors and induces a conformational change allowing translation to commence. The 5'UTR also binds to eukaryotic initiation factor 3 (eIF3) via the IRES and a liver-specific microRNA miR-122 (Jopling et al, 2005) initiating translation and then acting as a template for replication by an error-prone RNA-dependent RNA polymerase. Translation of the monocistronic RNA genome results in the formation of a polyprotein which is cleaved into core protein, the envelope proteins E1 and E2, p7 and the non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B. Cleavage of the structural proteins is carried out by host cell signalases and a signal peptide peptidase. The non-structural proteins are processed by the 2 viral proteases: NS2 which cleaves the NS2/NS3 junction and the NS3 serine protease which acts at all other cleavage sites in the non-structural polyprotein region.

The structural proteins consist of the core protein, envelope proteins E1 and E2 and the p7 ion channel. Core protein is released as a 191-residue precursor of 23 kDa and further processing yields the predominant form of 21kDa. It is polyfunctional, forming the nucleocapsid which is highly conserved amongst HCV strains but is also involved in modulation of signalling pathways, cellular and viral gene expression, cell transformation, apoptosis, and lipid metabolism (reviewed in Suzuki et al, 2005).

The envelope proteins E1 and E2 form heterodimers on the virus surface. In contrast to HCV core protein, HCV envelope proteins vary extensively in structure. The E2 protein contains two hypervariable regions (HVR-1 and HVR-2) where the amino-acid sequence varies considerably as a result of positive selection mediated by the adaptive (predominantly

antibody-mediated) immune response (Weiner et al 1991; Farci et al, 2000; Gaud et al, 2003). The E2 protein is involved in cell entry and contains a binding region for the ubiquitous CD81 co-receptor, required for entry into cells. Antibodies binding to epitopes within HVR1 inhibit binding of E2 to cells and block HCV infectivity, both *in vitro* and *in vivo* (Habersetzer et al., 1998; Shimizu et al., 1996; Zibert et al., 1999).

The P7 gene encodes 2 alpha helices which form a transmembrane ion channel (Patargias et al, 2006) the presence of which is essential for viral replication (Brohm et al, 2009). NS2 encodes a cysteine autoprotease that is separated from p7 by host cell peptidases and which cleaves the NS2/NS3 junction itself.

The NS3 protein has 2 domains. The N-terminal domain is a serine protease (Tomei *et al* 1993) requiring NS4A as a co-factor, while the C-terminal domain has helicase and NTPase functions (Suzich *et al* 1993; Wardell *et al* 1999). The structure of the NS3 protein complex has been well characterised. The NS3 serine protease requires NS4A to anchor the complex to cellular membranes via an N-terminal hydrophobic peptide (Wolk et al, 2000) and also for the insertion of a β strand which allows the NS3 protease to fold into an active conformation (Failla et al, 1994; Bartenschlagger et al, 1994; Lin et al, 1995). It may also cleave proteins which mediate interferon signalling. Inhibitors of the NS3/4 protease such as bocepravir and telaprevir, currently in clinical trials, are able to reverse this inhibition of the interferon pathway (Meylan et al, 2005; Li et al, 2005). The C domain of NS3 is a DExH/D-box RNA helicase (characterised by the DExH/D motif) which uses the energy of NTP hydrolysis to unwind double-stranded RNA and DNA homoduplexes and heteroduplexes in a 3' to 5' direction (Levin et al, 2005). It therefore plays a pivotal role in HCV-RNA replication.

The NS4B protein induces intracellular membrane alterations and results in the formation of a membranous web which supports RNA replication (Egger *et al* 2002).

NS5A is a membrane-associated protein which is phosphorylated and exists in two forms: hypo- and hyper-phosphorylated forms with 3 structural domains. It is likely to be polyfunctional, but these functions are not yet fully characterised. NS5A binds to and associates with lipid particles (Barba et al, 1997) via host proteins such as apolipoprotein E (Benga et al, 2010). An interferon sensitivity-determining region (ISDR) within NS5A has been described which may be associated with resistance to interferon. Resistance to IFNα may occur due to inhibition of the double-stranded RNA-dependent protein kinase (PKR) which is induced by IFN. Mutations within the NS5A PKR binding region which includes the ISDR render the virus sensitive to IFNα (Gale *et al* 1998). The E2 protein may also inhibit PKR (Taylor et al, 1999). NS5A is also associated with the production of IL8, which may potentiate viral replication (Polyak et al, 2001).

NS5B encodes the RNA-dependent RNA polymerase which replicates viral RNA. It lacks 5'-3' exonuclease (proofreading) capacity and therefore a large number of viral variants, the viral quasispecies are produced over time.

1.4 HIV-1 and HIV-2 discovery, structure and function

The human immunodeficiency viruses 1 and 2 were identified in 1983 (Barre-Sinoussi et al) and 1985 (Barin et al) respectively, following the emergence of unusual opportunistic infections and cancers (*Pneumocystis* pneumonia and Kaposi's sarcoma) in men-who-have-sex-with-men (MSM) in San Francisco in 1981. The illness was subsequently found to have been prevalent for a number of years in the USA and sub-Saharan Africa. HIV-1 was retrospectively isolated from patients in Kinshasa, Democratic Republic of Congo from 2 tissue samples obtained and stored in 1959 (Korber, 2000) and 1960 (Worobey, 2008). Phylogenetic analysis applying a molecular clock to multiple HIV sequences, including the 2 earliest isolated samples dates the origin of the HIV virus to the beginning of the 20th century (Worobey, 2008) having diverged from variants of chimpanzee simian immunodeficiency virus (SIVcpz; HIV-1) and sooty mangabee SIV (SIVsm; HIV-2).

HIV-1 and 2 are members of the *Lentivirus* genus within the Retroviridae family (Figure 1.2). The HIV virus binds to the CD4 receptor and the chemokine co-receptors CCR5 and CXCR4 via the viral protein gp120. Most primary HIV isolates bind CCR5 but changes in the structure of the highly variable V3 loop component of gp120 (Trkola et al, 1996) allow binding to CXCR4, usually during chronic infection. Gp120 represents a major target for neutralising antibody responses which may limit HIV viraemia. After binding to the host cell membrane, the viral gp41 fusion peptide is inserted into the membrane and viral RNA is deposited into the cytoplasm of the cell. Dendritic cells may also be infected via the receptor DC-SIGN and HIV may be disseminated by these cells as well as macrophages, particularly from mucosal surfaces.

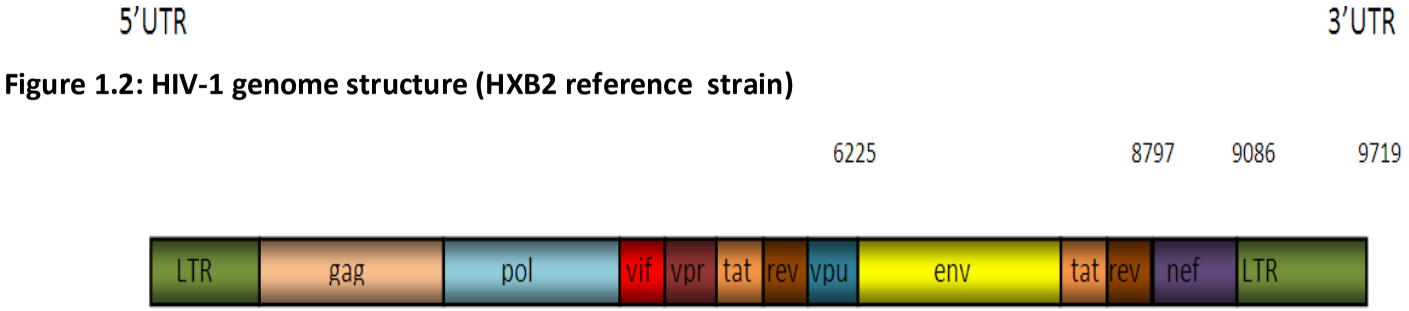


Figure 1.2: HIV-1 genome structure (HXB2 reference strain)

Adapted and simplified from the Los Alamos HIV database (http://www.hiv.lanl.gov/content/sequence/HIV/MAP)

Nucleotide positions are labelled above.

Once viral RNA has entered the cell, it is converted to double-stranded DNA by the viral enzyme reverse transcriptase. This proviral DNA is then integrated into the genomic DNA in the nucleus of the cell by another viral integrase. The viral genome encodes three structural protein genes gag, pol and env, 2 regulatory proteins tat and rev and 4 accessory proteins nef, vif, vpu and vpr. Once inserted into the host cell genome, proviral DNA is transcribed into mRNA and translated into a polyprotein which is then cleaved into functional proteins by the viral protease. The viral enzymes reverse transcriptase, integrase and protease as well as gp41 and CCR5 are all targets of highly active antiretroviral therapy (HAART).

The *env* gene encodes the gp160 protein that is then cleaved into gp120 and gp41.

The regulatory tat proteins activate transcription by binding the trans activation responsive (TAR) area of the long terminal repeat (LTR) promoter found at either end of the HIV genome (Gatignol et al, 1991). Rev promotes shuttling of viral mRNA from the nucleus and the cytoplasm by binding to the 200bp Rev-responsive RNA element (RRE; Felber et al, 1989) within mRNAs encoding the structural proteins.

The accessory proteins have a variety of functions. HIV viral infectivity protein (Vif) binds to the mRNA of the cellular cytosine deaminases APOBEC3G and APOBEC3F inhibiting their translation (Mercenne et al, 2010). Vpr protein arrests the cell cycle at the G2/M phase. This may occur via an interaction with E3 ligase and an unknown cellular factor whose ubiquitination (and subsequent proteasomal degradation) induces G2 arrest (Belzile et al, 2010). Nef protein down-regulates MHC class 1 and 2 molecule expression as well as reducing CD4 receptor expression by the endocytic pathway (Garcia and Miller, 1991; Stumptner-Cuvelette et al, 2001).

The Vpu protein also reduces expression of CD4 by increasing degradation via the ubiquitin-proteasome pathway. It also facilitates viral budding and appears to produce an ion channel which may aid this process (reviewed in Montal, 2003).

1.5 The clinical course of HCV in HIV-infected patients

Progression of HCV in HIV-positive individuals is accelerated with progression to fibrosis and liver cirrhosis occurring earlier and a higher risk of hepatocellular carcinoma which is more likely to have metastasised prior to presentation to hospital than in HIV-negative individuals (Singal & Anand, 2009; Benhamou et al, 1999; Graham et al, 2001; Giordano et al, 2004).

One study has revealed high levels of liver fibrosis during acute infection in HIV-positive individuals, but this has not been confirmed in other cohorts and was weakened by the unusually broad inclusion criteria used for diagnosing acute HCV which may have resulted in the inclusion of chronically infected patients in the analysis (Fierer et al, 2008). The 3 diagnostic criteria used in the study were 1) Seroconversion which is known to be delayed in HIV-positive individuals and is discussed in chapter 2 of this thesis, 2) Elevation in ALT level which could occur for a variety of reasons e.g. use of illicit drugs or HAART and 3) Fluctuation in the serum level of HCV RNA which may occur at any stage of HCV infection.

The picture is clearer however in individuals with chronic HCV and HIV. Immunosupression is associated with more rapidly progressive HCV-related liver disease. This has been recognized both in the setting of immunosuppressive therapy and in patients with HIV and HCV (Martin et al, 1989; Eyster et al, 1991; Telfer et al, 1994; Graham et al, 2001). In one

Spanish study carried out between 1989 and 1993, the outcome of HCV associated liver disease was assessed in 44 patients with HCV monoinfection and 32 patients with HIV and HCV infection (Sanchez-Quijano et al, 1995). Within 15 years 25% of those with HIV had cirrhosis compared with 6.5% of those who were HIV negative. Another study published in 1999 compared liver histology in HIV positive patients with HCV correcting for age, sex and alcohol consumption (Benhamou et al, 1999). CD4 count, HIV status and alcohol intake were predictive of outcome: An HIV positive individual with a CD4 count of <200 /mm3 and an alcohol intake of more than 50 g/day had a median expected time to cirrhosis of 16 versus 36 years for a patient with a CD4 count of more than 200 /mm3 and an alcohol intake of <50 g /day. Much of the first of these studies was carried out in the pre-HAART era, and many of the patients receiving HAART in the second study were treated with what are now regarded as hepatotoxic regimens (such as didanosine, stavudine and zalcitabine). Thus, following the advent of effective HAART, further studies investigated disease progression in treated individuals with HIV HCV coinfection. While the risk of hepatotoxic events are more frequent and serious in patients with HCV infection, immune restoration can slow down the rate of liver fibrosis towards that seen in HIV-negative individuals. One study compared disease progression in 85 HIV/HCV coinfected subjects with 296 patients with HCV who were HIV negative (Verma et al, 2006). The HIV-infected group were subdivided according to whether they had received no HAART, nucleoside analogue reverse transcriptase inhibitors (NRTIs) alone, or more effective combination therapy (generally those where therapy was started after 1996) and a third group who started on NRTIs and progressed on to more effective therapy as it became available. Serial liver biopsies showed that those who had received only more effective HIV combination therapy had similar disease severity to the HCV monoinfected group while more rapid progression was seen each of the other groups.

Another study of 285 HIV／HCV-infected patients revealed that both liver related and general mortality rates were decreased in patients who had taken HAART (Qurishi et al, 2003). Furthermore, in patients treated with HAART, rapid progression of liver disease is associated with poor control of HIV and correlates with high viral load and low CD4 count (Brau et al, 2006).

Thus, the availability of effective HAART has extended the life span of HIV and HCV co-infected patients resulting in a Pandora's box phenomenon; as the rate of mortality associated with HIV-related opportunistic infection has decreased, the morbidity and mortality rates of HCV-related liver disease have increased (Bica et al, 2001) and are likely to continue to do so.

1.6 The clinical course of HIV in HCV-infected patients

While the impact of HIV on the progression of HCV has been well studied, the impact of HCV on the progression of HIV is less clear. Hypothetically, it might be expected that the presence of HCV would be likely to subvert immune control of HIV by overwhelming, redeploying or subverting effective immune responses against the virus. This hypothesis is supported by observation disease progression in HCV-positive individuals monitored as part of the Swiss cohort study (1157 HIV and HCV positive patients compared with 1954 HIV positive patients). Coinfected individuals had a higher likelihood of progression to AIDS-defining events or to death (hazard ratio 1.7 (95% CI 1.26-2.30)). This was independently also associated with active intravenous drug use; HR 1.38 (95% CI: 1.02-1.88)). HCV

seropositivity was also associated with impaired CD4+ cell recovery following HAART (hazard ratio for a CD4-cell count increase of at least 50 cells/µl=0.79 (0.72-0.87)).

The association of accelerated disease progression in co-infected individuals is likely to reflect a disordered immune response and is the subject of discussion in Chapter 4 of this thesis.

1.7 Treatment of HCV in HIV-infected patients

1.7.1 Current treatments for HCV

At the time of writing, the only licensed treatments for HCV are conventional interferon alpha (IFNα), pegylated interferon alpha (pegIFNα) and ribavirin. These have variable success rates, dependent on a number of host and virus factors. Higher success rates in chronic HCV infection are obtained with combination therapy with pegIFNα and ribavirin. Both host and viral factors are likely to define the response to treatment and are studied and discussed further in chapter 2 of this thesis.

1.7.2 The treatment of chronic HCV in HIV-positive patients

The advent of HAART has significantly improved the outlook for HCV-infected HIV-positive patients. Initial trials with conventional non-pegylated IFNα as monotherapy or combination with ribavirin were disappointing with low sustained virological response (SVR) rates. However, the development of pegIFNα and ribavirin combination therapy has increased SVR rates considerably. In the RIBAVIC study 412 HIV/HCV-infected patients were randomized to receive standard IFNα and ribavirin *vs* pegIFNα and ribavirin (Carrat et al, 2004). The overall SVR was higher in the pegIFNα arm (27 *vs* 20%) but nevertheless

disappointing and thought to relate to a very high (42%) treatment discontinuation rate. A similar trial compared the combination of an escalating regimen of ribavirin in combination with standard or pegIFNα and gave very similar results (Chung, 2004).

The APRICOT study (Torriani et al, 2004) was the largest trial in this setting. This was an international study and enrolled 868 patients who were randomised to receive conventional IFNα three times weekly (3 MU) with ribavirin (800 mg/day), weekly pegIFNα monotherapy (180 µg) or a combination of weekly pegIFNα (180 µg) and ribavirin (800 mg/day) for 48 weeks. The SVR was significantly higher in those who received combination therapy and reached 62% in genotype 2 or 3 infection but only 29% in genotype 1 infection. Treatment was associated with an average, reversible CD4 decline of 140 cells/mm^3 and even in this highly selected group of patients, 25% of the patients in the combined arm of the study had to discontinue in view of side effects or other reasons. A serious concern was that liver failure occurred and was fatal in 6/14 patients. This was associated with cirrhosis with Child-Pugh scores of 5 or more at baseline and didanosine-containing regimens (Mauss et al, 2004).

The pre-treatment viral load did not influence the response rate in those with genotypes 2 and 3 infection but in pegIFNα/ribavirin-treated genotype 1 patients the SVR was 61% in those with HCV RNA levels of <800 000 IU/mL and 18% in those with higher levels.

When the APRICOT study was designed there were concerns that higher doses of ribavirin may not be tolerated by patients in whom anaemia is a common problem, particularly those treated with concomitant zidovudine. Studies in HCV monoinfected patients revealed that higher doses of ribavirin in patients with genotype 1 infection were more effective

(Hadziyiannis et al, 2004) and this appears to be the case in HIV/HCV-coinfected patients also. In the PRESCO study (Ramos et al, 2007), higher weight-based dosing of ribavirin was used and extension of treatment duration from 48 to 72 weeks also improved the SVR rate in genotype 1 infected HIV-positive patients (53% versus 31%). Premature treatment discontinuations were common, however, and occurred in 45% in the prolonged treatment arm (2.6% due to severe anaemia). Extension of treatment duration from 24 to 48 weeks in genotype 2/3 also resulted in a higher SVR (82% versus 67%).

Viral kinetics have become increasingly important in this setting and it has been suggested that in genotype 2 or 3 infected patients, 24 weeks of therapy may be suitable, particularly for those with a low baseline HCV RNA level who demonstrate a rapid virological response (RVR; undetectable VL at 4 weeks), can demonstrate good adherence and have no evidence of advanced hepatic fibrosis (Perez-Olmeda, 2003). An extended treatment course may also be considered when patients show a consistent but slow decline in the HCV RNA level. In future, it is likely that there will be more tailoring of treatment courses according to the baseline host and virological parameters and to the initial virological response.

The APRICOT study also showed that even if patients did not achieve an SVR that in those who agreed to have a follow-up liver biopsy, there was nevertheless an improvement in the liver histology (Lissen, 2006).

Depression rates as high as 40% have been recorded when treating patients with HIV and HCV with interferon therapy. There are also reports of significant weight loss (Bani-Sadr et al, 2008). As discussed above, severe anaemia, neutropenia and reductions in CD4 count are important dose-limiting factors when treating co-infected patients. It is therefore important

to counsel patients regarding these potential side effects and review the nutritional state of the patient prior to initiation of therapy.

1.7.3 Treatment of acute HCV in HIV-positive patients

Studies investigating the efficacy of treatment of acute hepatitis C infection have been limited in numbers because hepatitis C infection does not often present in the acute phase due to lack of a clinical syndrome. If recognized in the acute phase, treatment with IFNα monotherapy in HIV negative patients is usually successful (98% SVR in patients treated at 12 weeks from diagnosis) (Jaeckel et al, 2001). HIV positive patients have a less optimal response to treatment (summarized in Table 1.1).

Table 1.1 Treatment of acute HCV in HIV-infected patients

Study	Serpaggi et al, 2006	Vogel et al, 2005	Gilleece et al, 2005	Dominguez et al, 2006
Genotype	4d (10), 1a (1), 3a (1)	1 (8), 2 (1), 4 (2)	1 (31), non-1 (5)	3a (5), 4 (6), 1a (4)
SVR (%)	0	91	59	71
Length of treatment (weeks)	24	11-48	24	24
Median CD4 (cells/μL)	604	507	566(spontaneous clearers) 349 (progressors)	345
Type of treatment	Varied IFNα monotherapy (11) ribavirin + IFNα(1)	Varied IFNα monotherapy (6) IFNα + ribavirin (5)	pegIFNα2b (1.5 μg/kg/ week) + ribavirin (800–1200 mg/day)	pegIFNα2a (180 μg/week) + ribavirin (800 mg/day)
Time to treatment	49 ± 22 days	1–12 weeks Median 2.6 weeks	12 weeks	Median 14 weeks
Sample size	*n* = 12 (10 treated)	*n* = 11	*n* = 50 (27 treated)	*n* = 25 (14 fully treated)
Country	France	Germany	UK	France
Type of study	Retrospective	Retrospective	Prospective	Prospective

1.7.3.1 Interferon monotherapy

The use of IFNα monotherapy in HIV positive patients with acute HCV superinfection has not been extensively investigated. Two small retrospective studies (Gillece, 2005; ,Serpaggi, 2006) have been published showing markedly different results (Table 1.1). One revealed an SVR rate of 0% while another small retrospective analysis revealed a high SVR rate of 91%. This contrasting difference in response rates to treatment is likely to reflect small sample sizes and differences in patient population. Further studies would be required prior to drawing any conclusions from these data.

1.7.3.2 Interferon and ribavirin combination therapy

Combination therapy with therapy with pegIFNα and ribavirin given 12 weeks from diagnosis has a reported success rate of 59–71% (Gillece et al, 2005; Dominguez et al, 2006) significantly less than in HIV negative patients (Serpaggi et al, 2006). Variation in patient population and timing of diagnosis and treatment from study to study may partially explain these differences in outcome. Patients presenting with a clinical syndrome associated with infection may increase the likelihood of response to treatment in contrast with those identified as a result of deranged liver function tests taken at routine HIV follow-up appointments. It is likely, however, that the major difference in SVR rates is likely to be due to perturbation of the immune response associated with HIV infection. Cell-mediated immunity to HCV is of key importance in controlling infection. HCV-specific antibody responses in HIV-infected patients are known to be impaired in patients with a CD4 count <500 cells/mm^3 (Netski, 2007). This

may limit control of the virus itself and may also impair early diagnosis of infection. Some HIV positive patients fail to produce an antibody response to HCV (Stramer, 2004) and therefore studies which include patients with a preceding negative antibody test followed by a positive polymerase chain reaction test may in fact include patients who are chronically infected with HCV but have not developed antibody seroconversion.

1.7.3.3 Treatment limitations

HIV positive patients with acute HCV may wish to elect for prompt treatment with IFNα and ribavirin in view of a higher likelihood of response during early infection. However, eligibility for treatment may be limited by comorbidity, lifestyle and access to specialized treatment centres. One study estimated that 70% of patients chronically infected with both viruses were ineligible for treatment (Fleming et al, 2003). The main reasons for exclusion were missed clinic visits, active psychiatric illness, active drug or alcohol use, decompensated liver disease or active medical illness. Ongoing IDUs with HCV represent a particularly challenging group of patients to treat because of increased clinic non-attendance and associated poor health. Intervention strategies targeting IDUs may occur outside of the hospital, for example, in the prison setting.

Patients who are considered eligible for treatment may require HAART or antidepressant medication prior to starting IFNα and this may result in a longer diagnosis treatment time period. Depression is a significant concern when treating such patients as it reaches a high prevalence in patients with dual infection (up to 42%) (Ryan, 2004). IFNα may cause depression itself and has been associated with suicide attempts while on therapy (Janssen, 1994). Many patients, therefore, require assessment by psychiatric services and antidepressant medication prior to initiating treatment. Specialist clinics treating

such patients benefit from a close psychiatry link in order to assess patient suitability for treatment and provide follow-up assessment and care during therapy.

Patients with CD4 counts <350 cells/mm^3 may benefit from the initiation of HAART prior to IFNα therapy in order to increase the likelihood of successful treatment and to avoid the risk of the CD4 count descending below 200 cells/mm^3 with the potential for opportunistic infection while on therapy.

In summary, treatment of HIV positive individuals infected acutely with HCV may achieve higher SVR rates with pegIFNα and ribavirin than those with chronic infection. However, a significant proportion of patients does not respond or qualify for treatment. Results of trials of novel therapeutic agents, e.g. polymerase and protease inhibitors are eagerly awaited.

Early studies with standard IFNα therapy were disappointing in this group of patients. Decreases in the CD4 count associated with treatment occurred (Vento et al, 1993; Soriano et al, 1995), and in some cases, resulted in life-threatening opportunistic infection (Pesce, 1993).

1.7.4 Prescribing antiretroviral therapy in HCV-infected individuals

Many HAART agents are potentially hepatotoxic and there have been concerns that there may be an increased risk of hepatotoxic reactions in those with hepatitis C. The Swiss cohort study showed that the number of treatment changes was similar in HCV positive and negative individuals when prescribed HAART (Greub et al, 2000). Several other studies, however, suggest that the incidence and possibly severity of HAART-associated hepatotoxicity is increased in patients with concomitant HBV or HCV infection (Sulkowski et

al, 2002). However, many reports of hepatotoxicity relate simply to biochemical liver function tests and a more detailed histological study (Mehta et al, 2005) found no evidence of increased long-term liver damage in patients with HIV/HCV who had been exposed to HAART.

There have also been concerns regarding impaired drug metabolism in patients particularly with advanced liver disease. One study showed that higher levels of efavirenz and nevirapine were seen in patients with advanced disease, but that there was very little effect on the drug levels of protease inhibitors (Barreiro et al, 2007). Overall the benefit of judicially selected HAART therapy appears to outweigh the benefit (Tural et al, 2003).

The situation becomes more complex when patients already taking HAART require HCV therapy and particular care is required in selecting the antiviral regimen – particularly the choice of nucleoside analogues.

The nucleoside analogues used to treat HIV infection undergo intracellular phosphorylation. Ribavirin, a purine guanosine analogue, also undergoes this process towards the triphosphate-active form. Ribavirin, *in vitro*, inhibits the intracellular phosphorylation of zidovudine, stavudine and zalcitabine. This does not seem to impair the efficacy of the HAART regimen. However, didanosine and abacavir phosphorylation is increased and it is thought that the increased intracellular levels of the active triphosphate form of didanosine contributed to the cases of mitochondrial toxicity/lactic acidosis reported in the APRICOT (51) study. A multivariate analysis of cases of spontaneous hepatic decompensation in the RIBAVIC study also found a significant association with didanosine use (Bani-Sadr et al, 2005).

Patients receiving a didanosine-containing regimen should therefore be switched to an alternative HAART regimen. There have also been concerns that patients with more severe liver disease taking stavudine-containing regimens may be at risk of lactic acidosis and many physicians also avoid stavudine-containing regimens in this setting.

Zidovudine and ribavirin can both cause anaemia so the concomitant use of both of these agents may require dose reductions of ribavirin or the use of erythropoietin.

A recent report suggests that abacavir may interact with ribavirin and that patients taking an abacavir-containing regimen have a reduced SVR, possibly as both are guanosine analogues (Bani-Sadr et al, 2007; Vispo et al, 2008) although this remains controversial (Amorosa et al, 2010; Laufer et al, 2008).

It has been suggested that an ideal time to treat the HCV infection is at an early stage in the HIV disease when the patient has a reasonable CD4 count and does not require HAART. This is supported by a study which found that the risk of hepatotoxicity with HAART was reduced in patients who had achieved an SVR with antiviral therapy for HCV (Labarga et al, 2007).

1.8 The natural history of acute HCV infection in HIV-negative and positive populations

Twenty-one years after the characterisation of the HCV virus (HCV) genome (Choo et al, 1989), the asymptomatic nature of early infection has meant that its natural history has only been partially described. Exposure to the virus is usually unnoticed by patients until they develop cirrhosis of the liver or hepatocellular carcinoma following many years of silent infection. Thus, while large cohorts of patients with chronic infection have been extensively

described, insights into early host-virus dynamics have largely been limited to follow-up of point-source outbreaks, for example, rhesus-negative women who received contaminated immunoglobulin during pregnancy (Power et al, 1995; Wiese et al, 2005) or case series including large proportions of patients with symptomatic disease (Farci et al, 2000). These latter studies are small-scale and may not represent the most usual course of early infection. Young women (such as those infected with immunoglobulin) and patients with symptomatic infection are known to have a better prognosis which is likely to have biased these studies. Even less is known about early HCV in HIV-infected individuals, in whom HCV is emerging as a key concern, with liver disease now a major cause of mortality in patients with access to HAART (Bica et al, 2001). An emerging epidemic of acute HCV infection in HIV-positive men in urban centres in Europe (Gillece et al, 2005; Dominguez et al, 2006; Vogel et al, 2005), USA (Fierer et al, 2008) and Australia (Matthews et al, 2009) has provided the opportunity to prospectively study the progression of early disease in a large number of individuals. Patients are identified early in infection, despite lack of symptoms, due to the detection of abnormal liver function tests at routine clinic follow-up.

In chimpanzees, acute HCV infection is characterized by positive viraemia by day 7 of infection and peak viral loads of 10^5-10^7copies/ml. These are higher and decrease more slowly in animals which progress towards chronic infection than those which resolve (Major et al, 2004; Bukh, 2004). Vigorous intrahepatic CD4+ and CD8+ responses correlate with viral load descent and spontaneous clearance (Major et al, 2004; Thimme et al, 2001; Cooper et al, 1999; Shoukry et al, 2004).

Delayed seroconversion, higher ALT levels and later IFNγ responses correlate with persistent infection. Interestingly responses to the HCV HVR-1 are associated with viral persistence, suggesting that this region could serve as an immunological decoy (Major et al, 2004; Table 1.2).

Table 1.2: Predictors of spontaneous clearance of HCV in experimentally infected chimpanzees (adapted from Major et al, 2004)

	Acute Resolving infection (n=4)	**Persistent infection (n=6)**
Appearance of viremia	Week 1	Weeks 1-2
Peak titres	10^5-10^6 genome copies/mL	10^6-10^7 genome copies/mL
Initial decrease in titres	Weeks 6-9	Weeks 7-10
Peak ALT	200-250 IU/mL (weeks 8-9)	200-650 IU/mL (weeks 9-17)
Seroconversion (ELISA 3.0)	Weeks 6-9	Weeks 7-14
Anti-HVR1	None detected	Weeks 11-15 (none detected in one animal)
IFNγ	Weeks 6-10	Weeks 8-12
Monocyte-induced protein 1	Weeks 2-20	Detected in only 1 animal (week 8)

While providing important insights into the early host-virus dynamics of HCV infection, it is unlikely that the chimpanzee model is entirely representative of the situation in humans. Viral load dynamics have not been well described in humans and while many studies have

investigated the role of B and T cell responses in small groups of patients, longitudinal responses in a larger group of patients have not been published to date. In humans, a number of predictors of spontaneous clearance are known (Table 1.3).

Table 1.3: Predictors of spontaneous clearance of acute HCV in humans

Host	Favourable	Unfavourable	Reference
Sex	Female	Male	Micallef et al 2006
Bilirubin	High	Normal	Micallef et al 2006
T cell response	Multispecific CD4 and CD8	Narrow weak response	Lauer et al 2005, Gruner et al 2000, Gerlach et al1999
HLA	HLA-B27 HLA-DQB1*0301 HLADRB1*01	HLA-DRB1*0701 HLA-DRB4*0101	Neumann et al 2007, Thursz et al 1999, Barrett et al 1999
KIR/HLA C	KIR2DL3/KIR2DL3-HLA-C1C1		Khakoo et al 2004
HIV	Negative	Positive	Thomas et al 2000
Route of transmission	Sexual	IDU	Shores et al 2008
IL28B (IFN-λ3) promoter single nucleotide polymorphism rs12979860	C/C genotype	T/T genotype	(Thomas et al, 2009)
Virus			
Viral diversity	Low	High	Farci et al 2000
dN/dS ratio	Low (<1)	High(>1)	Ray et al 1999, Farci et al 2000

1.9 Aims and hypotheses

In this thesis, a cohort of 110 HIV-positive MSM with acute HCV and a background of HIV-1 infection was followed longitudinally in order to address the following research hypotheses:

1. HCV diversification and evolution predicts the outcome of acute infection in a cohort of HIV-infected individuals
2. Diversification occurs early during the course of infection and defines the later viral population and response to treatment
3. The evolution of acute HCV in this cohort is defined by the adaptive cellular immune response

We aimed to:

1. Characterise the natural history of acute HCV in an HIV positive MSM cohort (Chapter 2)
2. Determine the diversification and immune-mediated selection of HCV in HIV co-infected patients over time by phylogenetic analysis of the HCV E2 HVR-1, NS5A and NS5B regions (Chapters 2-3)
3. Characterise the adaptive immune response during acute HCV infection (Chapter 4)

Chapter 2: Predicting outcome in HIV-positive MSM with acute HCV

2.1 Background

2.1.1 The phyloepidemiology of acute HCV in HIV-positive men

In 2004, three reports of acute HCV infection occurring in HIV-positive men in London, Paris and Amsterdam appeared in the scientific literature (Gilleece et al, 2005; Ruys et al, 2004; Ghosn et al, 2004). All of the affected individuals were MSM and it was considered likely that the route of transmission was sexual. A detailed case-control and phylogenetic study carried out by Danta and colleagues (2007) later prospectively investigated routes of transmission in 111 HIV-positive patients with acute HCV recruited from 3 centres in the UK. In this study, the main risks for infection were found to be sexual. Intranasal and rectal recreational drug use were also significantly more common in cases than in controls when univariate statistical tests were used to analyse each risk factor separately, but became insignificant following multivariate logistic regression. Interestingly only 1 patient in the case group admitted to using intravenous drugs, although 57% admitted to using crystal methamphetamine which is commonly injected but may also be smoked, suggesting the possibility of under-reporting of parenteral drug use, even when using a confidential questionnaire. Other cohorts have reported higher rates of parenteral drug use; 4/11 in one study which examined the first cases in New York (Fierer et al, 2008). The sample size and resultant statistical robustness of this study was not comparable, however, to that presented by Danta et al. Furthermore, the inclusion criteria in this study for the diagnosis of acute HCV were extremely broad. It is highly likely, therefore, that the primary mode of transmission of HCV in this group of patients is sexual. Intranasal mucosal exposure and

parenteral drug use also remain a possible route of transmission in at least a minority of these individuals.

Case control evidence that transmission events are interrelated is further supported by genetic sequence analysis providing evidence of a new predominantly sexually-transmitted emerging epidemic. Phylogenetic analysis of a region of the HCV E2 gene, including the HVR-1, revealed several distinct monophyletic lineages in MSM when compared with reference strain sequences obtained from IDU patients, indicating a number of interrelated introductions of HCV into the MSM population (Danta et al, 2007). A subsequent cross-sectional phylogenetic analysis of HCV NS5B sequences from multiple centres in Australia and Europe also revealed several distinct monophyletic lineages (van de Laar et al, 2009). Each lineage contained interrelated sequences obtained from different countries, revealing evidence of a global transmission network in MSM.

2.1.2 Methods used to assess viral diversity

Clonal sequencing is a powerful research tool, providing a sample of viral population diversity and has been the gold standard approach for a number of years, providing key insights into host-virus interactions. It has a number of limitations, however. A particular concern is that errors may be introduced during the PCR, particularly when *Taq polymerase* is used, rather than a proof-reading enzyme. If an error is generated early on in the PCR reaction, it will be introduced into many cDNA amplicons and give an unrealistic picture of diversity. Unfortunately, cost may limit the use of proof-reading enzymes in large-scale

analyses. A limiting dilution PCR approach can help to overcome some of this error, but is laborious and also unsuitable for large-scale studies. Reverse transcription and amplification in *E coli* may also result in the introduction of error as can the sequencing reaction itself. Bidirectional sequencing may reduce error rates at this stage, but again has cost implications. Another problem is primer selection where the primers are designed to anneal to a region of high viral diversity e.g. the HVR-1 and will not amplify all variant strains. Use of multiple primer sets or degenerate primers may help to reduce this problem.

While clonal sequencing has been traditionally used in the past to analyse quasispecies diversity, it is usually used to generate 10-20 sequences at a time. Thus minority variants may be undetected. The recent development of ultradeep pyrosequencing is starting to revolutionise analysis of viral populations as it may be used to analyse tens of thousands of variants in a single step. Pyrosequencing is discussed further in Chapter 3.

Another technique, designed to overcome the more laborious or expensive analyses described above is the heteroduplex diversity assay. This assay is a PCR assay, designed to identify DNA heteroduplexes which occur as a result of quasispecies diversity. In order to carry it out, a PCR reaction is set up, following which ethylenediaminetetra-acetic acid (EDTA) is added to the reaction mix to deactivate DNA polymerase. A denaturation step to separate the cDNA into single strands is then carried out at 95°C followed by slow cooling to allow annealing of homologous and heterologous strands. This technique can detect up to

2% variation in viral quasispecies when compared with sequence analysis. The duplexes are then counted under ultraviolet light following gel electrophoresis (Shire et al, 2006).

2.1.3 Predicting spontaneous clearance of acute HCV using measures of viral evolution

Although the cross-sectional phyloepidemiology of acute HCV in this population has been well characterised (Danta et al, 2007; van de Laar et al, 2009), the evolution of viral populations over time in individual patients has not. As the virus has an extremely rapid generation time and each viral genome is copied imperfectly due to the lack of proof-reading function of the HCV viral RNA polymerase (Neumann et al, 1998), within any individual, a swarm of related, but genetically different viral sequences circulate at any given time; the viral quasispecies. The ability of the host immune system to recognize and eliminate each variant strain is highly likely to influence the final outcome. In a small, well-characterized cohort (n=12) of HIV-negative individuals, it has been shown that those who progress to chronicity have a higher rate of viral diversification than those who clear the virus spontaneously (Farci et al, 2000). In this study, patients were selected based on outcome and divided into 3 clinical groups: 3 patients had fulminant hepatitis, 3 spontaneously cleared infection and 6 had acute hepatitis that progressed to chronicity. The progressor group were further subdivided into slow and rapid progressors (mild stable disease versus severe and rapidly progressive disease leading to death within 5 years). The number of viral variants and the genetic diversity of the HCV quasispecies were assessed by examining viral sequences spanning the envelope genes (*E1* and *E2*) both within and outside the hypervariable region 1 (HVR1). Genetic diversity was calculated using the mean Hamming distance which is a measure of the mean number of amino acid differences

between paired sequences. While the genetic diversity and the number of viral variants within the HCV quasispecies in early samples did not differ significantly between patients who resolved their infection and those who developed chronic disease, later samples, obtained after antibody seroconversion revealed significant differences between groups. Patients with spontaneous clearance had a decrease in genetic diversity of the HVR-1, whereas those with progressing hepatitis showed a marked increase in diversity. The patients with fulminant hepatitis (a rare complication of acute HCV infection) showed the lowest degree of genetic diversity, both before and after seroconversion, despite high HCV RNA levels. These results suggest that the immune system is key both in eradication of the virus and in the pathogenesis of disease. To analyse differences in genetic sequence further, a calculation of the ratio of nonsynonymous (amino acid replacement; dN) nucleotide substitutions per nonsynonymous site to synonymous (silent; dS) nucleotide substitutions per synonymous site was carried out. The dN/dS ratio was highest in progressor patients; evidence of ongoing positive selection and low in spontaneous clearers and patients with fulminant hepatitis.

The relationship between viral sequence diversity and spontaneous clearance of acute HCV in HIV-positive patients has not previously been described and is the subject of investigation in this chapter.

2.1.4 Response to treatment

The disparity in SVR rates between HIV-negative and HIV-positive patients treated with pegIFNα and/or ribavirin is not fully understood (Jaeckel et al, 2001; Gilleece et al, 2005; Serpaggi et al, 2006, Dominguez et al, 2006; Table 1.1) with only one small study (n=11)

reaching a sustained virological response (SVR) rate comparable with that achieved in HIV-negative individuals (Vogel et al, 2005). Early diagnosis and treatment of HIV-infected patients with pegIFNα and ribavirin results in improved SVR rates [59% in acutely versus 40% in chronically infected patients (Gilleece et al, 2005; Torriani et al, 2004)], but does not reach the 98% treatment success rate reported in HIV-negative individuals (Jaeckel et al, 2001).

Impaired immune control of HCV secondary to HIV-infection may result in a higher rate of viral evolution and the formation of multiple variants. A number of studies have been carried out using either clonal sequencing or heteroduplex diversity assays to assess variation within the HCV quasispecies and response to treatment during chronic HCV infection, mainly in HIV-negative populations, but also more recently in HIV-positive patients. Two regions of the genome have been analysed in multiple studies; the E2 HVR-1 and the interferon sensitivity determining region (ISDR) within NS5A.

Pre-treatment quasispecies complexity within the E2 HVR-1 and NS5A region is negatively associated with viral clearance rate and SVR during chronic HCV infection using heteroduplex (Shire et al, 2006) and clonal sequence analysis (Ueda et al, 2004) in both HIV-positive and negative patients. These studies focussed primarily on genotype 1 infection. A recent study confirmed similarly that interferon non-responders with genotype 3 infection, normally associated with higher SVR rates in chronically infected patients also had evidence of higher viral complexity within the E2 HVR-1 (Moreau et al, 2008). Hypothetically, high

viral diversity may result in a higher likelihood of IFN-resistant strains within the quasispecies.

A number of studies have shown that certain ISDR prototype sequences are associated with resistance to pegIFNα. The phenomenon has been well described in Japanese genotype 1b isolates (Enomoto et al, 1995; Enomoto et al, 1996) where 'mutant type', meaning 4 or more nucleotide differences between the 1b isolate and the HCV-J reference strain within the ISDR, are associated with a high SVR rate. A more recent analysis revealed that as few as 2 differences from the HCV-J 1b reference sequence increase the chances of SVR with an odds ratio of 5.18 (Nakagawa et al, 2010). Another study carried out in Japan used a sliding window approach (discussed further in Chapter 3) following a full genome analysis to identify a specific mutation within HCV core - the R (arginine) 70Q (glutamine) substitution, which was found to be strongly associated with resistance and lack of early virological response (EVR). The predictive value of the ISDR in European and American genotype 1b isolates has revealed varying results, possibly due to small study populations, but a metanalysis suggested that the phenomenon extended to isolates from around the globe (Pascu et al, 2004). A similar phenomenon has been reported also in genotype 1a infection (MacQuillan et al, 2004). A region upstream of the ISDR within NS5A has also been associated with interferon treatment response (Durante Mangoni et al, 2003) with a higher prevalence of mutations in responder-relapsers versus non-responders ($p<0.001$).

The mechanism behind increased resistance to pegIFNα and ribavirin in certain HCV strains is not yet fully understood. Previous studies in subgenomic replicon systems revealed that both E2 and NS5A may inhibit the double-stranded RNA-dependent protein kinase (PKR)

which is induced by IFN (Gale et al 1998; Taylor et al, 1999). However, a recent publication making use of the JFH-1 infection system revealed that IFN strongly induces interferon stimulated gene mRNA transcription in HCV-infected cells, reaching higher levels than in uninfected cells, indicating that HCV may not in fact block IFN signalling. However, despite high mRNA levels, IFNα-induced, interferon stimulated protein expression is attenuated in HCV-infected cells in parallel with the strong phosphorylation of PKR and eIF2α (Garaigorta & Chisari, 2009) Thus the phenomenon of reduced interferon stimulated protein expression is well-described, but the mechanism remains unclear.

Although the correlation between high viral diversity and low SVR rate is well described, the time at which the diversity reaches a level less likely to be amenable to treatment is not known. It is not clear for example, if the diversity level at transmission defines the outcome, or if there is a gradual accumulation of mutations over time reflecting the window period during acute disease when the infection may be treated more easily. If transmission diversity is a factor, it might be expected that patients with haemophilia as a route of transmission would have greater diversity and lower SVR rates than intravenous drugs users, who in turn might have greater diversity than those with mucosal exposure through sexual or intranasal exposure.

If the time at which the quasispecies diversity reaches a threshold level defines suitability/timing of treatment, this could potentially be used to target treatment appropriately in individual patients.

2.2 Hypotheses

HCV diversification and evolution predicts the outcome of acute infection in a cohort of HIV-infected individuals

2.2.1 Viral diversification of HCV in HIV-positive patients during early infection determines the likelihood of spontaneous clearance, and

2.2.2 Viral diversification of HCV in HIV-positive patients during early infection determines the likelihood of SVR following treatment with pegylated interferon alpha and ribavirin

2.3 Aims

To characterise the natural history of acute HCV in an HIV positive MSM cohort

2.3.1 To identify the best diagnostic test for acute HCV in HIV-positive patients

2.3.2 To characterise the viral dynamics and clinical outcomes of the acute HCV cohort, and

2.3.3 To quantify viral diversity and rate of evolution of the HCV E2 HVR-1, NS5A and NS5B during acute infection

2.4 Methods

2.4.1 Patient cohort

A cohort of 110 patients with HIV and acute HCV (100 with chronic HIV/acute HCV and 10 with acute HIV/acute HCV coinfection) were prospectively recruited from 2005-2010 in a single centre (the St Mary's Acute HCV Cohort; Figure 2.1). Plasma and peripheral blood mononuclear cell (PBMC) samples were obtained prospectively at 1-3-monthly intervals. Stored frozen samples (taken for the routine measurement of HIV viral load between 1994 and 2010) were also analyzed retrospectively.

Informed consent in writing was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. The study was approved by St Mary's Hospital Research Committee and ethical approval granted by the Riverside Research Ethics Committee (Reference number 05/Q0401/17). Patients gave written consent for participation in the study.

Patients were offered 48 weeks of treatment with pegIFNα and weight-based ribavirin (800-1200mg) 12-24 weeks following HCV diagnosis unless this was contraindicated or spontaneous clearance occurred.

2.4.2 Case definition and end-points

Acute HCV diagnosis was defined by a positive HCV RNA result by RT-PCR within 6 months of a preceding negative RT-PCR or antibody test. The date of infection was estimated as the midpoint between the last negative and first positive test.

Spontaneous clearance of HCV was defined as 2 successive negative RT-PCR tests (<12IU/ml) 3 months apart.

Sustained virological response (SVR) was defined as a negative HCV RNA by RT-PCR (<12IU/ml) 24 weeks after the end of 48 weeks of treatment with pegIFNα and ribavirin.

Samples for HCV viral load and liver function (ALT, bilirubin, and albumin) were taken at monthly intervals for 3 months following diagnosis and at 3 monthly intervals thereafter. CD4 count and HIV viral load were assessed at 3 month intervals. Viral diversity within the E2 HVR-1, NS5A, NS5B and the 5'UTR was determined by clonal analysis at 1-3 monthly intervals.

2.4.3 Storage of blood samples

Blood samples were obtained in EDTA-containing vacutainer tubes. Plasma and PBMCs were separated on the day of clinic attendance and stored at -80^{o}C and in liquid nitrogen (-180 ^{o}C) respectively. All samples were processed in a designated category 3 laboratory.

2.4.4 Anti-HCV Enzyme Linked Immunosorbent Assays (ELISAs)

Stored plasma samples were tested for the presence of antibodies to HCV core, NS3 and NS4 using a third generation enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (Monolisa, BioRad). A second kit designed to detect core antigen as well as antibodies was also used (Monolisa AgAb Ultra, Biorad). Assays were carried out in duplicate in a 96-well plate. Positive and negative controls were included in each assay. Diluent (80µl) was added to each well followed by 20µl of plasma, covered with cling film and incubated for 1 hour at 37^{o}C. The film was removed and plasma in the wells washed 3 times with 370µl of washing solution. Conjugate solution (100µl) was then added

to each well and incubated at 37^{o}C for 30 minutes. The contents of the wells were washed 4 times, 100µl development solution added and incubated in darkness at room temperature for 30 minutes. Stopping solution (100µl) was added and absorbance readings were obtained at 450 and 620nm. A cut-off value (CO) for positive and negative samples was obtained according to the formula:

$$\text{CO} = \frac{\text{mean positive control A450}}{5}$$

Borderline samples were those within 10% of the CO value. The date of infection with HCV was taken as the date of the first positive PCR result. The date of antibody seroconversion was the date of the first positive antibody result.

2.4.5 Extraction of viral RNA from plasma samples

Plasma stored at -80^{0}C was thawed and ultracentrifuged at 25,000g for 1 hour at 4^{o}C. RNA was extracted using the QIAamp Viral RNA kit (Qiagen). An aliquot (140µl) of plasma was added to 560µl cell lysis solution (buffer AVL) containing 5.6µl carrier RNA and incubated for 10 minutes. Ethanol (560µl) was then added and the sample applied to columns and centrifuged at 8,000g. Two further centrifugation steps were carried out using 500µl of wash buffers AW1 and AW2. Finally, extracted viral RNA was eluted in 60µl of buffer AVE (RNase-free water containing 0.04% sodium azide).

2.4.6 Quantitative RT-PCR assays

Quantitative HCV RNA levels were measured using automated real-time RT-PCR (Abbott) in the clinical virology laboratory at St Mary's Hospital. Backdated plasma samples were

diluted 1:10 in PBS prior to analysis. The ABBOTT Real Time HCV assay is an in vitro RT-PCR kit for the quantitation of HCV RNA in human serum and plasma from HCV infected individuals. An RNA sequence that is unrelated to the HCV target sequence is simultaneously amplified by RT-PCR and serves as an internal control (IC) to demonstrate that the process has proceeded correctly. The target sequence for the Abbott Real Time HCV assay is in the 5′ UTR of the HCV genome, which is highly conserved. The primers are designed to hybridise to the 5′ UTR region with the fewest possible mismatches among HCV genotypes 1–6. The limit of detection of the assay is 12 IU/mL (48 copies/mL).

2.4.7 Amplification of the HCV 5'Untranslated Region (5'UTR)

Following clinical diagnosis of acute HCV, stored plasma samples (obtained for HIV viral load testing) were retrospectively tested for the presence of HCV RNA using nested RT-PCR with primers 26, 27, 28 and 29 (Table 2.1) from the 5' UTR. This region was chosen as it is one of the most conserved between isolates.

Table 2.1: Primers used for RT-PCR amplification of the 5'UTR

Outer (5'-3')		**Inner (5'-3')**	
26	GTCTAGCCATGGCGTTAG	28	GTGTCGTGCAGCCTCCAG
27	GCACGGTCTACGAGACCT	29	GGGGCACTCGCAAGCACC

First-round PCR reactions were carried out using a OneStep RT-PCR kit (Qiagen) with a reaction mix as follows: 2-10µl of template RNA, 10µl of RT-PCR buffer (final $MgCl_2$ concentration of 2.5mM), 10µl of Q solution, 2µl of dNTP Mix (containing 10 mM of each

dNTP), 2µl of enzyme mix, 1µl of each primer (0.3µM/reaction) and RNase-free water to a final total volume of 50µl. The PCR cycling conditions were as follows: Reverse transcription 50^{o}C 30 minutes; reverse transcriptase inactivation 95^{o}C 15min; 40 cycles of denaturation at 94^{o}C for 30seconds, annealing at 55-60^{o}C for 30second and elongation at 72^{o}C for 30seconds. A final extension was carried out for 10 minutes at 72^{o}C. Second round PCR was carried out using a PCR mastermix (Megamix, Helena Biosciences). An aliquot (10µl) of round 1 PCR product was added to 40µl mastermix and cycled as above.

An end-point dilution assay was carried out to estimate the sensitivity of the 5'UTR assay.

2.4.8 Amplification of the HCV Hypervariable region-1 (HVR-1)

Amplification of the HCV E2 HVR-1 was optimised using nested PCR with genotype-specific primers (Table 2.2) and PCR cycling conditions, as described above.

Table 2.2: Primers used in RT-PCR for amplification of the E2 HVR-1

Outer (5'-3')		Inner (5'-3')	
E4	GGTGTGGAGGGAGTCATTGCAGTT	E3	GGGCAGTGCTGTTGATGT
E5	GCTTGGGATATGATGATGAACTGGTC	E3	AGGCCGTGCTATTGATGT
E6	CGCCACTGGACCACCCAGGATTGCAATTGTTC	E3	GGGCAGTCCTATTTATATGCC
E7	CCCAGTGTTTAAGCTGTCATTGC	E1U	GGGATATGATGATGAACTGG
		214	CACTGGGGAGTCCTGGCGGGC

PCR was carried out using genotype-dependent primers, as shown above. These were adapted from previously published primers (Gaud et al 2003) by aligning with genotype 1a, 4d and 3a isolates and selecting a 17-28bp region with a GC content of 50-60% and similar melting temperatures (Tm; 55-80^{O}C) using Primalign (http://hcv.lanl.gov/content/hcv-db/PRIMALIGN/PRIME.html). Each primer name is followed by the genotype it may be used to amplify in parentheses. Primer design was carried out using the primer design programme Primer3 available at http://primer3.sourceforge.net/ which predicts potential problems with secondary structures such as hairpin formation which may reduce subsequent amplification.

2.4.9 Amplification of NS5A by RT-PCR

Amplification of NS5A was optimised using semi-nested PCR with primers (Table 2.3) and PCR cycling conditions as described above. Primers were adapted for use from previously published primer sequences designed for genotype 1b HCV (Durante Mangoni et al 2003) by aligning with genotype 1a, 4d and 3a isolates, as above.

Table 2.3: Primers used for amplification of NS5A

Outer (5'-3')		Inner (5'-3')	
378	GTGTTGACGTCCATGCTCACTG	379	CCCATATAACAGCAGAGGCGGC
382	GTTGCCGCCCATCTCCTGCCTCC	382	GTTGCCGCCCATCTCCTGCCTCC

2.4.10 Amplification of NS5B by nested PCR

Amplification of a 376bp region of NS5B was optimised using semi-nested PCR with degenerate primers (Table 2.4; Enomoto et al, 1990). The PCR reaction was carried out using a blend of Taq polymerase and the proof-reading enzyme Tgo polymerase as part of the Titan One-Step PCR Kit (Roche). Reaction mixes were prepared on ice. Master mix 1 consisted of 10.5µl sterile water, 4µl dNTP, 2.5µl DTT solution, 1µl (5U) RNase Inhibitor, 1µl primers (0.4 µM) and 5µl RNA and was added to master Mix 2 consisting of 14µl water, 10µl 5x RT-PCR buffer and 1µl of enzyme mix. Reverse transcription was carried out at 50°C in a thermocycler. 40 cycles of denaturation at 94°C for 2 min, annealing at 50-72°C for 30 s and elongation at 68°C for 1 minute. PCR samples were then analysed on a 2% agarose gel.

Table 2.4: Primers used for amplification of NS5B

Outer (5'-3')		Inner (5'-3')	
ENO2	TGGGSTTYKCSTATGAYACYCGMTGYTTTGA	NS5S3	TATGATACCCGCTGCTTTGACTCCAC
ENO4	ARTACCTRGTCATAGCCTCCGTGAA	NS5A5	GTCATAGCCTCCGTGAAGGCTC

2.4.11 Gel Electrophoresis and DNA extraction

PCR products were run on a 1% agarose gel containing ethidium bromide with lane markers and a 100bp ladder (Promega). DNA bands visualised under ultra-violet light and bands of appropriate size were cut out using a clean scalpel blade. These bands were purified to remove extraneous products by column centrifugation using a QIAquick gel extraction kit (Qiagen), and resuspended in 30µl of dH20.

2.4.12 Cloning

Purified PCR products were ligated into a plasmid vector (pCR®4-TOPO®; Appendix 1) using a TOPO-TA Cloning kit, Invitrogen). An aliquot (2-4µl) of PCR product was added to 0.5-1µl of salt solution (1.2M NaCl plus 0.06M MgCl2) and 0.75-1µl of pCR®4-TOPO® vector and incubated at room temperature for 5-30 minutes. Ligation of the PCR product disrupts expression of the lethal *Escherichia coli* suicide gene, *ccd*B permitting growth of only positive colonies upon transformation in TOP10 or Mach-1 cells (Invitrogen).

The cloning reaction (2µl) was added to a vial of TOP10 or Mach-1 *E. coli* cells and incubated on ice for 5-30 minutes. The cells were then heat-shocked for 30 seconds at 42°C in a water bath and transferred back to ice. A volume (250µl) of room temperature S.O.C. medium (2% tryptone, 0.5% yeast extract 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose) was added to each vial, capped and shaken horizontally (250rpm) at 37°C for 1-2 hours. An aliquot of 30-100µl from each transformation was spread on a pre-warmed agar plate containing 100µg/ml ampicillin or 50 µg/ml kanamycin and incubated overnight at 37°C. Incorporation of the insert into the plasmid disrupts transcription of a suicide gene, enabling selection of clones containing the product.

2.4.13 Preparation of DNA following clonal amplification

Twenty bacterial colonies per plate were selected and transferred to 10ml Falcon tubes containing 2ml of LB medium (10g tryptone, 5g yeast extract, 10g NaCl in 800ml of distilled water) and 100g/ml ampicillin. These were cultured in a horizontal shaker (250rpm)

overnight at 37 ^{o}C. Bacterial cultures were transferred to 1.5ml Eppendorf tubes or a 96-well plate and centrifuged for 1 minute at 8,000g. The supernatant was removed and cells resuspended. DNA extraction was carried out using a Qiagen miniprep kit. Plasmid DNA was resuspended in 30µl of dH_20. Spectrophotometry was carried out at an absorbance (A) of 260nm in order to quantitate the concentration of DNA. The ratio of values obtained at A260/A280 was calculated to estimate the purity of DNA produced. Successful transformation was confirmed by digestion using the enzyme EcoR1, which cuts the vector at two sites located on either side of the insert, generating 2 linear strands of DNA. Digested products were run on a 1-2% agarose gel stained with ethidium bromide and visualised under ultraviolet light.

2.4.14 Sequencing

Envelope products were sequenced using the ABI BigDye Terminator v3.0 system (Applied Biosciences, UK). The reaction mix consisted of an 8µl reaction mix, 1µl 3.2µM sequencing primer (Table 2.5) with 500ng of DNA template. dH_2O was added to make a total volume of 20µl. Cycling conditions were as follows: 25 cycles of 10 sec at 96^{o}C, 15 sec at 50^{o}C and 4 min at 60^{o}C. 2µl of 3M sodium acetate and 50µl of 95% ethanol were added to each sample and left on ice for 30 minutes. The sample was then centrifuged at 13,000g for 30 min. The supernatant was removed and a further 200µl 70% ethanol added. The samples were centrifuged for 5 min at 13,000g, the supernatant aspirated and left to air dry for 10 min. T7, T3, M13 forward (M13F) and M13 reverse (M13R) primers were tested; these bind specifically to the vector, allowing the insert to be sequenced in both directions. M13F was

associated with superior results and was used for the majority of experiments due to a longer distance from the DNA insert sequence.

Table 2.5: Sequencing primers

Forward (5'-3')		Reverse (5'-3')	
T3	ATTAACCCTCACTAAAGGGA	T7	TAATACGACTCACTATAGGG
M13F	GTAAAACGACGGCCAG	M13R	CAGGAAACAGCTATGAC

2.4.15 Estimated PCR error rate

Universal precautions to avoid PCR contamination were followed at all times. PCR mixes were prepared in an allocated room separate from clinical samples. In order to confirm the possibility of multiple superinfection events and to rule out PCR contamination error, PCR amplification of the HCV NS5B region (in addition to E2 HVR-1 amplification) was carried out.

An estimate of PCR error rate was carried out using the QIAgen one-step PCR kit and MegaMix Blue PCR kit (Helena Biosciences), containing *Thermus aquaticus* (Taq) DNA polymerase. In order to calculate the actual PCR and cloning error rate, a subcloning experiment was carried out. Firstly, the 5'UTR was amplified by nested PCR using primers 25, 26, 27 and 28 (Table 2.1). The PCR product was then inserted into the pCR®4-TOPO®vector (Invitrogen) and amplified. One colony was picked and grown in LB medium containing ampicillin (50µg/ml). Following centrifugation, the DNA was extracted by column centrifugation (QIAgen miniprep kit). An end-point dilution was carried out and 10 copies of cDNA product was reinserted into the vector in order to avoid PCR saturation. 175 colonies were picked, grown in LB medium, and DNA extracted following centrifugation.

Recombination of DNA may occur in vitro during PCR amplification. In order to assess the presence of recombination in samples, similarity plots and bootscanning was carried out using Simplot and PHYLIP software. In order to distinguish between recombination occurring in vitro and in vivo, end-point dilution PCR is necessary in order to amplify single DNA molecules. In order to minimise the necessity for multiple PCR reactions, the presence of possible recombination was assessed as above for all cloned sequences from each PCR reaction.

2.4.16 Sequence analyses and construction of phylogenetic trees

Sequences were aligned using Clustal W. Phylogenetic trees were constructed with MEGA 4.0 using the neighbour-joining method and Kimura two-parameter distance for all substitutions, and the inferred phylogenies were tested with 1,000 bootstrap replications. Genetic diversity was calculated using the Hamming distance, which is defined as the number of amino acid differences between two sequences using the formula (1 - s) x 100, where s is the fraction of shared sites in two aligned nucleotide sequences. Genetic distance was calculated as the mean percentage difference between all samples at any time point. Mean intra-strain corrected genetic distance and Hamming distances were also calculated (separate strains were defined as those originating from distinct branches following phylogenetic analysis with >10% difference from other strains derived from the same patient). dN/dS ratios were calculated using MEGA 4.0. dN/dS values >1 were considered as evidence of positive selection.

2.4.17 Statistical analyses

Univariate logistic regression, students t-test, Mann-Whitney, Chi square and Fisher exact tests were used to analyze the significance of observations using the statistical computer package STATA 10.0. Odds ratios were calculated with 95% confidence intervals. P values of less than or equal to 0.05 were considered to be statistically significant.

2.5 Results

2.5.1 Patient cohort diagnosis, treatment and follow-up

Patients were recruited following referral to a dedicated acute HCV clinic on the basis of a new positive HCV antibody or PCR test. The diagnosis was confirmed by retrospective PCR testing of stored plasma obtained for routine measurement of HIV viral load in the clinical laboratory as described below and the date of infection was estimated as the midpoint between the last negative and first positive PCR test. Patients were followed up at 4 weekly intervals for 3 months and 3-monthly thereafter. Treatment was offered at 3-6 months following diagnosis unless contraindicated (Figure 2.1).

2.5.2 Diagnosis of acute HCV

Three tests were assessed and compared with RT-PCR for the diagnosis of acute HCV on a subset of 43 patients who had had frequent plasma sampling prior to inclusion in the study, allowing retrospective dating of infection by RT-PCR; ALT (ALT), the Monolisa HCV Antibody assay and the Monolisa HCV Antigen–Antibody Ultra assay. In this group, the median HCV viral load was 1 836 595 IU/ml. A variety of HCV genotypes were identified; 1a in 75%, 4d in 17.5%, 1b in2.5% and 3a in 2.5% while 2.5% were coinfected with genotypes 1a and 4d. The median CD4 cell count at the time of HCV acquisition was 570 cells/µl. The median nadir CD4 cell count was 325 cells/µl. Those patients who spontaneously cleared infection had a trend towards a higher nadir CD4 cell count (median 440 cells/µl versus median 320 cells/µl; *P*=0.07).

Figure 2.1: Patient cohort diagnosis, treatment and follow-up

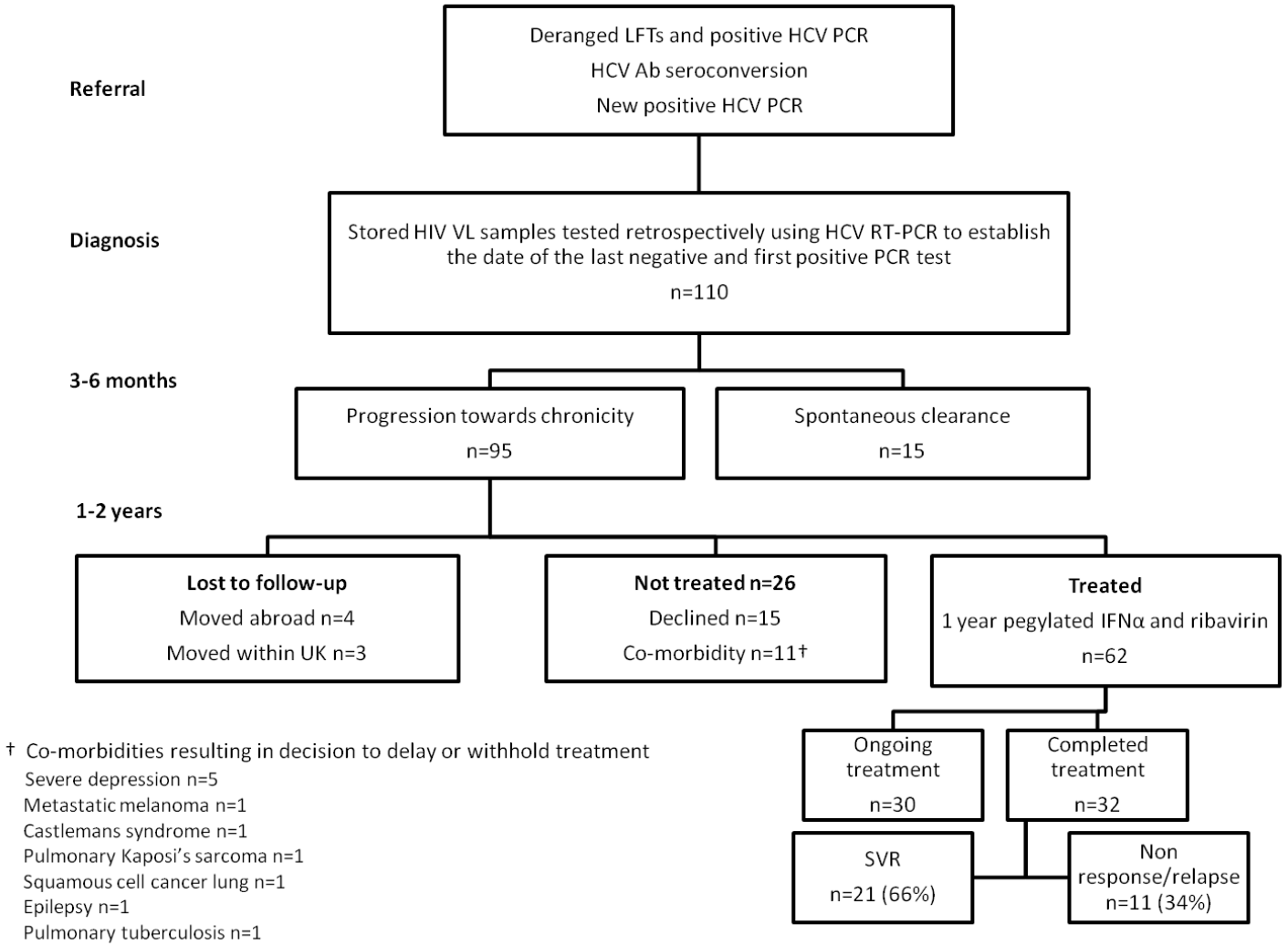


Figure 2.1 Patient cohort, diagnosis and follow-up. All patients were treatment with interferon alpha and ribavirin unless this was contraindicated. Reasons for withholding or delaying treatment are given.

2.5.2.1 Seroconversion time

The median time from HCV RNA detection by PCR to the development of anti-HCV antibodies was estimated to be 91 days (range 0–1206 days; Thomson et al, 2009). An analysis of seroconversion time in a subset of patients where the last negative and first positive RT–PCR were less than 30 days apart (*n*=8) revealed a median seroconversion time of 158 days (range 47–209 days). Two patients (4.7%) developed anti-HCV antibodies more than 1 year after the initial infection, one of whom did not become antibody positive until after 3 years. Seroconversion in one of these patients was associated with a superinfection with genotype 1a virus on a background of genotype 4 infection. No correlation between CD4 cell count at the time of HCV diagnosis and seroconversion time was found (*P*=0.96) although a weak negative association between nadir CD4 cell count and seroconversion time was observed that did not reach statistical significance (p=0.09). Seroconversion time in spontaneous clearers and progressors was not significantly different (*P*=0.88). However, seroconversion time was significantly longer in patients with a low HCV viral load (*P*=0.04) or low ALT (*P*=0.0005) at the time of the first positive PCR. A low ALT level at 3 months into HCV infection was also weakly associated with prolonged seroconversion time (*P*=0.08). The early development of anti-HCV antibodies did not correlate with patient age (*P*=0.78), HCV genotype (*P*=0.51) or HIV viral load (*P*=0.22).

Figure 2.2 Time to seroconversion

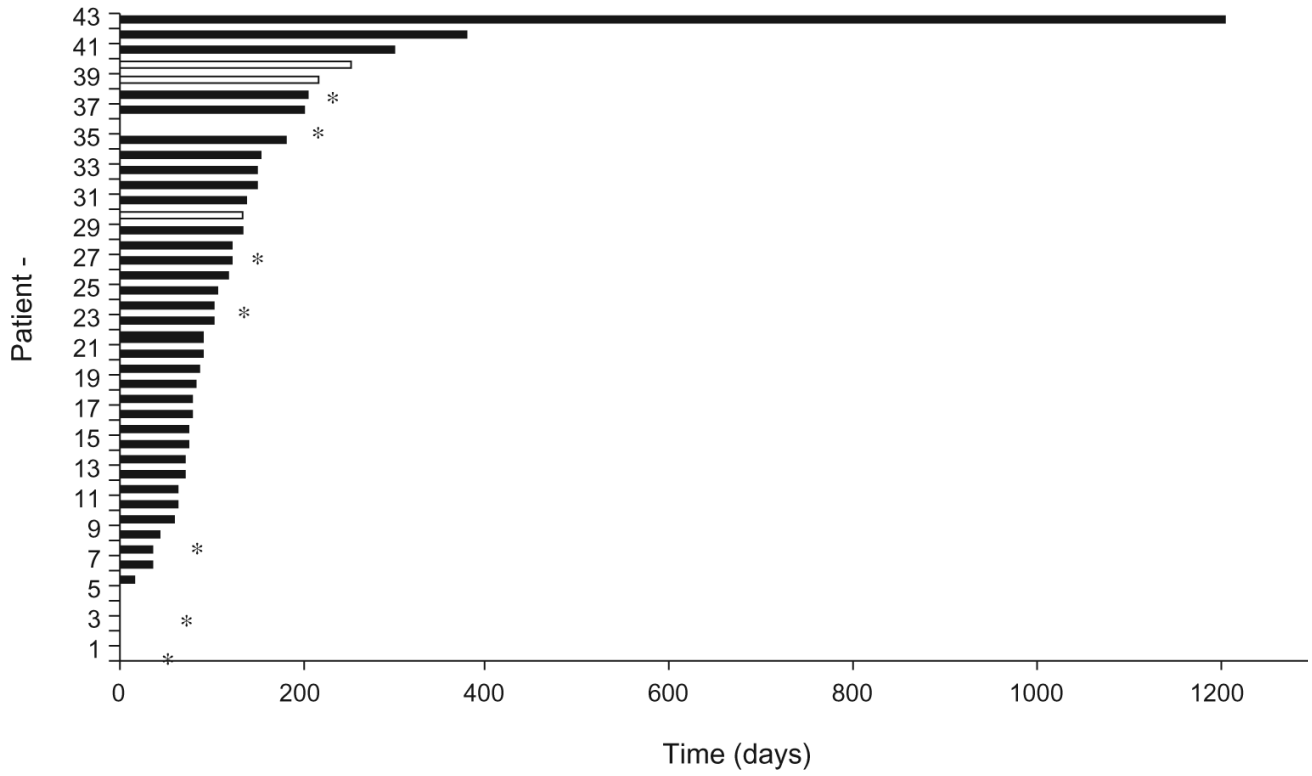


Figure 2.2: Seroconversion time (days) was calculated as the time from the first PCR positive sample to the first positive antibody in 43 patients (range 0–1206 days). Four patients (patients 30, 36, 39 and 40 shown in white) did not produce an antibody by 133, 183, 205 and 218 days of follow-up. Seven patients (asterisked) spontaneously cleared the infection, two of whom did not produce an antibody response.

2.5.2.2 Diagnostic testing

The sensitivity of antibody, antibody-antigen and ALT testing was assessed at 1–3 monthly time-points.

On first amplification of HCV, only 25% of patients were serologically positive. Antibody detection rose from 63% by 3 months to 87% at 6 months, 90% at 9 months and 95% by 12

months (Figure 2.2). A combined assay detected HCV antigen–antibody in 52% of HCV RNA positive baseline samples (Nastouli et al, 2009). In 18% cases the assay gave an equivocal result and a negative result was obtained in 32%. In practice, equivocal results would trigger HCV RNA testing and can, therefore, increase the overall sensitivity of the assay.

The median ALT at the time of the first positive PCR test (baseline) was 65 IU/ml (normal range <40 IU/ml). Elevated ALT levels were present in 76% of patients at baseline and 74% at 3 months. Most patients (88%) had an elevated ALT either at baseline or 3 months post infection. An elevated ALT level was significantly more sensitive than antibody testing (*P*=0.017). When a lower normal value of less than 30 IU/ml was used (recently proposed as normal in men (Prati et al, 2002)), the sensitivity of ALT testing increased to 79% at baseline and 82% at 3 months. As many as 93% of patients had an ALT level higher than 30 IU/ml at either baseline or 3 months postinfection.

2.5.3 Viral load dynamics during early HCV infection

Sequential viral load (VL) measurements were analysed in the whole cohort (n=-110) revealing 3 patterns of infection (Figure 2.3); progression to chronicity with an initial peak viraemia and chronic plateau viraemia (PV) phase (48%), fluctuating viraemia (FV) defined as a fall followed by a rise in VL of >1 $\log_{10}$ (38%) and spontaneous clearance (SC; 14%).

Figure 2.3 : Median HCV viral load, bilirubin and ALT levels over time in a) the whole cohort and b) representative individual patients

a) Median HCV viral load, bilirubin and ALT levels over time in the whole cohort

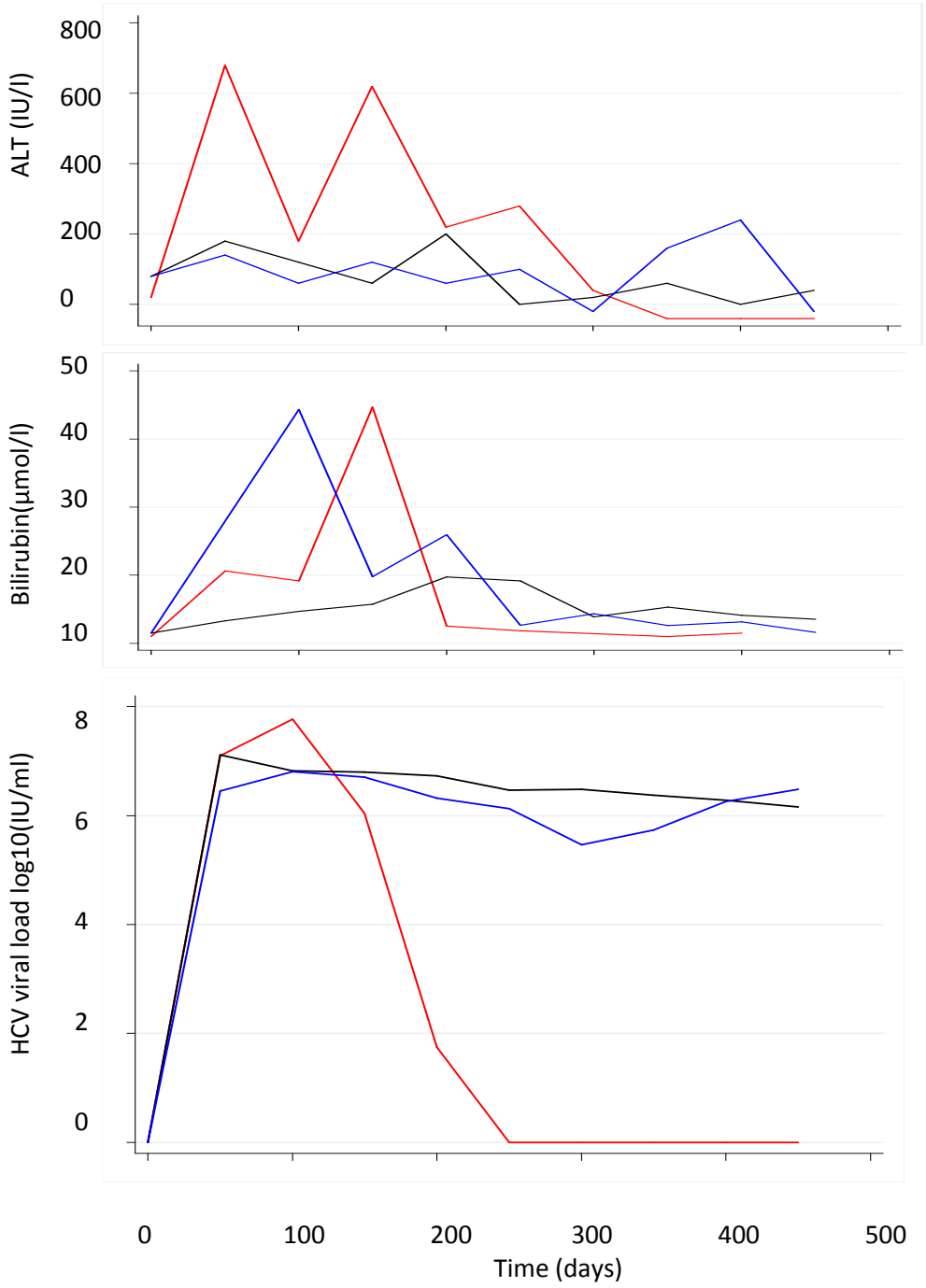


Figure 2.3 continued

b) Median HCV viral load, bilirubin and ALT levels over time in representative individual patients

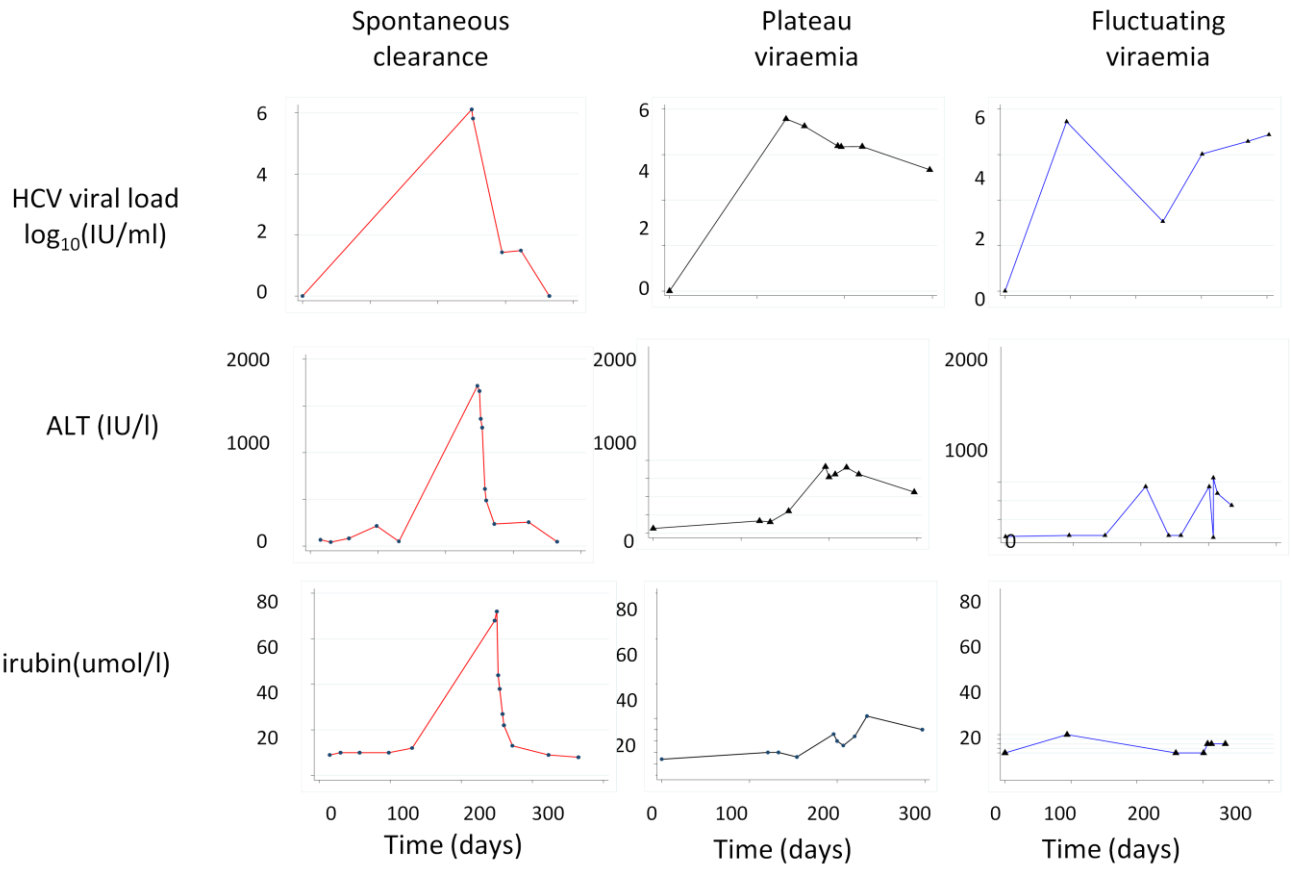


Figure 2.3: Longitudinal HCV viral load measurements taken prior to treatment revealed 3 patterns of infection: spontaneous clearance (SC;14%; red) and 2 patterns of progression (86%); peak and plateau (PV;48%; black) or fluctuating viremia (FV;38%; blue) defined as a viral load peak followed by a decline (>1log_{10}) and subsequent rise in viremia (>1log_{10}). Group data is shown in panel a while representative individual patients are shown in panel b.

Spontaneous clearance was associated with an initial increase followed by a rapid fall in VL (1.7 $\log_{10}$ drop within 100 days and 4.03 $\log_{10}$ drop within 200 days; Table 2.6) leading to clearance at a median of 108 days (95% CI: 38, 170 days). Thirteen FV patients became transiently PCR negative, but took on average 154 days to reach negativity (95% CI=113, 323 days; p=0.04) and 129 versus 27 days (p=0.009) to achieve a 1.7 $\log_{10}$ drop in VL.

Table 2.6: Sequential viral load measurements in untreated spontaneous clearers and progressors

	Spontaneous clearers (95% CI)	**Progressors (plateau) (95% CI)**	**Progressors (fluctuating) (95% CI)**	**Odds ratio (95% CI)**	**p value**
Maximum HCV VL drop 100 days from first positive PCR[a]	1.70 (1.39, 5.87)	0.001 (-0.47, 0.21)[b]	0.57 (0.25, 0.96)	2.76 (1.55, 4.90)	0.001*
Maximum HCV VL drop 200 days from first positive PCR	4.03 (1.70, 7.32)	0.13 (-0.22, 0.34)[b]	0.85 (0 .45, 1.77)	2.48 (1.56, 3.97)	<0.0001*

2.5.4 Cohort characteristics

Demographic and clinical parameters are shown in Table 2.7. Early infection in progressors (PV and FV groups) and spontaneous clearers (SC) was associated with a rise in ALT and bilirubin. The peak SC bilirubin was significantly higher than in the progressor groups; in

particular, a peak bilirubin level of ≥40 µmol/l was associated with a hazard ratio of spontaneous clearance of 5.04. Elevation in bilirubin levels was temporally associated with a fall in HCV VL (Figure 2.3). Elevated ALT was also associated with spontaneous clearance (ALT ≥1000 IU/ml; HR=2.62; Table 2.7). Low peak and baseline HCV VL were associated with spontaneous clearance as was a baseline CD4 count of ≥650x10^6/l. Age, HIV VL and the use of HAART were not associated with outcome. All patients were men-who-have sex-with-men and had had recent unprotected sex recorded in the medical notes while 31% also admitted to the use of intravenous or intramuscular drugs.

Table 2.7: Clinical measures and outcome (spontaneous clearance versus progression)

Clinical measure (units, normal range)	**Spontaneous clearance (median, 95% CI)**	**Progression**		**Range**	**Hazard ratio (95% CI)**	**p value†**
		Plateau viraemia (median,95% CI)	Fluctuating viraemia (median, 95% CI)			
Number	15 (14%)	53 (48%)	42 (38%)	-	-	-
Age (years)	43 (32, 45)	39 (37, 42)	39 (37, 40)	HR represents change in hazard per year of age	1.00 (0.94, 1.06)	1.00
Baseline HCV VL log_{10} (IU/ml)	6.42 (1.70, 7.37)	6.25 (5.71, 6.44)	5.89 (5.06, 6.13)	HR represents change in hazard per log_{10} change in VL	0.75 (0.55, 1.01)	0.06
Peak HCV VL log_{10} (IU/ml)	6.42 (1.70, 7.37)	6.52 (6.36, 6.88)	6.21 (5.87, 6.72)		0.61 (0.46, 0.80)	<0.0001**
HIV VL (copies/ml)	<50 (<50, 7044)	<50 (<50, 1185)	87 (<50, 6847)		1.00 (1.00, 1.00)	0.40

Table 2.7 continued

Clinical measure (units, normal range)	Spontaneous clearance (median, 95% CI)	Progression		Range	Hazard ratio (95% CI)	p value†
		Plateau viraemia (median, 95% CI)	Fluctuating viraemia (median, 95% CI)			
Elevated bilirubin **(0-17 µmol/l)**	30 (12, 60)	16 (14, 19)	14 (12, 17)	<20 20-40 ≥40	1[a] 1.64 (0.48, 5.6) 5.04 (1.60, 15.92)	 0.43 0.006**
Peak ALT **(0-40 IU/l)**	340 (127, 1814)	308 (204, 420)	500 (316, 659)	<1000 ≥1000	1[a] 2.62 (1.01-6.80)	 0.048*
CD4 count (300-1400 x10^6/l)	630 (484, 854)	510 (439, 640)	520 (455, 617)	<650 ≥650	1[a] 2.66 (1.02-6.91)	 0.045*
Nadir CD4 (300-1400 x10^6/l)	340 (200, 519)	310 (280, 350)	315 (270, 367)	≥200 <200	1[a] 1.51 (0.52-4.35)	 0.45
Transmission						
IDU[b]	3/13 (23%)	13/44 (30%)	13/36 (36%)	All patients were men-who-have sex-with-men and had had recent unprotected sex.	1.63 (0.27, 2.75)	0.36
INDU[c]	9/12 (75%)	31/42 (74%)	27/34 (79%)		0.92 (0.25, 3.46)	0.94
Sexual	14/14 (100%)	46/46 (100%)	39/39 (100%)		-	-
HAART	11/15 (73%)	31/53 (58%)	23/42 (55%)		1.63 (0.57, 4.70)	0.36

† p values shown represent all progressors (FV and PV) versus clearer (SC) groups
* Significant p value<0.05
a Median values are given for all variables
b Intravenous, intramuscular or subcutaneous drug use (crystal methamphetamine, cocaine, anabolic steroids, heroin)
[c] Fisher's exact test
[d] Intranasal drug use (ketamine, cocaine)

2.5.5 HVR-1 sequence analysis

2.5.5.1 Cross-sectional phylogenetic analysis of the HIV-positive MSM population with acute HCV

Phylogenetic analysis of majority quasispecies compared with reference sequences revealed several monophyletic clusters of infection within genotype 1a (77%), 4d (15%), 3a (7%) and 1b (4%) indicating several introductions of the virus into the men-who-have-sex-with-men (MSM) population (Figure 2.4). Six percent were infected with 2 genotypes and 2% (a transmitting couple) had 3 genotypes (1a, 3a and 4d).

Figure 2.4 Multiple strains of HCV are circulating in the HIV-positive MSM population

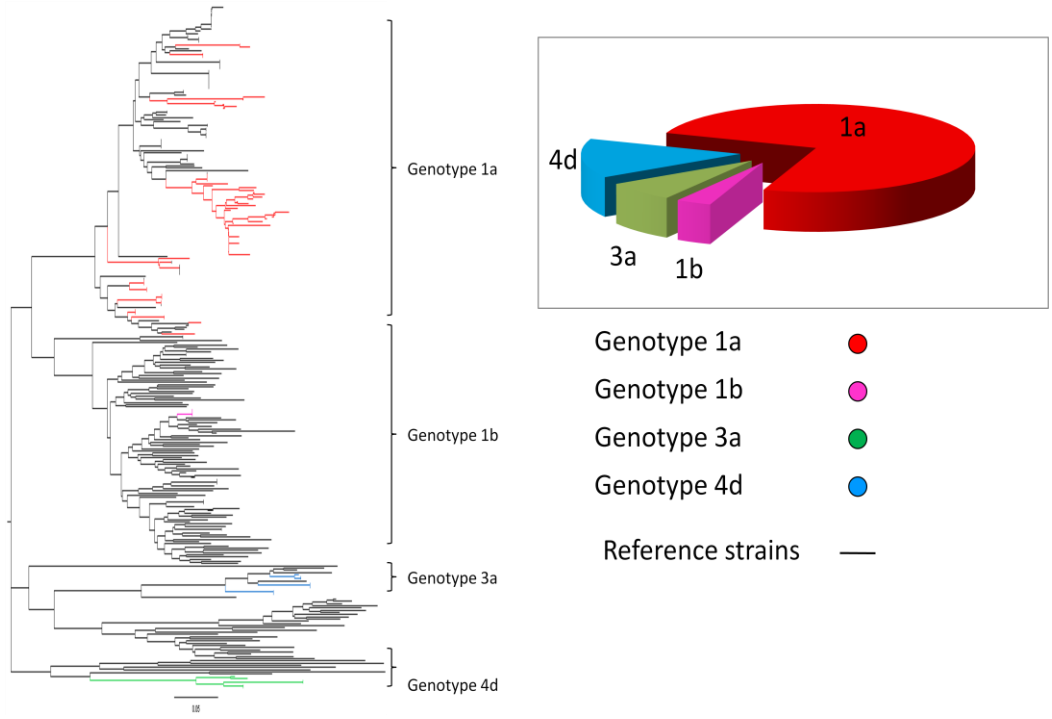


Figure 2.4 A neighbour-joined phylogenetic tree was constructed (using MEGA 4.0 and Figtree; Kimura 2-parameter method) with patient majority quasispecies sequences (n=97; coloured lines) and reference sequences (n=212; black lines) obtained from the Los Alamos database. This revealed several monophyletic clusters of infection suggesting several introductions into the MSM population. The majority of infections were genotype 1a but genotypes 1b, 3a and 4d were also identified.

2.5.5.2 E2 HVR-1 sequence evolution within patients and prediction of spontaneous clearance

In order to determine which phylogenetic factors predicted spontaneous clearance, Hamming distance (diversity), number of quasispecies and dN/dS ratios were calculated in 50 patients (40 progressors and 10 clearers) at multiple (median=3) time points. Initially, genetic diversity was noted to vary considerably over time in all groups (Figure 2.5) but did not correlate with VL.

Figure 2.5: Genetic diversity within HVR-1 (uncorrected) over time

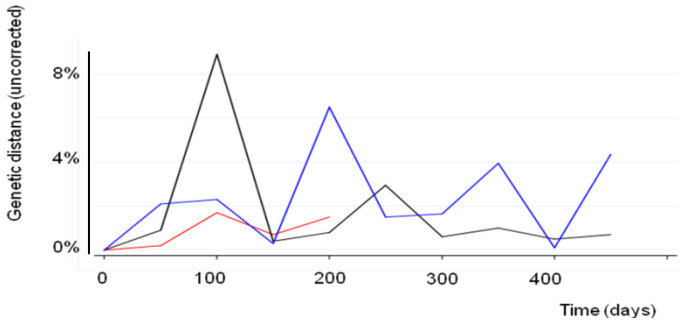
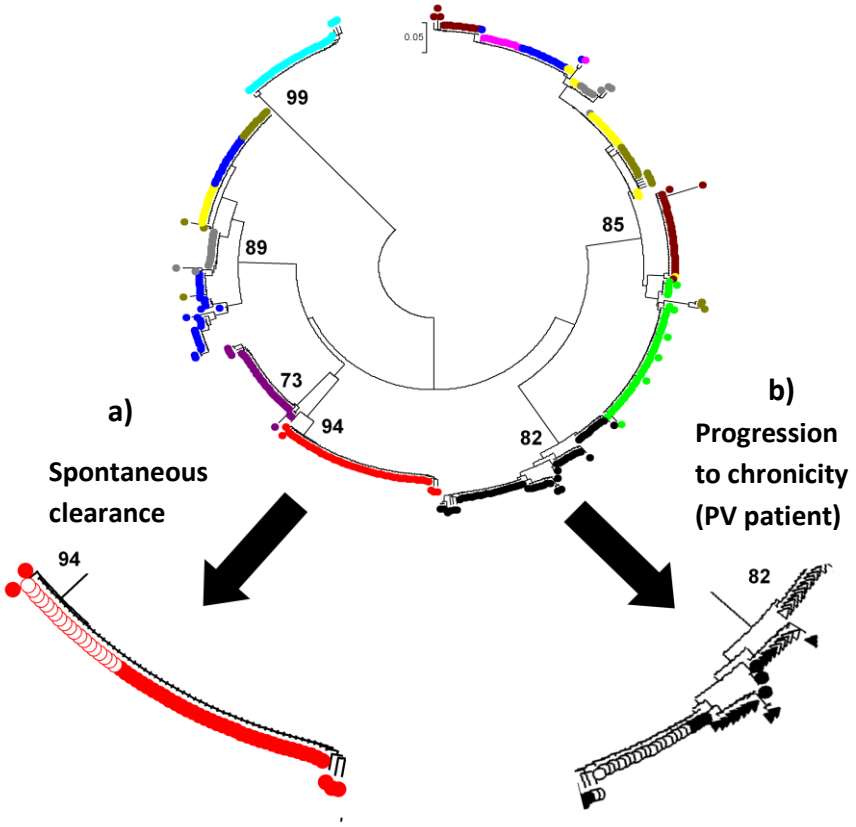


Figure 2.5 Cumulative genetic distance was calculated for each group of patients (plateau progressors; black, fluctuating viraemia; blue, spontaneous clearers; red) at multiple time points (range 0-450 days) showing marked non-linear variation over time.

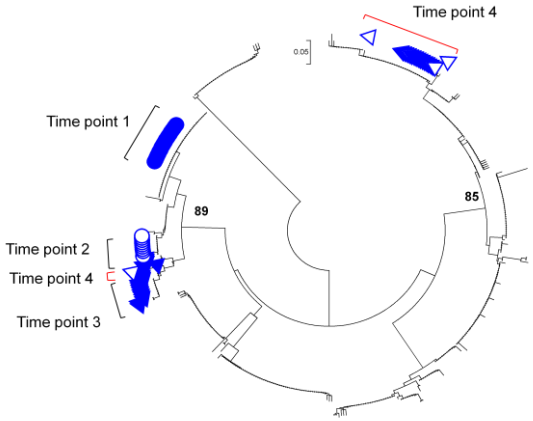
In order to investigate if this variation was the result of superinfection events, neighbour-joining phylogenetic analyses of all longitudinally acquired E2 HVR-1 sequences obtained from the first 50 recruited patients (n=2295) were carried out, revealing 3 groups of patients (Figure 2.6); those who spontaneously cleared infection (n=10; 20%), those who progressed

to chronicity with increasing quasispecies diversity but no evidence of superinfection (n=20; 40%) and those who progressed to chronicity with evidence of superinfection events (n=20; 40%). Superinfecting strains were evident both of the same genotype (30%) and with new genotypes (10%). A gradual increase in genetic diversity can be seen in the illustrated PV and FV progressor patients, while clones derived from the spontaneous clearer reveal a narrower repertoire. A single case of possible HCV reactivation is shown in Figure 2.6d. The patient acquired acute HCV following an episode of anonymous unprotected sex and was treated with 48 weeks of pegIFNα and ribavirin, following which he had an SVR. One year later, he developed a rapid decrease in CD4 count (nadir $106x10^6$/l) and was hospitalised with *Pneumocystis jeroveci* pneumonia. During this time, his ALT became elevated and HCV RNA was once again detected in his blood. He denied any risk factors for reacquisition of HCV. Branches derived from patient HVR-1 sequences (n=56) are coloured in filled green (baseline sample) and open green (3 years later). These samples derive from the same lineage. A paraffin-embedded liver biopsy taken at the time of hospital admission and PBMCs were later examined for compartmentalisation of HCV (Chapter 3).

Figure 2.6: Patterns of acute HCV based on phylogenetic analysis a). Spontaneous clearance, b) Progression to chronicity (PV patient) c) Superinfection (FV patient) and d) Reactivation



c) Superinfection (FV patient)



d) Reactivation of HCV

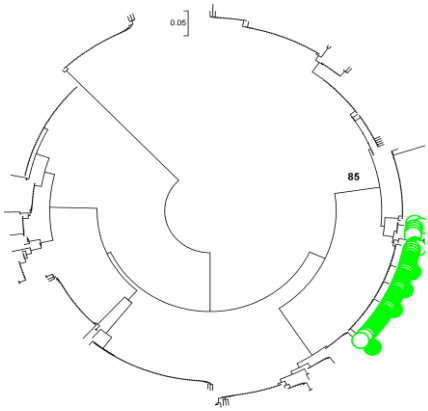


Figure 2.6 Circular neighbour-joined phylogenetic tree analysis (Kimura 2-parameter model) of 305 HVR-1 sequences derived from 10 representative patients at 2-4 time points. Clones derived from each patient are given a different colour and filled circles represent time point 1, clear circles time point 2, filled triangles time point 3 and clear triangles time point 4. Bootstrap values greater than 70% are shown. **2.6a** OTUs from clonal sequences derived from two timepoints in a patient with spontaneous clearance are highlighted in red. **2.6b** OTUs from clonal sequences derived from a patient with plateau viraemia at 4 timepoints are highlighted in black. **2.6c** A patient with evidence of superinfection (blue) is highlighted. Clones derived from timepoints 1, 2 and 3 (filled circles, clear circles and filled triangles respectively) are derived from the same branch on the phylogenetic tree. Sequences from time point 4 (clear triangles, red brackets) consist of a dominant superinfecting strain (one clone related to the previous lineage is also present). This patient had a recrudescent viral load, with a negative HCV PCR (limit of detection=12 IU/ml) between time points 3 and 4. **2.6d** A case of probable HCV reactivation is shown.

In order to assess the role of positive selection and viral variation over time, intrastrain calculations of genetic diversity and dN/dS ratio were used for patients found to have multiple strains of infection. Following this correction, diversity and dN/dS ratios increased gradually with time in progressors, but decreased in clearers (Figure 2.7, Table 2.8). Both progressor groups were combined in order to assess measures of genetic diversity compared with SC patients to see if they had predictive value which might have potential clinical value (Table 2.8). While baseline measures of diversity were not significantly different in progressors (FV and PV groups combined) versus SC patients (baseline Hamming distance 0.64 versus 0.39, p=0.38), by 150 days, evidence of higher diversity in the progressors was apparent (2.045 versus 0.03; p=0.02) with a higher dN/dS ratio in the progressor group indicating positive selection (dN/dS ratio 1.02 versus 0.17; p=0.03). The number of quasispecies, genetic distance, Hamming distance and dNdS ratios were not significantly different between PV and FV progressor groups. However, differences were observed when FV patients were divided into those with a single-strain infection and those with evidence of superinfection based on longitudinal clonal sequence analysis.

Figure 2.7: Genetic variation within E2 HVR-1 over time by group

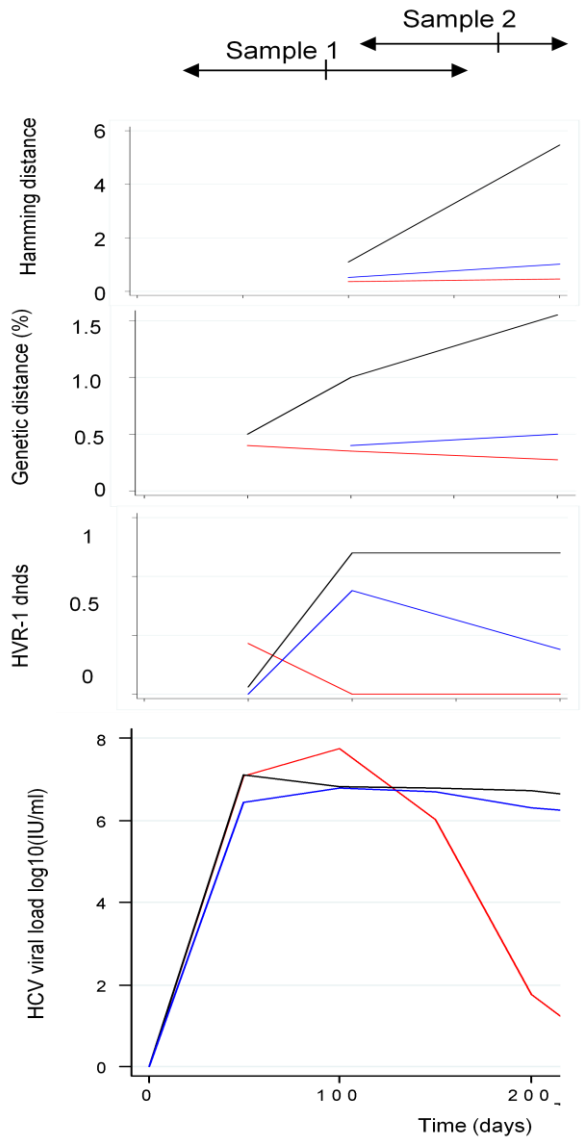


Figure 2.7 Viral load and measures of genetic diversity; genetic distance, Hamming distance and dN/dS ratios are plotted over time in each patient group (plateau progressors; black, fluctuating viraemia; blue, spontaneous clearers; red).

Table 2.8: Viral sequence analyses and predictors of spontaneous clearance

	Clearers **(95% CI)**	**All progressors FV + PV (95% CI)**	**p value**
Number	10	40	-
Baseline quasispecies	3 (2, 5)	4 (3, 6)	0.21
Cumulative Quasispecies (2 samples taken <150 days apart)	7 (7,8)	9 (8, 12)	0.24
Baseline Hamming distance	0.39 (0.22, 0.92)	0.64 (0.36, 2.97)	0.38
Cumulative Hamming distance (2 samples taken <150 days apart)	0.03 (0, 0.06)	2.045 (0.81, 10.47)	0.02*
Baseline HVR-1 dN/dS	0 (0, 0.325)	0.28 (0, 0.95)	0.18
Cumulative HVR-1 dN/dS (2 samples taken <150 days apart)	0.17 (0, 0.35)	1.02 (0.71, 1.84)	0.03*

Fluctuating Viraemia

Patients with a fluctuating VL were more likely to have evidence of superinfecting strains than those with plateau persistence (63% versus 29% p=0.04; Figure 2.6). However, not all FV patients had evidence of superinfection. Amongst those with single strain infection, persons with recrudescent viraemia had similar dN/dS ratios at baseline but higher cumulative ratios following a second sample than plateau progressors (1.78 versus 0.76;

p=0.02). At baseline, those with single strain recrudescent viraemia had higher viral diversity (Hamming distance 2.84 versus 0.49; p=0.04). Peak bilirubin, ALT and number of quasispecies did not differ between these groups. One patient showed evidence of reactivation of HCV 2 years after successful treatment (Figure 2.6d).

2.5.5.3 E2 HVR-1 sequence evolution and prediction of SVR

Thirty-two patients completed treatment with 48 weeks of IFNα and ribavirin within the study period (Figure 2.1). Twenty-one of these (70%) achieved an SVR. We analyzed the same clinical, virological and phylogenetic variables as above in relation to response to therapy (Table 2.9). Clinical parameters did not significantly differ between groups and pre-treatment viral loads were not significantly different. However, the diversity (Hamming distance) at baseline and within 150 days from infection was significantly lower in those who achieved an SVR (p=0.008 and 0.004 respectively). The E2 HVR-1 dN/dS ratio did not differ at baseline, but was subsequently higher in those who did not respond to treatment (p=0.007).

Table 2.9: Viral sequence analyses and predictors of SVR

	SVR (95% CI)	No SVR (95% CI)	p value
Number	14 (70%)	6 (30%)	-
Baseline quasispecies	3 (2, 5)	4 (3, 11)	0.23
Cumulative Quasispecies (2 samples taken <150 days apart)	8 (6, 12)	9 (8, 9)	0.63
Baseline Hamming Distance	0.44 (0.07, 0.59)	4.56 (1.88, 15.50)	0.008*
Cumulative Hamming distance (2 samples taken <150 days apart)	2.05 (0.70, 12.61)	7.815 (0.4, 21.5)	0.004*
HVR-1 dN/dS baseline	0.26 (0-1.04)	0.28 (0-5)	0.77
Cumulative HVR-1 dN/dS (2 samples taken <150 days apart)	0.76 (0.35-0.99)	4.76 (4.3-6.5)	0.007*
Baseline VL (log_{10}IU/ml)	6.17	5.83	0.64
Week 4 drop (log_{10}IU/ml)	3.78	0.63	0.24
Week 12 drop (log_{10}IU/ml)	6.17	0.04	0.01*

2.5.6 NS5B sequence analysis

In order to confirm the results found following amplification of the E2 HVR-1, amplification of NS5B was carried out in 12 patients at 2 time points (11 clones/patient/timepoint=total 274 sequences). The mean genetic distance between all strains using NS5B was lower than that of the HVR-1 (0.14 versus 0.25; p=0.01).

The median genetic distance within patients with spontaneous clearance was lower than progressor patients but did not reach statistical significance (0.009 versus 0.015; p=0.59). Similarly the dN/dS ratio was higher in progressors than clearers (0.22 versus 0.04; p=0.59).

Neighbour-joined phylogenetic trees were next constructed using sequences derived from 6 patients at 2 time points following amplification of HVR-1 and NS5B. The genetic distance between NS5B sequences was 14% and HVR-1 sequences was 25% and thus branches were shorter on the tree constructed using NS5B sequences. However, trees derived from sequences obtained at the same time points showed similar results on both trees.

Figure 2.8: Phylogenetic tree using HVR-1 and NS5B sequences derived from the same time points in a) a patient with evidence of 3 circulating strains at baseline and b) a patient with similar strains at 2 time points

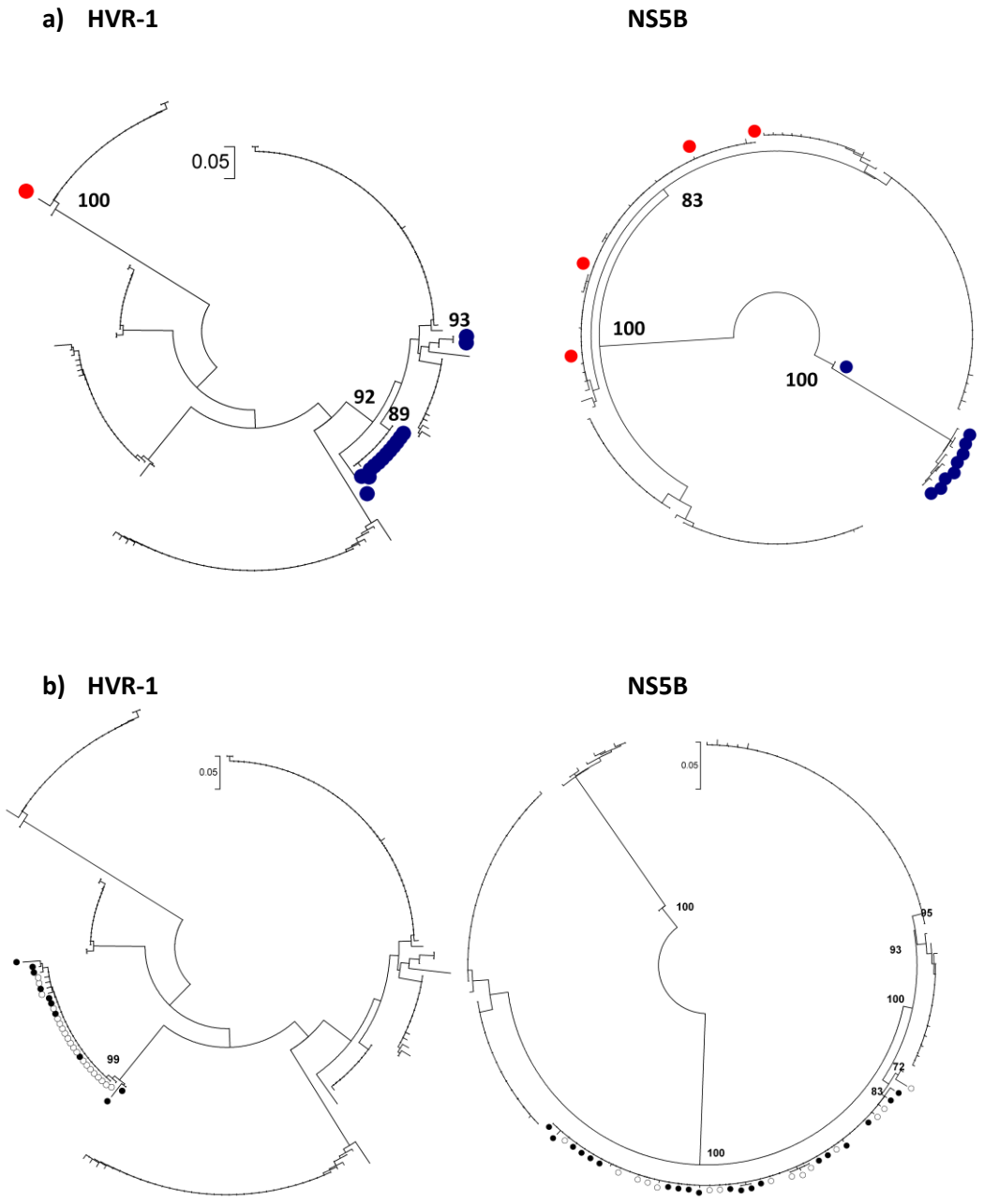


Figure 2.8: Bootstrap scores >70% are indicated for the relevant branches. Figure 2.8a shows branches derived from a patient with 2 genotypes at baseline (genotype 1a in red; genotype 4d in blue). Figure 2.8b shows samples taken at baseline (filled circles) and 3 months later (open circles).

2.5.7 NS5A sequence analysis

A region of NS5A (including the ISDR) was amplified in 20 patients (mean =16 sequences/patient= total 314 sequences). The mean genetic distance between all strains using NS5A was 0.17. However, this variation was accounted for by a large amount of variation within 3 patients. It did not vary significantly within patient groups over time (0.002 in progressors versus 0.005 in clearers; p=0.63). The median dN/dS ratio was 0 in both groups suggesting that the region is not under positive selection during early infection.

Figure 2.9: Phylogenetic tree using NS5A sequences derived from 20 patients at 2 timepoints

a) **b)**

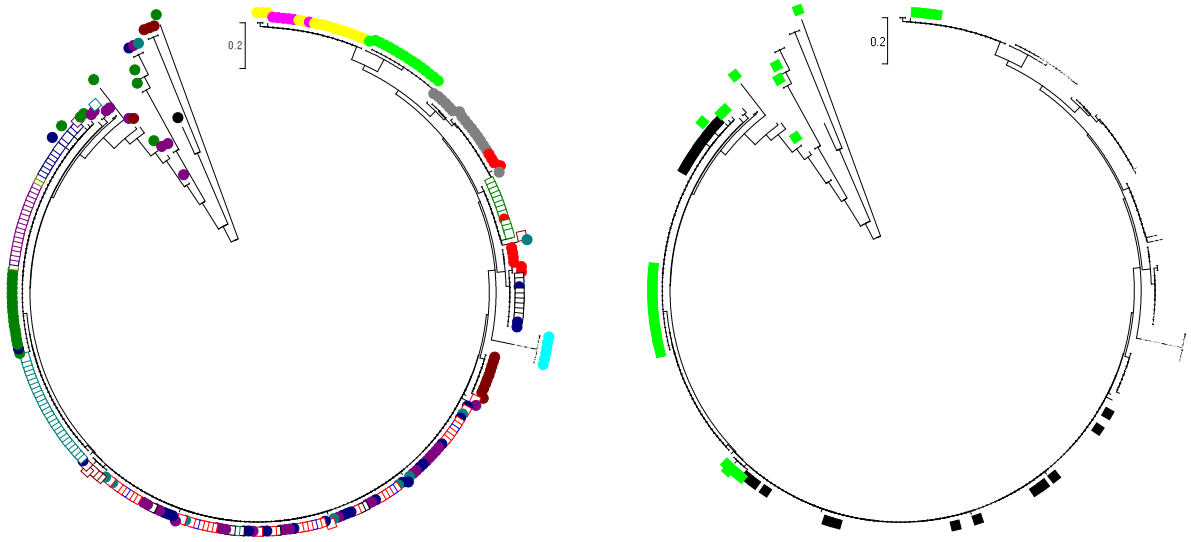


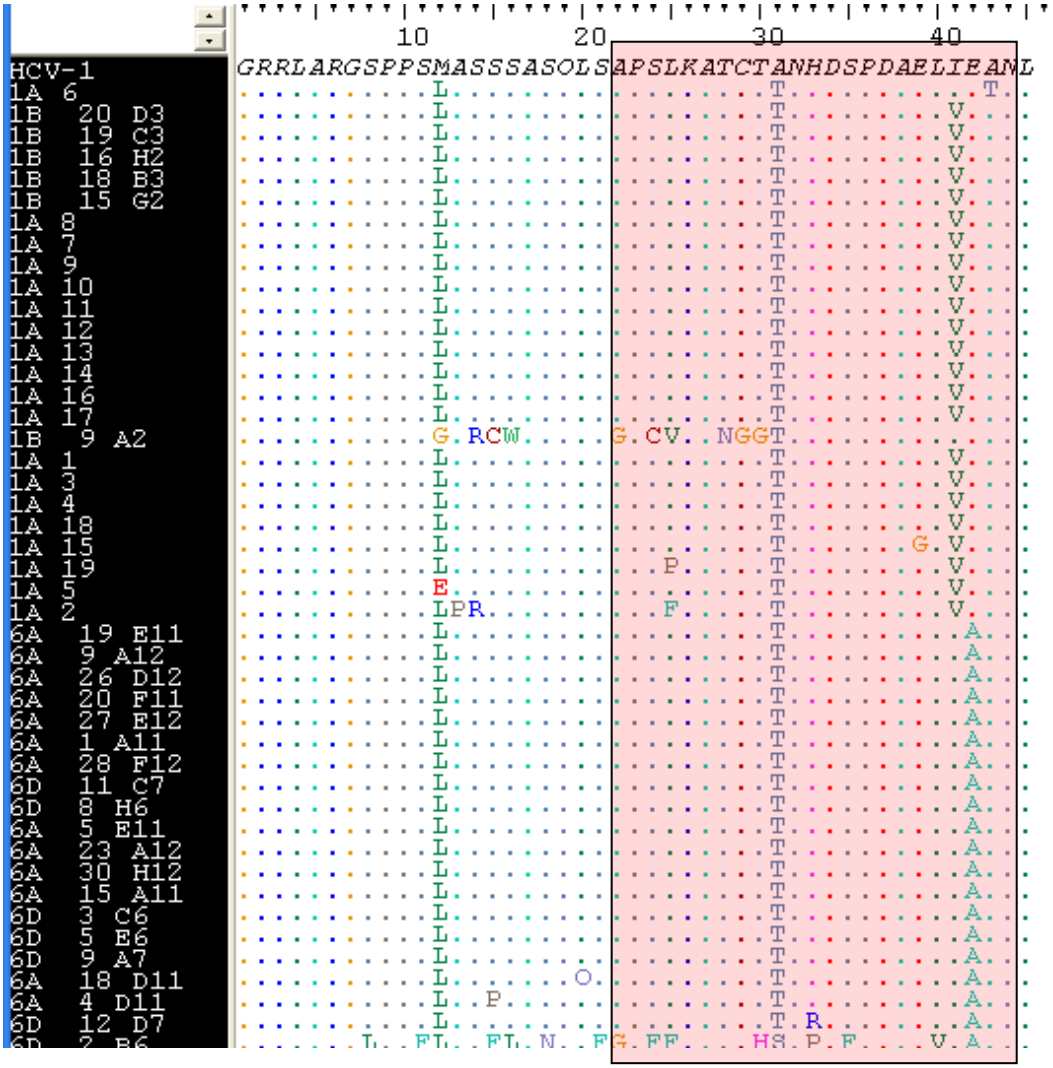
Figure 2.9: A neighbour-joined phylogenetic tree was constructed using MEGA 4.0 and the Kimura 2 parameter. a) Sequences derived from individual patients are allocated a different colour b) Sequences derived from patients with an SVR are shown in green while sequences derived from non-responders are shown in black.

NS5A and SVR

Sequences derived from NS5A were aligned with genotype 1a prototype sequence HCV-1 and the median number of amino acid differences counted for each patient (MacQuillan et al, 2004; Figure 2.9; Table 2.10).

Figure 2.10 Alignment of NS5A sequences in a) Patients with SVR and b) Non-responders/relapsers

a) **Patients with SVR**



b)Non-responders/relapsers

a)

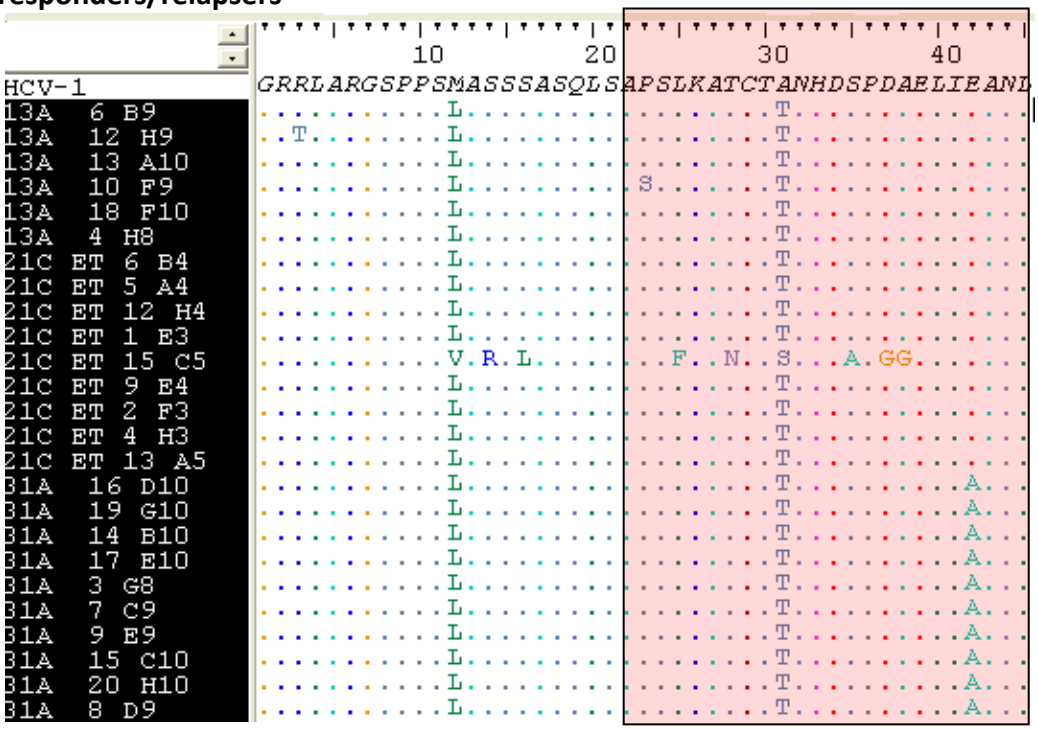


Figure 2.10 NS5A sequences are aligned in a) Patients with SVR and b)Non-responders/relapsers. The putative ISDR is highlighted in pink. More variation in the ISDR region can be seen in the SVR group.

Table 2.10: Amino acid differences within the ISDR compared with the HCV-1 reference strain

Number of ISDR mutations per sequence	No SVR	SVR
1	13	0
2	11	38
3	0	5
6	1	1
8	0	1
Total	25	45

The number of mutations within NS5A from patients with an SVR was significantly higher than the number of mutations in sequences derived from patients without an SVR when compared with the HCV-1 reference sequence strain ($p<0.0001$). This was accounted for by the number of mutations within the ISDR region (Figure 2.10, Table 2.10; $p<0.0001$). Mutations in the region of NS5A upstream of the ISDR were not significantly different in sequences derived from patients with or without an SVR ($p=1.0$).

2.5.8 PCR and cloning error rate

2.5.8.1 Sensitivity of PCR assays

5'UTR: An end-point dilution PCR was carried out to assess the sensitivity of the 5'UTR assay for detecting genotypes 1a, 3a and 4d by serial dilution of samples with a known HCV VL (determined by quantitative RT-PCR). All 3 genotypes were detected with a sensitivity of <10 IU/ml.

HVR-1: The sensitivity of the HVR-1 PCR was 693 IU/ml using the Roche One Step PCR kit containing Taq polymerase. Detection of positive samples was reduced by 60% when using the proof-reading enzyme Pfu.

2.5.8.2 Estimated PCR error rate

Taq polymerase has previously been estimated to have an error rate of $0.2\text{-}2\times10^{-4}$ to 10^{-5} errors/bp/cycle. Proof-reading enzymes are thought to be more accurate with error rates of around 10^{-6} but are significantly more expensive than Taq-containing preparations. Using Taq polymerase to determine variation would add a maximum expected error rate of 220 base pairs x 65 cycles x 2 x 10^{-4}= 2.86 errors/sequence = 1.3% variation/sequence. Reverse

transcription adds an expected error rate of 6.4 x 10^{-5} nucleotide substitutions/site. Thus 220 base pairs x 6.4 x 10^{-5} =0.01 errors/sequence= 0.005%

2.5.8.3 Actual PCR/cloning error rate

A positive band was visible at up to 10IU/ml. This was excised and extracted by column centrifugation as above and the concentration of DNA measured using spectrophotometry giving a concentration of 90ng/ul. The molecular weight of one copy of plasmid with incorporated NTR DNA (3956bp plasmid + 217bp cDNA) was estimated to be 2578790 Da using an online molecular weight calculator available at http://www.changbioscience.com/genetics/mw.html. The total number of molecules of DNA present was calculated thereafter using Avogadro's constant.

$$\text{no. grams per molecule DNA} = \frac{\text{molecular weight (grams per mole)}}{\text{Avogadro's constant (molecules per mole)}}$$

$$\text{no. grams per molecule DNA} = \frac{2578790 \text{ grams per mole}}{6.022\text{x}10^{-18} \text{ molecules per mole}} = 4.28\text{x}10^{-18}\text{g}$$

Thus for 10 molecules of plasmid DNA, 4.28x10^{-17}g was obtained and used in the PCR reaction, following serial dilutions with sterile water. The NTR PCR product was then gel extracted and re-ligated into pCR®4-TOPO® vector. Twenty clones were then sequenced in order to calculate actual PCR error.

Figure 2.11: End-point dilution of the 5'UTR

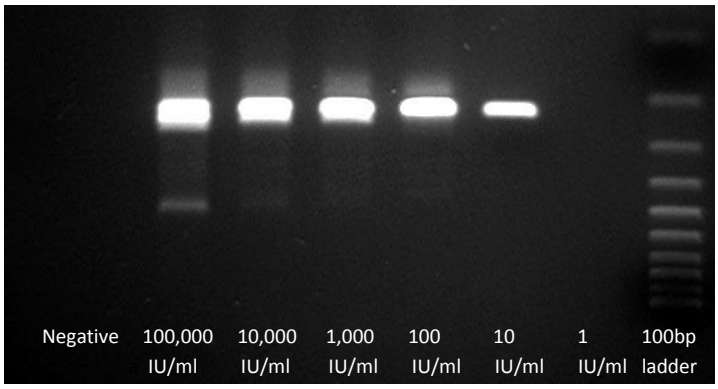


Figure 2.11: An end-point dilution PCR of the 5'UTR region was carried out and run on a 2% agarose gel at concentrations of 1-100,000 IU/ml.

175 clones were selected and sequenced. Following this, a total of 50 introduced errors were calculated over 175x185 base pairs=50/32375 total= 0.0015/bp/80 cycles = $1.9x10^{-5}$ errors/bp/cycle. This is likely to reflect the upper end of possible error, as PCR saturation is likely to have prevented the incorporation of as many errors due to higher starting concentrations of DNA pre-amplification.

2.6 Discussion

An emerging epidemic of acute HCV in HIV-positive men-who-have-sex-with-men (MSM) has for the first time allowed characterization of the natural history of early HCV infection in a large number of HIV-positive patients. Unravelling the determinants of spontaneous clearance of HCV is crucial in order to increase our understanding of the biological mechanisms which define protective immunity and to improve the management of patients who face a lengthy course of treatment which is toxic, expensive, and not always effective. HCV infection in HIV-positive individuals is a growing clinical problem associated with an increased rate of disease progression and reduced response to treatment (reviewed by Klenerman et al, 2007). In this longitudinal study of 110 patients with acute HCV and HIV infection, the key predictors of spontaneous clearance were VL dynamics, liver function and measures of viral diversity including Hamming distance and dN/dS ratio.

2.6.1 Diagnosis of acute HCV in HIV-positive patients

Early treatment for HCV improves outcome in HIV-positive patients (Gilleece et al, 2005; Torriani et al, 2004) and early diagnosis is therefore crucial. Tests available for the diagnosis of HCV infection in the acute phase vary in sensitivity. Third generation antibody ELISA tests which detect multiple antigenic determinants (core, NS3, NS4, NS5) are the current diagnostic test of choice in HIV-positive patients (Brook et al, 2010). HIV-negative patients generally produce an antibody to HCV within 6 weeks of infection (Netski et al, 2005). We have shown in this study that in HIV positive individuals, however, screening with a third-generation antibody assay in the first 6 months of infection is inadequate in most patients.

Furthermore, at 1 year, nearly 5% of patients remain seronegative. The use of a combined antigen-antibody test in HIV-positive individuals increases screening sensitivity but may not be cost-effective and is not as sensitive as PCR (Nastouli et al, 2009). This delay in the formation of antibodies may result in significant delay in diagnosis of HCV in HIV-positive individuals and patients may lose the opportunity to have their infection treated successfully. An accurate estimate of seroconversion time (time of infection to the development of antibodies) in this group of patients is limited by the 3 monthly sampling window, and therefore, a subgroup of eight patients who had been sampled at monthly intervals (<30 days) were identified, allowing a more precise assessment of seroconversion time. In this group, the median time from infection to the development of antibodies was estimated as 158 days (IQR 47–209 days). Factors found to correlate with a prolonged seroconversion time included low ALT level and low HCV viral load at baseline, perhaps reflecting reduced B cell recognition and lower antigenic load, respectively. The CD4 cell count at the time of HCV infection was not associated with seroconversion time, although patients with a lower nadir CD4 cell count had a slightly increased likelihood of a delayed antibody response, suggesting that prolonged CD4 dysfunction may occur, despite an increased CD4 cell count following HAART. Delayed anti-HCV antibody responses have also been reported in other immunosuppressed groups, such as solid-organ transplant and haemodialysis recipients (David-Neto et al, 1997; Schroeter et al, 2005; Preiksaitis et al 1997). The phenomenon has also been described previously in a case series of three immunosuppressed HIV-infected individuals (CD4 cell counts 14–266 cells/µl; John et al, 1998), with formation of anti-HCV antibodies following treatment with HAART and resultant immune restoration. This phenomenon was not observed in our cohort of patients, perhaps because of the higher median CD4 cell count in this patient population (Table 2.7). Thus,

delayed anti-HCV seroconversion may also occur frequently in HIV-positive patients who have normal CD4 cell counts. The association between nadir CD4 cell count and delayed seroconversion time, as well as likelihood of clearing HCV spontaneously, is likely to reflect impaired CD4 function, despite the restoration of absolute counts. The role of T cell function during acute disease is explored further in Chapter 4 of this thesis.

In conclusion, elevated ALT levels in at-risk HIV-positive patients should indicate testing for HCV by PCR in order to diagnose infection early. Furthermore, HIV-infected patients diagnosed with acute HCV should have a preceding negative HCV RNA test (performed retrospectively) to accurately establish the timing of infection.

2.6.2 Predicting spontaneous clearance of acute HCV in HIV-positive men based on viral dynamics, liver function and other clinical measures

Three courses of infection were observed, based on VL changes over time (Figure 2.3). Spontaneous clearance (SC; 14%) was associated with a rapid decrease in VL. Patients who progressed towards chronicity exhibited 2 patterns of viraemia; the majority (48%) had an initial rise in VL followed by a plateau viraemia phase (PV), analogous to that seen in other chronic viral infections when the rate of viral clearance equilibrates with the rate of viral production. Some patients, however, developed a pattern of fluctuating viraemia (FV; 38%) characterized by an initial decline and subsequent rise in VL (>1$\log_{10}$). This pattern of

viraemia has been observed in HIV-negative individuals and chimpanzees but has not previously been correlated with outcome in a longitudinal study.

We aimed to employ the viral kinetics of early HCV infection to predict subsequent outcome. SC patients had a 1.7log_{10} VL drop versus a 0.001log_{10} drop in PV and 0.57 log_{10} drop in FV within 100 days of infection (OR=2.76, p=0.001; Table 2.6), increasing to a 4.03 versus 0.13(PV)/0.85(FV) log_{10} drop by 200 days (OR=2.48, p<0.0001). While the likelihood of clearance is low if a 1log_{10} drop is not achieved by 100 days, some patients will not subsequently clear the virus even if they do achieve this, and therefore require careful ongoing monitoring. Such patients fall by definition into the fluctuating pattern of progression towards chronicity.

A difficulty encountered in predicting the outcome of infection is in distinguishing between those who will develop a fluctuating viraemia and those who will go on to spontaneously clear infection, particularly those patients who become transiently RNA negative. Examination of the VL gradient in such patients (the FV group) revealed a slower decline in VL (median 129 versus 27 days to a 1.7log_{10} drop; p=0.009); thus the rate of decline of viraemia is key in predicting outcome.

Other clinical measures were also predictive of outcome; an elevated serum bilirubin level of ≥40µmol/l was associated clearance with a hazard ratio of 5.04 while elevated ALT also predicted outcome (≥1000IU/l HR 2.62; Table 2.7). This may reflect the presence of a protective immune response in the liver. Finally, a higher CD4 count at the time of infection

was also associated with a higher likelihood of spontaneous clearance (≥650cells/mm^3 HR 2.66, p=0.045). The role of the CD4 response in controlling acute HCV is discussed further in Chapter 4.

2.6.3 Predicting spontaneous clearance of acute HCV in HIV-positive men based on sequence analysis

A detailed phylogenetic and genetic sequence analysis of 2295 longitudinally-acquired clonal E2 HVR-1 sequences allowed us to track the impact of superinfection, and to assess whether measurements of viral diversity and selection had predictive value. Several monophyletic clusters were present (Figure 2.4), in keeping with multiple transmission events in the MSM population, as previously described (Danta et al, 2007; van de Laar et al, 2009).

2.6.3.1 Superinfection with new strains of HCV

Within individuals, the presence of superinfection was suggested by marked non-linear variation in genetic distance over time (Figure 2.5). In order to investigate if this variation was the result of superinfection events, neighbour-joining phylogenetic analyses of all longitudinally acquired E2 HVR-1 sequences (n=2295) were next carried out, revealing 3 groups of patients (Figure 2.3); those who spontaneously cleared infection (n=10; 20%), those who progressed to chronicity with increasing quasispecies diversity but no evidence of superinfection (n=20; 40%) and those who progressed to chronicity with evidence of superinfection events (n=20; 40%). The introduction of new infecting strains occurred in

40%, both of the same genotype (30%) and with new genotypes (10%). This high rate of superinfection is in keeping with the likely ongoing high risk behaviour of this group of patients (Danta et al, 2007) and the observation of reinfection events in other cohorts (Herring et al, 2004). The majority of patients with fluctuating viraemia had evidence of reinfection and superinfection with new strains of HCV (63%).

This observation could be explained in 3 other ways. Firstly, the observation could be artefactual as a result of PCR contamination error or Taq polymerase-related error introduction. Secondly, the phenomenon could be explained by the resurgence of minority quasispecies, present in plasma at baseline but undetected by clonal sequencing (<5%). Thirdly, unique strains could have been compartmentalised in the liver, PBMCs or the brain at the time of initial infection. The technicalities of reducing PCR-related error are discussed in the next section, and the possibility of undetected minority variants in Chapter 3 of this thesis.

The presence of superinfecting strains was a confounding factor when analysing the evolution of HCV strains over time, producing wide variations in genetic diversity and dN/dS ratios (Figure 2.5). A correction was therefore introduced to investigate the evolution of related strains by calculating the average diversity/dN/dS ratio over branches of the phylogenetic trees separated by >10% variation.

2.6.3.2 Viral diversification within the E2 HVR-1 is predictive of spontaneous clearance

In order to determine which genetic factors predicted spontaneous clearance, mean intrastrain Hamming distance (diversity), number of quasispecies and dN/dS ratios were calculated in 50 patients (40 progressors and 10 clearers) at multiple (median=3) time points (Table 2.8). Genetic diversity did not correlate with VL but was found to be significantly higher in progressors (both FV and PV) by 150 days of infection compared with SC patients, consistent with similar observations in HIV-negative individuals (Farci et al, 2000). In the main, a gradual increase in viral diversity and dN/dS ratio was observed over time in PV patients as a result of generation of positively-selected variants. In contrast, viral diversity and dN/dS ratio decreased gradually in SC patients (Figure 2.7). We hypothesised that this would be likely to occur in association with a strong, multi-specific T cell response (Chapter 4).

2.6.3.3 Fluctuating Viraemia; superinfection versus recrudescence

In the FV group, when patients with superinfection were excluded from the analysis, genetic diversity at baseline was greater than the PV group (Hamming distance 2.84 versus 0.49; p=0.04), possibly suggesting a more diverse infecting inoculum. The dN/dS ratio in FV patients with single strain infection was <1 suggestive of purifying selection at this stage. However, by 150 days, positive selection was evident, with a significantly higher median dN/dS ratio in FV than PV patients (1.78 versus 0.76; p=0.02). One unusual case within the cohort suggested that reactivation of HCV may rarely occur in immunosuppressed individuals after successful treatment. This patient had evidence of reactivation of HCV 2

years after successful treatment for acute disease (Figure 2.6d). This coincided with a period of severe immunosupression and hospitalization and may have been the result of loss of T-cell control of a virus present in the liver but not in the bloodstream or extremely low level viraemia below the limit of detection. Such cases are rare but have been previously reported in the literature (Kim et al, 2006).

This characteristic variation in viral diversity and VL in FV patients would be in keeping with a model of partial immune control and may reflect within some individuals a delayed partially effective immune response and in others the emergence of escape variants or an unsustained protective immune response due to B or T-cell failure, for example, as a consequence of PD-1 receptor upregulation (a marker of T-cell exhaustion and disease progression both in HIV-positive individuals and in acute HCV infection (Day et al, 2006; Urbani et al, 2006). Alternatively, it could represent reinfection with the same strain of virus, which has been artificially demonstrated in the chimpanzee model (Bukh et al, 2008). A third explanation would be the persistence and re-emergence of virus from sites outwith the bloodstream e.g. the liver, PBMCs or the central nervous system (Maggi et al, 1997; Forton et al, 2004). These possibilities are investigated further in Chapters 3 and 4.

2.6.4 Predicting SVR in HIV-positive men with acute HCV

Patients with acute HCV are usually offered early antiviral treatment. This is known to be more effective than treatment given in the chronic phase, reaching success rates of 98% (Jaeckel et al, 2001) versus 54-56% (Manns et al, 2001; Fried et al, 2002) in HIV-negative individuals. Co-infection with HIV results in lower success rates; 59%-70% in the acute phase

versus 40% in chronic infection (Gilleece et al, 2005; Serpaggi et al, 2006, Dominguez et al, 2006; Torriani et al, 2004). In this study, patients who did not achieve spontaneous clearance 12 weeks following diagnosis were offered treatment with 48 weeks of IFNα and ribavirin and 70% achieved an SVR. We hypothesized that those who had accumulated a higher diversity over time would be less likely to respond to treatment. In keeping with this hypothesis, an SVR was associated with lower viral diversity as early as the baseline sample, increasing in significance with successive measurements. Thus it is possible that the diversity within the initial inoculum may partially define the likelihood of SVR. A gradual increase in diversity over time may also explain the disparity between treatment during the acute and chronic phases of HCV infection. Once a threshold diversity has been reached, the likelihood of SVR is markedly reduced (Hamming distance at 150 days 2.05 in responders versus 7.82 in relapsers/non-responders and dN/dS 0.76 versus 4.76; p=0.004 and 0.007 respectively). It remains to be seen if patients with high viral diversity at baseline would benefit from very early anti-viral therapy.

The mechanism of resistance to IFNα and ribavirin is not known, but a trend towards increased variation in the ISDR region of NS5A in patients with an SVR has been described in patients with chronic genotype 1a, 1b and 3a infections (MacQuilan et al, 2004; Pascu et al, 2004). In this study, there was a trend towards greater variation in this region in treatment responders also, with a highly significant p value (<0.0001). If sequences were present with ≤1 amino acid difference from the prototype reference strain, the SVR was 0%. Further work to identify variation in this region and SVR is indicated.

2.6.5 Artefactual error during PCR and clonal sequence analysis

In order to reduce the likelihood of PCR contamination occurring, samples were processed in strict aseptic conditions as detailed in the methods section and a negative control was always included. Variation in genotype or HCV strain was noted on multiple occasions, in multiple specimens from the same patients and by the clinical laboratory in separately processed samples. Another cohort (of IDUs), tested separately in another laboratory using the same primers also revealed evidence of multiple circulating HCV strains over time (Smith et al, 2010). Amplification of a region of NS5B was next carried out as a control experiment to verify these results. Although shorter branches were observed secondary to lower genetic diversity within NS5B, similar phylogenetic trees were produced, confirming that the appearance of new strains was unlikely to be secondary to contamination error.

Nucleotide error may occur during PCR amplification (up to $2x10^{-4}$ errors/bp/cycle; Tindall et al, 1988) due to the lack of 3' to 5' exonuclease activity of Taq polymerase and to a lesser extent during RNA reverse transcription (6.4×10^{-5} nucleotide substitutions/site). Proof-reading enzymes such as Tgo DNA polymerase or Pfu DNA polymerase are superior with error rates in the 10^{-6} range (Cline et al 1996), but are significantly more expensive than Taq-containing preparations.

In order to calculate the actual PCR error rate, the 5'UTR was subcloned following end-point dilution PCR and $1.9x10^{-5}$ errors occurred/bp/cycle (0.3 errors/sequence). This is likely to reflect the upper end of possible error, as PCR saturation is likely to have prevented the incorporation of as many errors due to higher starting concentrations of DNA pre-

amplification. If errors are incorporated early on in the PCR reaction, the error rate could be much higher. Thus the error range lies between 0.14-1.3% per sequence. We used a cut-off of 10% for defining different strains, well above this level. Phylogenetic trees and sequence analyses were compared with a control experiment following amplification of NS5B using the proof-reading enzyme Tgo DNA polymerase with comparable results.

2.7 Conclusions

This study is the first longitudinal and detailed analysis of clinical outcome of acute HCV in a large cohort of HIV-positive patients, incorporating viral dynamics and detailed viral sequence evolution. Acute HCV infection is an emerging global epidemic in HIV-infected men and requires new public health planning and treatment strategies. These data suggest that the outcome of infection can be predicted by viral kinetics, CD4 count, liver function and genetic diversity. Diversification of HVR-1 of the E2 protein, which contains both B and T-cell epitopes was associated with progressive disease, while a narrowed quasispecies repertoire occurred in patients who subsequently spontaneously cleared infection. SVR was also related to viral diversity at baseline and over time. Targeting of high risk patients e.g. with no evidence of rapid decline in VL and higher viral diversity at baseline may increase the efficacy of treatment while those more likely to clear infection spontaneously could be monitored for a longer period of time thus avoiding a rigorous and expensive treatment.

The study has a number of limitations. Firstly, it is restricted to an HIV-positive population, although patterns of evolution during early infection may also apply to those with HCV

monoinfection. Steps were taken to minimise error rates attributable to PCR contamination and clonal analysis, as detailed above. In order to overcome the problem of delayed HCV seroconversion in HIV-positive individuals (to avoid the inclusion of chronically infected patients), we used retrospective PCR testing on stored HIV VL samples to date the time of infection. More work is needed to further define specific T cell targets and Class I/II restriction of these responses, as well as the impact of viral escape. The role of the immune response in containing HCV infection is discussed in detail in Chapters 3 and 4.

Chapter 3: The viral evolution of acute HCV

3.1 Background

3.1.1 Evolutionary theory

The observation of variation in the phenotypic characteristics of animals and plants led Charles Darwin to conclude that speciation was not a fixed but a dynamic process (Darwin, 1859). He realised that this variation occurred with the generation of progeny displaying new characteristics by observing the breeding of pigeons and other animals (variation under domestication) and subsequently under the forces of nature, a process he named natural selection. Charles Darwin argued that the mechanism for variation in species was a tree-like branching process, with each new generation diverging from the previous, which he illustrated with a diagram remarkably similar to current phylogenetic trees, constructed on the basis of sequence data. Nearly 100 years later, the discovery of the genetic code in 1953 (Watson & Crick) paved the way towards a deeper understanding of genetic variation and new theories of evolution based on genotypic rather than phenotypic observations.

The dynamic nature of variation in genetic sequences is now clearly apparent and a number of evolutionary forces have been defined all of which have been identified in observations of viral evolution. The first, positive (Darwinian) selection reflects change in the phenotype of an organism as a result of environmental positive pressure. Purifying selection is the selection of genes with a structure essential to survival and the favouring of the status quo. Neutral selection is the continued generation of variants in a population with no selection pressure. These 3 models can be defined in mathematical terms by examining the ratio of non-synonymous to the number of synonymous substitutions.

Synonymous substitutions (those that do not result in a change in amino acid) are neutral and a measure of random genetic drift while non-synonymous substitutions (resulting in a change in amino acid sequence) are subject to positive or negative selection pressures. A comparison of the rate of nonsynonymous (r_N) to synonymous (r_S) substitutions may indicate whether or not a region of interest is under positive selection pressure ($r_N > r_S$) or negative selection pressure ($r_S > r_N$) (Hughes and Nei, 1988, 1989). Both r_N and r_S can be estimated by comparing two nucleotide sequences and the number of synonymous substitutions per synonymous site (d_S) compared with the number of non-synonymous substitutions per non-synonymous site (d_N); the dN/dS ratio; Figure 3.1.

Figure 3.1: Models of evolution

$$\text{Positive selection: } \frac{dN}{dS} > 1 \qquad Purifying\ selection\ \frac{dN}{dS} < 1 \qquad Neutral\ selection \frac{dN}{dS} = 1$$

Figure 3.1: Models of evolution: positive, purifying and neutral selection are displayed as ratios of dN to dS

These models rely on the assumption that silent synonymous mutations are not subject to selection pressure. While they do not result in amino acid changes, it is highly likely that variations in synonymous regions of genes have secondary structural functions. HCV core and NS5B RNA, for example, contain multiple stem–loops that are found in all HCV genotypes throughout both regions, as well as several strikingly conserved single-stranded regions, one of which coincides with a region of the genome to which ribosomal access is required for translation initiation (Tuplin et al, 2004). The impact of variation in RNA structure is one which is relatively unexplored at the present time.

3.1.2 The evolution of HCV

The red queen hypothesis of evolution is based on Lewis Carroll's quotation that "it takes all the running you can do, to keep in the same place[1].". As a model of evolution this refers to the continued struggle between host and virus to maintain fitness over time. A characteristic feature of RNA viruses is their ability to diversify rapidly; 10^{12} HCV particles are produced every day in an infected individual due to the lack of proof-reading function of viral RNA polymerase (Neumann et al, 1998). This presents the immune system with an immense task; a host-virus arms race which in the majority of cases continues without resolution for the lifetime of the host. The evolution of the viral quasispecies is emerging as key in defining the outcome of infection and response to treatment (Chapter 2; Farci et al 2000; Sheridan et al 2004).

Variation within the 28-amino acid structure of the HCV E2 HVR-1 is extensive (Weiner et al 1991) and contains both antibody (Farci et al 2000) and T cell epitopes (Sarobe et al 2006). As discussed in Chapter 2, evolution of HVR-1 is associated with progressive disease, while a narrowed quasispecies repertoire occurs in patients who subsequently spontaneously clear infection (Farci et al 2000; Sheridan et al 2004). Agammaglobulinaemic patients (who cannot form antibodies) with HCV develop chronic infection with a narrow repertoire of quasispecies (Gaud et al 2003) suggesting that this diversification of virus arises at least in part as a result of antibody-mediated immune pressure. Detailed studies of longitudinal evolution in this region and outside it during acute infection (Farci et al 2000; Sheridan et al 2004) have been limited by small sample size and have not previously been carried out in HIV-positive patients. The point at which such diversity arises after acute infection has not

[1] Exert from "Through the Looking Glass" by Lewis Carroll

previously been fully established. We discussed in Chapter 2 that the emergence of divergent strains correlates with progression to chronicity by 150 days of acute infection and also with the likelihood of treatment success. A number of factors may impact this diversification of virus over time. We aimed to investigate the impact of transmission diversity, superinfection with new viral strains and of the adaptive immune response on viral diversity in further detail.

3.1.2.1 Transmission diversity

A gradual accumulation of viable viral strains within an infected individual over time correlates with a decreasing likelihood of response to treatment (Chapter 2). The diversity at the onset of infection is also predictive of outcome. While all patients in this cohort were exposed to HCV by the sexual route, a significant minority also used intravenous or intramuscular drugs (most commonly crystal methamphetamine). We hypothesised that the route of transmission would impact on viral diversity and final outcome (spontaneous clearance or SVR) due to the likelihood of high diversity within the infecting inoculum in IDUs. Initially, a phylogenetic analysis of IDUs within the cohort was carried out to see if they represented a distinct population or were dispersed throughout the cohort. Measures of viral diversity were next compared in IDUs and non-IDUs. Finally, molecular clock techniques were used to reconstruct phylogeny within individual patients and 2 transmitting couples (both with dual genotype infection) to identify possible population bottlenecks at the time of transmission.

3.1.2.2 Superinfection or recrudescence

A major confounding factor in this cohort was the appearance of new dominating quasispecies populations at different time points in individual patients (Figure 2.6). While a compelling explanation for this is reinfection or superinfection with other strains, there are other alternative explanations. The analysis of variant strains using clonal sequencing was limited to 20 per time point. As the mean number of viral copies at any time point in this cohort was $3x10^6$/ml, each sample of 140µl of plasma could be expected to contain approximately 20,000 viral copies. This ratio of sampled viral strains was thus approximately 1:1000 of the viral population present in each sample and $1:5^{11}$ produced within the host in a single day (20 per 10^{12} viral copies). A minority variant present and amplified by PCR could thus remain undetected using the clonal sequencing technique. We aimed to investigate this possibility further by designing strain-specific primers for the superinfecting strain to test on the baseline sample to see if the majority quasispecies present at a later time point originated from the minority acquired during initial infection. Another approach would be to use ultra-deep pyrosequencing which can detect tens of thousands of sequences within a single cDNA sample.

This problem of detection within the bloodstream is compounded by fact that viral strains may also be compartmentalised within the liver (Sobesky et al, 2007), PBMCs (Maggi et al, 1997; Roque Alfonso, 1999) or the central nervous system (Forton et al, 2004). The role of compartmentalised virus acting as a reserve for future viral recrudescence is controversial and relatively unexplored. One patient in this cohort who denied reinfection risk had evidence of reactivation of HCV 2 years after successful treatment (Figure 2.6d). This coincided with a period of severe immunosupression and hospitalization and may have been

the result of loss of T-cell control of a virus present in the liver but not in the bloodstream or extremely low level viremia below the limit of detection. We aimed to examine the case in more detail by looking for evidence of viral strains present in an archived paraffin-embedded liver biopsy taken prior to viral recrudescence and in PBMC samples taken just after the re-emergence of infection. We also looked for evidence of viral compartmentalisation in PBMC samples in another 27 patients, including a transmitting couple with dual genotype infection.

3.1.2.3 The adaptive immune response

In Chapter 2, we attempted to correct for the impact of superinfection in each patient by using the mean intrastrain diversity in order to assess the impact of the immune response on viral populations. A number of more sophisticated techniques have been designed to overcome this problem by calculating the dN/dS ratio firstly over the entire phylogenetic tree and then for each branch of the tree. It is therefore possible to identify specific codons under positive, negative or neutral selection pressure. Several population-based tests may be used to ascertain if sequences are evolving due to random genetic drift (neutrality) or are under positive selection. Taijma's Test of Neutrality (Tajima, 1989) compares the number of segregating sites with nucleotide diversity. Fu and Li's related neutrality test (1993) compares segregating sites with singletons (D test) or average pairwise divergence versus singletons (F test). Both the D test and dN/dS ratio can be averaged over a stretch of 25-100 nucleotide sequence in a sliding window thus reducing the calculation to a smaller length and providing a visual assessment for sections of the alignment in a sliding window.

3.2 Hypotheses

The diversification of viral quasispecies predictive of final outcome is defined by

$H_{1,}$ Transmission diversity as a consequence of the route of transmission

$H_{2,}$ Superinfection events

H_3 ,Positive or negative selection pressures as a consequence of the adaptive immune response, or

H_0, Chance

3.3 Aims

1. To identify epitopes under selection at the codon-specific level during early HCV infection
2. To determine if superinfection contributes to viral diversity using PCR and clonal sequence analysis
3. To compare viral diversity over time in patients depending on route of transmission

3.4 Methods used to analyse viral sequence diversification

3.4.1 Measuring nucleotide diversity (π)

Nucleotide diversity (π) in a population is defined as the number of nucleotide substitutions per site between sequences and may be calculated as follows:

$$\pi = \sum_{ij} x_i x_j \pi_{ij}$$

where x_i=frequency of i^{th} type of sequence and π_{ij}=proportion of nucleotide differences between the i^{th} and j^{th} type of sequences.

The evolution of viral diversity can be measured using a variety of phylogenetic and statistical techniques based on RNA or DNA sequence differences. Kimura (1968) introduced the theory of "neutral evolution" following the observation that many genetic mutations are random events and may not confer phenotypic change. This model may be applied to the analysis of variation in HCV viral quasispecies which occur largely as stochastic (random) events due to the lack of proof-reading function of the RNA-dependent RNA polymerase. Provided the population size is large enough, some mutations may occur which are beneficial and increase in frequency in a population as a result of natural selection. Others may be neutral and persist due to random genetic drift - these changes however take longer to arise than adaptive mutations. This phenomenon may be measured by comparing genetic sequences and taking into account whether or not RNA sequence changes encode changes in the translated amino acid sequence.

3.4.2 Inferring phylogeny

This section on the construction of phylogenetic trees is a summary of information obtained from Felenstein Inferring Phylogenies 2004, The Phylogenetic Handbook 2nd edition 2009 and Phylogenetic Trees Made Easy, Hall, 3rd edition, 2008).

3.4.2.1 Nucleotide substitution models

Phylogenetic tree analysis may be carried out using a variety of models. Sequences are compared and the genetic distance between them (represented by branch length) used to construct a tree. The observed number of nucleotide substitutions between sequences is called the p distance. Substitution of nucleotides was originally assumed to be an entirely random (stochastic) process and thus the first nucleotide substitution model introduced by Jukes and Cantor in 1969 (the JC69 model) was based on the assumption that all nucleotides have the same likelihood of being replaced by any other (that the nucleotide frequencies [π] are equal and that $\pi_A = \pi_T = \pi_C = \pi_G$). A second assumption made was that substitutions only occur once at each site, when in fact multiple substitutions at the same site may occur (multiple hits), a substitution may revert to the original nucleotide (back mutation) and parallel substitutions may occur in two or more sequences. Lastly, in an open reading frame, third nucleotide substitutions in a codon are more likely to become fixed as they are less likely to result in an amino acid substitution than first or second nucleotide substitutions due to the redundancy of the genetic code. The observed genetic distance p may therefore differ substantially from the true genetic distance (d).

In order to correct for this difference, newer model systems introduce a formula which takes into account the frequency of transitions (T_S) (purine ↔ purine or pyrimidine ↔ pyrimidine) which is usually greater than the frequency of transversions (T_V)(purine ↔ pyrimidine) due to steric hindrance. The ratio of T_S to T_V (κ) and the ratio of purine transitions (T_{SR}) to pyrimidine transitions (T_{SY}), denoted by γ, and may also be incorporated into the equation as in the Tamura-Nei (TN93) model (Tamura & Nei, 1993). If γ=1 and the base frequencies are uniform ($\pi_i = 0.25$), the TN93 model reduces to the Kimura 2-parameter (K80) model (Kimura, 1980). When κ=1, the F81 model is generated (Felenstein, 1981). Choosing an appropriate model is essential as inappropriate models may produce inaccurate tree topologies.

3.4.2.2 Methods for the construction of phylogenetic trees

Phylogenetic trees may be produced using exhaustive search methodology or stepwise clustering. Exhaustive search methods evaluate all possible tree topologies and apply criteria to select the most likely candidate. However, with increasing numbers of sequences, increasingly large numbers of possible trees may be produced, resulting in a lengthy analysis. Stepwise clustering methods overcome this problem by grouping sequence data together to form clusters with each new sequence compared to a cluster, thereby reducing computing time. Examples of exhaustive search methods are maximum parsimony (selects a tree with the smallest number of mutations required to generate internal nodes for each sequence) and maximum likelihood.

3.4.2.3 Stepwise methods

In order to reduce computation time, stepwise clustering methods do not compare every possible tree phylogeny, but instead reconstruct phylogenies in a stepwise manner.

The unweighted pair group method with arithmetic means (UPGMA) is the oldest technique developed for inferring phylogeny. A distance matrix is constructed, quantifying differences between pairs of operational taxonomic units (OTU; each representing a sequence). OTU clusters are constructed based on similarity within the distance matrix. The remaining OTUs are then sequentially paired with the cluster. This method assumes a constant evolutionary rate between OTU cluster pairs and does not identify different rates of evolution along different branches.

The neighbour-joining (NJ) method also uses a distance matrix to identify similarity between sequences. However, instead of clustering OTUs, it involves the calculation of distance from OTUs to each internal node. The distance from each paired OTU to the internal node need not be the same and thus different rates of evolution along the branches of the tree are incorporated. A tree is constructed sequentially by the introduction of OTUs based on minimisation of the length of internal branches.

The accuracy of trees may be evaluated by conducting a bootstrap analysis. This is a resampling technique and is carried out by constructing a new hypothetical alignment based on resampling of columns from the original alignment and reconstructing phylogenetic trees for each. The proportion of each clade among the bootstrap replicates is provided as a measure of the validity of each branch of the tree.

3.4.2.4 Exhaustive methods

Maximum parsimony and maximum likelihood phylogenetic methods are exhaustive methods in which multiple tree topologies are constructed and the best tree selected from all calculated trees. Maximum parsimony finds the tree which is constructed with the smallest number of nucleotide substitutions. For each tree topology, a score called the parsimony length is calculated as the sum of all substitutions required to construct the tree. The tree with the lowest parsimony length is selected as the best tree.

Maximum likelihood involves the construction of multiple phylogenetic trees and the calculation of likelihood based on all possible nucleotide or amino acid states in the hypothetical internal nodes. As well as the number of substitutions required to construct the tree, the validity of a particular evolutionary model is applied to the data.

3.4.2.5 Bayesian analysis

Bayesian analysis incorporates the principle of accommodation of previous evidence (posterior distribution) based on Bayes theorem (Figure 3.2) into the phylogenetic modelling process. The prior probability (or prior) is the likelihood before the posterior distribution was known. In phylogenetic analysis, posterior distributions may be obtained using Markov Chain Monte Carlo (MCMC) sampling. In mathematical modelling, a Markov chain is a discrete random process which depends only on the current state of the system and not on the probability distribution of the system at previous steps. Monte Carlo methods are computational algorithms (finite sequence of instructions) reliant on repeated random sampling to compute results. A domain of inputs is defined and generated randomly, given a

specified probability distribution. A deterministic computation is performed thereafter using the inputs and these computations are aggregated into the final result.

Figure 3.2: Bayes theorem

a) Bayes theorem

$$\text{Conditional probability of A given B} = \frac{\text{Conditional probability of B given A x Prior probability of A}}{\text{Prior probability of B}}$$

$$P(A|B) = \frac{P(B|A)P(A)}{P(B)}$$

b) The Monty Hall problem solved using Bayes theorem

The Monty Hall problem was published in 1975 (Selvin, 1975) and takes the form of a TV games show. There are 3 doors—red, green, and blue, one of which has a prize hidden behind it. The contender guesses the red door. The presenter (who knows where the prize is) opens the blue door which is empty. He then asks if the contender wishes to change his choice from the initial selection of red. Thus, the Monty Hall question is which choice is better; to choose the green or the red door? Intuitively, the answer may seem to be either, with a 0.5/0.5 chance of each. However, following the application of Bayes theorem, this is not the case where P(Ar)=likelihood of the prize being behind the red door, P(B)=presenter opens the blue door = 0.5 (one of two doors without the prize), P(B|Ar)=if the prize is behind the red door, the likelihood of picking green or blue=0.5, P(B|Ag)=if the prize is behind the green door, the presenter must pick the blue door=1, P(B|Ab)=if the prize is behind the blue door=0. Thus,

$$P(Ar|B) = \frac{P(B|Ar)P(Ar)}{P(B)} = \frac{0.5 \text{ x } 0.33}{0.5} = 0.33$$

$$P(Ag|B) = \frac{P(B|Ag)P(Ag)}{P(B)} = \frac{1 \text{ x } 0.33}{0.5} = 0.66$$

$$P(Ab|B) = \frac{P(B|Ab)P(Ab)}{P(B)} = \frac{0 \text{ x } 0.33}{0.5} = 0$$

The contender should always choose the green door, doubling the chance of winning.

3.4.2.6 Application of the molecular clock hypothesis to the evolution of HCV

In 1962 and 1965, Zuckerkandl and Pauling published 2 papers revealing that the coding sequence for the same protein varied over time; showing a gradual linear increase in genetic diversity between species over time. They then hypothesised that this linear increase in genetic diversity with time could be used to estimate a molecular clock model of divergence times between species, with different proteins displaying different but constant genetic variation over time. This molecular clock hypothesis fits with the neutral theory of evolution described by Kimura in 1963 and 1983. Although a linear increase in sequence variation occurs in some proteins, it does not occur in a linear fashion in others (Jenkins et al, 2001). "Relaxed" models based on a non-linear accumulation of genetic mutations have been designed to overcome this problem (Pybus, 2006). Both relaxed and strict (linear) molecular clock models can be incorporated into a Bayesian analysis (Figure 3.4)

Using the clock-like theory of sequence evolution, it is estimated that HCV diverged from a common ancestor shared with GB Virus-B (GBV-B); a virus found in tamarins and chimpanzees (Ohno et al, 1996; Adams et al, 1998) between 500 and 2000 years ago (Smith et al, 1997). Within the last 300 years, six genotypes have evolved and within the last 100 years, multiple subgenotypes emerged. This reconstruction of the history of HCV must however be interpreted with caution (Simmonds, 2001) as ancestral viral sequences have not been identified from more than 50 years ago, thus the reliability of the molecular clock model has not been verified. The diversity of HCV in westernised countries is considerably less than that in sub-Saharan Africa and South-East Asia. This would suggest that the variation seen in HCV in western countries is an example of founder effect and that HCV

originated either in sub-Saharan Africa or South East Asia (Simmonds, 2001). Expansions of transmission and evolution of the virus have occurred at various key historical time points; the onset of the industrial revolution coincides with an increase in diversity of genotype 1b HCV, likely to be related to urbanisation and the use of parenteral therapies on an extensive basis. While this hypothesised expansion is based on theoretical mathematical techniques, assuming rates of evolution based on sequence data obtained within the last 30 years, other expansions have been well-documented in real-time. Two notorious well-documented transmissions of HCV in recent years occurred in North Africa. The first occurred in Egypt, where a genotype 4a epidemic paralleled an ambitious project to eradicate schistosomiasis using intramuscular and intravenous treatment in the 1960s and 1970s (Frank et al, 2000). Another outbreak in hospitalised Libyan children was investigated by the use of molecular phylogenetic techniques, revealing using Bayesian inference that spread of viral strains of HCV and HIV had occurred prior to the arrival of foreign nursing staff who stood accused of deliberately infecting the children (de Oliveira et al, 2006).

3.4.2.7 Estimating effective population size

Using Bayesian analysis, an estimate may be made of effective population size (N_e). This is the estimated size of a regenerating population with a variation rate equal to the studied sample. It becomes smaller than the overall population size (N) when some strains do not replicate and when natural selection is operating. When N_e is small, the rate of diversification reduces also. A decrease in N_e is known as a genetic bottleneck and may occur at the time of viral transmission, as a result of the immune response or as a result of treatment.

3.5 Laboratory methods

3.5.1 Extraction of viral RNA from formalin-fixed, paraffin-embedded (FFPE) liver biopsy specimens

Liver biopsy specimens stored in paraffin blocks were obtained retrospectively from the clinical pathology department. RNA was extracted using the RNeasy FFPE kit (Qiagen). Freshly cut sections (5-10µm thick) of FFPE liver tissue were provided by Dr Rob Goldin (St Mary's Hospital Pathology Department) and placed immediately into a sealed microcentrifuge tube. Xylene (1ml) was added to the sample which was vortexed vigorously for 10 seconds and centrifuged at 13,000g for 2 minutes. The supernatant was then removed using a pipette and 1 ml of 100% ethanol was added to the pellet in order to remove excess xylene, mixed by vortexing and centrifuged at 13,000g for 2 minutes. The supernatant was again removed by pipetting and the pellet allowed to air dry. The pellet was then resuspended in 150µl of Buffer PKD and 10µl Proteinase K, and incubated at 55ºC for 15 minutes, then at 80ºC for 15 minutes. 320µl of Buffer RBC was added and the sample transferred to a spin column and centrifuged for 30 seconds at 8,000g. A volume of 720µl of 100% ethanol was added to the flow-through and the sample added to an RNeasy spin column and centrifuged for 15 seconds at 8,000g. 500µl of Buffer RPE Wash Buffer was added and centrifuged for 15 seconds at 8,000g. The RNA was eluted using 30µl of RNase-free water.

3.5.2 Extraction and concentration of RNA from PBMCs

RNA was extracted from PBMCs was carried out using the QIAgen RNA Blood Minikit with a modified protocol. $2x10^6$ cells were centrifuged at 300g and the supernatant removed by aspiration. 350µl of buffer RLT containing 10ul/ml of β-mercaptoethanol was added to each

sample. This was then vortexed and added to a QIAshredder spin column in a 2ml collection tube and centrifuged at full speed for 2 minutes. The column was then discarded and the lysate added to 350µl of 70% ethanol and then placed in a QIAamp spin column and centrifuged for 15 seconds at 8000g. The lysate was then discarded and 700µl of buffer RW1 added to the column which was inverted and then centrifuged for 15 seconds at 8000g. 500µl of buffer RPE was then added to the spin column and centrifuged for 15 seconds at 8000g. A further 500µl of buffer RPE was added and centrifuged at 20000g for 2 minutes. This step was repeated with a further centrifuge step of 1 minute carried out prior to elution with 30µl of RNase-free water. The RNA concentration and 260:230 and 260:280 absorbance ratios were then measured using a nanodrop machine.

3.5.3 Strain-specific PCR

Semi-nested strain-specific PCR was carried out using primers designed to amplify specific HVR-1 sequences in order to determine if low copy numbers of virus (not detected by clonal analysis) were present in preceding plasma samples of 11 patients thought to be superinfected with new strains and used to amplify HCV RNA baseline samples. PCR products were sequenced in order to rule out non-specific primer-binding.

3.6 Computer-based methods for viral sequence analysis

3.6.1 Program for cropping sequence files using Python

Primer sequences were produced in separate sequence (.seq) files. A computer program was designed using the programming language Python to crop primers and vector sequence leaving the desired sequence behind in the correct orientation in fasta format (Appendix 2).

3.6.2 Sequence alignment

Sequences were next translated and aligned in Fasta format in an open reading frame using Clustal W (www.ebi.ac.uk/Tools/clustalw) and MUSCLE www.ebi.ac.uk/muscle). Chromatograms were visualised and sequences edited manually using BioEdit.

3.6.3 Construction of neighbour-joined phylogenetic trees

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al 2007) and the Kimura-2 parameter (K80). All trees were inferred using 1000 bootstrap replicates. Branches with bootstrap values > 70 were considered to be reliable.

3.6.4 Construction of maximum likelihood trees

Data files were converted from MEGA format to nexus format and imported into Phylip v3.68 and Garli v0.96 for the calculation of maximum likelihood trees.

3.6.5 Path-O-Gen v1.2

Path-O-Gen is a tool designed for analysing trees that have not been inferred under a molecular-clock assumption to see how valid this assumption may be. It can also root the tree at the position that is likely to be the most compatible with the assumption of the molecular clock. Maximum likelihood trees were constructed using GARLI and then analysed using this tool in order to assess their suitability for application in a molecular clock model.

3.6.6 Estimating tree topologies using a molecular clock model

Initially sequences aligned in nexus format were uploaded to the data manipulation and model selection programme BEAUTI v1.4.8. Sequences were assigned a number in days based on the number of days from the earliest detected sequence. Initially a strict molecular model was implemented (less computationally intensive) and subsequently 2 relaxed models (exponential and logarithmic) applied to sequences derived from each patient at a minimum of 3 time points. The resulting xml programme file was implemented in BEAST v1.4.8 for a predetermined number of MCMC analyses. Two files were then produced – a log file and a tree file. The log file was examined in Tracer v1.4.1 in order to analyse the output to check performance and accuracy. An appropriate burn in was selected on the basis of the trace file (Figure 3.3) and effective sample size scores (ESS) of >100 were considered to be reliable.

Each phylogenetic reconstruction was repeated twice and uploaded into Tracer and LogCombiner v1.4.8 to maximise accuracy. Finally a consensus tree was created in TreeAnnotator v1.4.8. The resulting consensus tree was then viewed and edited in FigTree v1.2.2.

Figure 3.3 Tracer file output

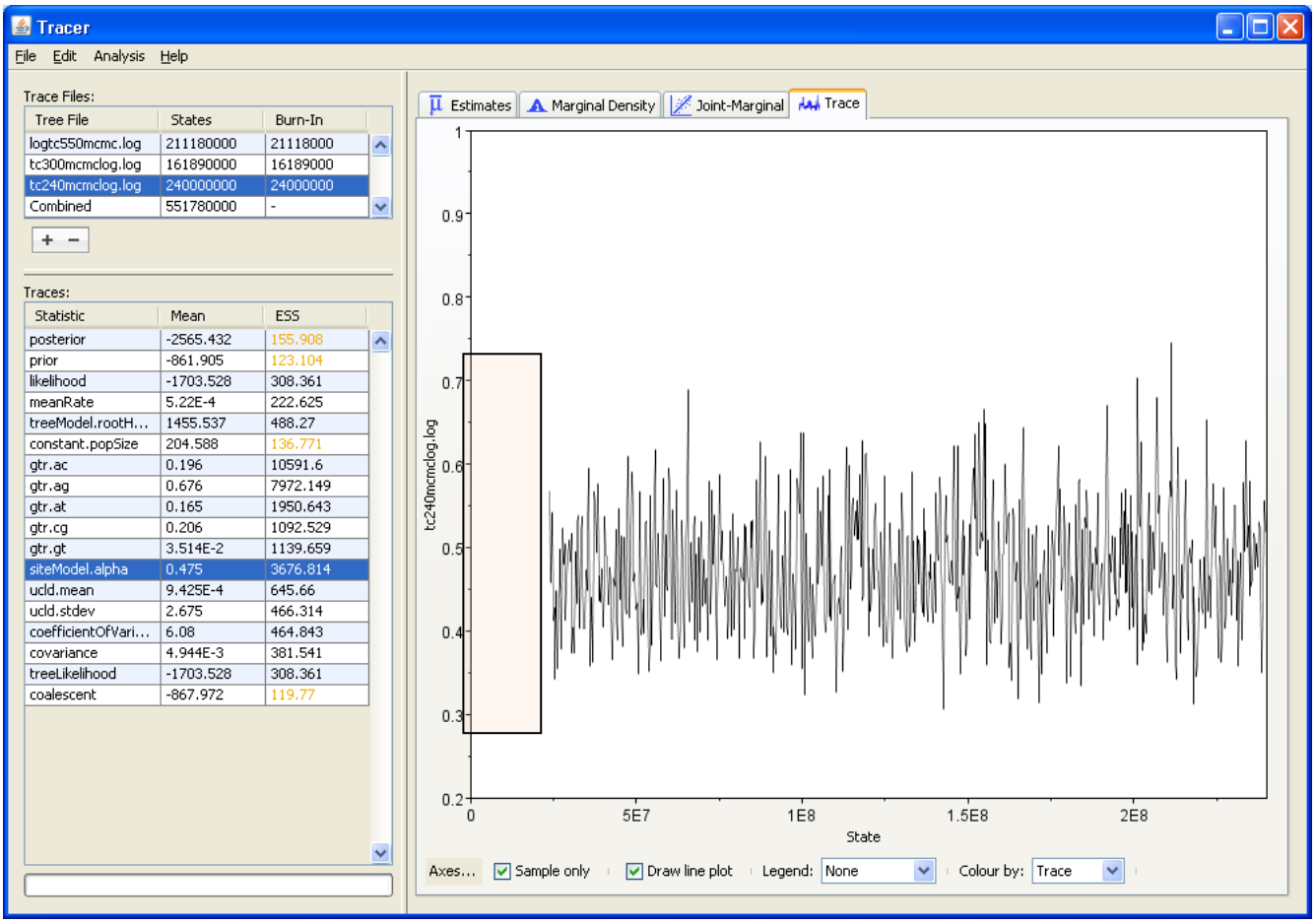


Figure 3.3 Tracer file output A trace file is shown with each variable tested against the length of MCMC. The excluded burn in region (highlighted) was highly variable while the selected region illustrated adjacent regions of similarity

3.6.7 Detecting evidence of purifying or positive section by pairwise comparison of sequences using the codon-based Z test

In order to identify sequence pairs under positive selection or purifying selection pressure, the codon=based Z test was implemented using MEGA 4.0. Two hypotheses were tested:

$$H_1 = \text{purifying selection: } \mathrm{Z} = \frac{(\mathrm{dS} - \mathrm{dN})}{\text{variance}(\mathrm{dS} - \mathrm{dN})}$$

$$H_2 = \text{positive selection: } \mathrm{Z} = \frac{(\mathrm{dN} - \mathrm{dS})}{\text{variance}(\mathrm{dN} - \mathrm{dS})}$$

$$H_0 = \text{neutral selection: } \mathrm{dN} = \mathrm{dS}$$

If Z>1 for the purifying selection hypothesis H1 (dS>dN), this indicates evidence of purifying selection, where amino acid changes to the sequence have been eliminated. If Z>1 for the positive selection hypothesis H1 (dS>dN), this indicates evidence of positive selection, where amino acid changes to the sequence have been selected. The variance of the difference between these two quantities is estimated by the bootstrap method. In MEGA, a matrix is constructed and p values obtained for each pair of sequences indicating which are under purifying or positive selection respectively.

3.6.8 Testing for neutrality using Tajima and Fu and Li's D test in DNAsp v5

In order to test for neutrality, sequences were opened in nexus format in DNAsp v5. A sliding window was created for sequence alignments from each patient in order to view the d score across 50 nucleotide stretches with a corresponding p value for each region examined.

3.6.9 Codon-specific models of positive, neutral or purifying selection

3.6.9.1 Detecting codon-specific selection using HyPhy

In order to identify specific regions of the genome under positive or negative selection, initially, sequences obtained from patients over time were examined using HyPhy (Kosakovsky et al, 2005) on the server located at www.datamonkey.org. Sequence alignments were entered in adjusted MEGA format. Three different codon-based maximum likelihood methods, SLAC, FEL and REL, can be used estimate the dN/dS (ω) ratio at every codon in the alignment. Each model involves the construction of a nucleotide model maximum likelihood (ML) fit, then the construction of a codon model ML fit and codon ancestral sequences are next reconstructed site by site using maximum likelihood by using the data generated from both ML models. Finally, for every variable site, the expected and observed dN and dS values are compared and codons under selection identified by a variety of statistical modelling techniques. In this study, the REL method was used.

3.6.9.2 Detecting codon-specific selection using CODEML

The computer software package PAML may be used to produce evidence of codon based selection (using CODEML). Sequences alignments were converted to interleaved Phylip 4.0 format from MEGA 4.0 (.phy). Maximum likelihood trees were constructed using the PHYLIP dnaml programme and saved in Phylip format (.tree). The CODEML control file which contains the specified model script was adjusted for each set of sequences analysed in order to identify the input Phylip sequence alignment, the input Phylip ML tree and the output file. The model was next selected. Initially, Model 0 was used, which calculates a dN/dS ratio averaged across the whole tree (each branch is assigned the same ratio). Next, Model 1 was used which calculates an independent dN/dS ratio for each branch of the tree. The log likelihood of the 2 models was next compared, to see if the variation in Model 1 was >Model 0 indicating that variation was not the same across the phylogenetic tree. The likelihood ratio test is next carried out using a chi square analysis by comparing the log likelihoods (lnl) for each model. The chi square value is taken as 2*(lnl1-lnl2). The degrees of freedom are calculated as the number of parameters estimated under the 2 models -1. Under model 0, 1 parameter is measured (the mean dN/dS ratio) while under model 1, each branch is counted and the degrees of freedom are the number of branches-1.

3.7 Results

3.7.1 The impact of transmission diversity on outcome

In order to address the hypothesis that route of transmission impacts diversity and final outcome, we constructed a neighbour-joined phylogenetic tree using majority quasispecies

from the entire cohort in order to identify if IDU patients were dispersed throughout the cohort or were represented by one or several monophyletic lineages (Figure 3.4). This revealed that IDU patients were dispersed throughout the cohort.

Figure 3.4: Neighbour-joined phylogenetic tree by route of exposure

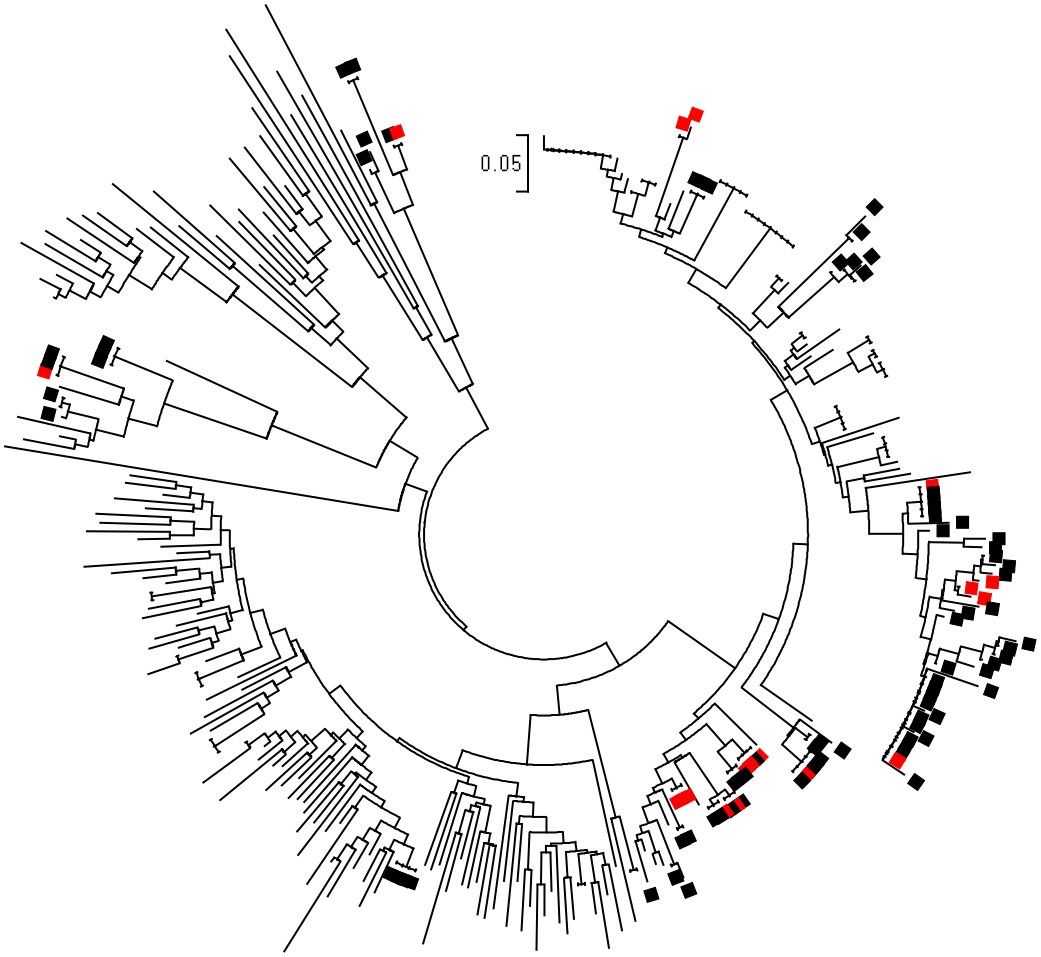


Figure 3.4 Neighbour-joined phylogenetic tree identifying patients by route of exposure: A neighbour-joined phylogenetic tree (K80 model) was constructed using 95 majority quasispecies strains from IDU patients (red squares) and non-IDU patients (black squares) and 214 reference sequences derived from patients from Europe and the USA available on the Los Alamos database.

Sequence diversity was next compared in the IDU and mucosal exposure populations (Table 3.1).

Table 3.1 Clinical and virological measures and route of exposure

Transmission route	IDU + mucosal	95% CI	Mucosal	95% CI	P value
Number of patients	29	-	69	**-**	**-**
Baseline HCV VL (log_{10})	6.39	5.97, 6.89	5.79	5.24, 6.08	**0.05***
Peak HCV VL (log_{10})	6.91	6.49, 7.22	6.22	5.82, 6.45	**0.001****
Baseline ALT	68	42, 149	86	48, 140	0.57
Peak ALT	585	401, 1480	311	234, 467	**0.03***
Baseline bilirubin	9	6, 12	12	9, 16	**0.05***
Peak bilirubin	17	15, 19	15.50	14, 20	0.84
Baseline quasispecies	4	3, 5	4	3,6	0.55
Total quasispecies (all samp combined)	10	7, 14	11	8, 14	0.75
Baseline Hamming distance	3.38	0.28 ,11.28	0.49	0.36, 1.16	**0.05***
Hamming distance at 3 months	1.89	0.31, 15.32	1.95	0.94, 4.94	0.26
Mean Hamming distance	12.29	2.67, 17.08	2.52	1.41, 9.33	**0.03***
Spontaneous clearance	3/30 (10%)		9/64 (14%)		0.75
SVR	2/9 (22%)	-	18/21 (86%)	-	**0.002***

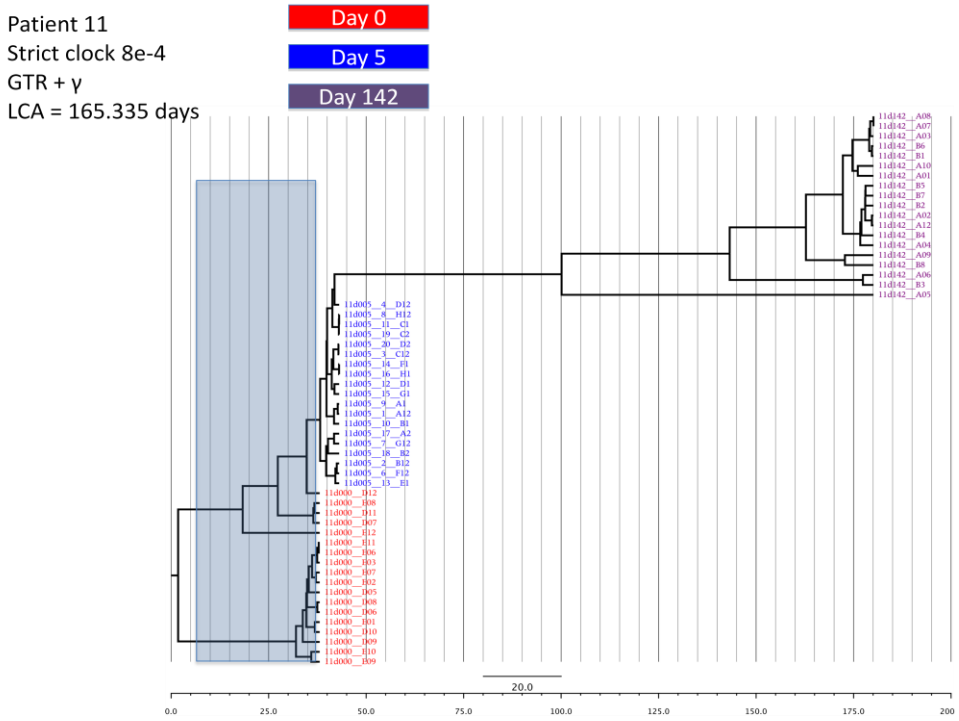
In order to further investigate diversity at the time of transmission, Bayesian phylogenetic trees were constructed using a molecular clock model in 22 patients (Figure 3.5). Patients in

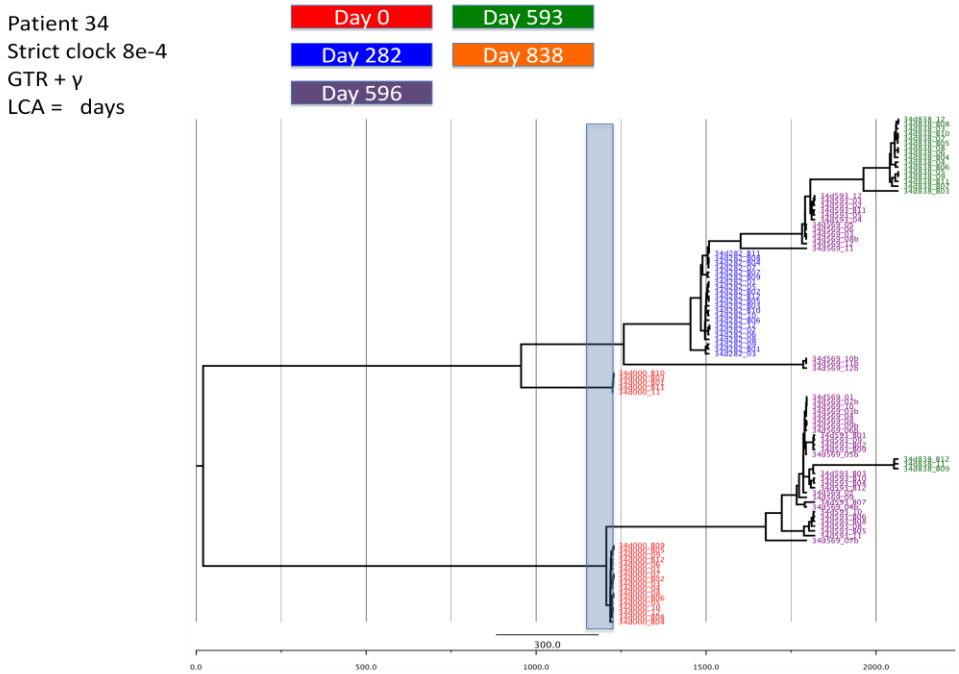
the IDU group had a higher viral diversity at the time of transmission (transmission window highlighted in blue) than patients in the mucosal exposure group showing that although the number of quasispecies was not significantly different in the 2 groups, patients were infected with more diverse quasispecies; each of the IDU patients is infected with 2 groups of quasispecies which originate from 2 different branches, while the quasispecies in patients with mucosal exposure derive from the same branch, suggesting that a bottleneck at the time of transmission occurs in this group of patients.

Two transmitting couples within the cohort were identified. Both had dual infection with genotypes 1a and 4d. Bayesian phylogenetic trees were constructed for each pair using a logarithmic relaxed molecular clock model. Both showed multiple probable transmission events over time.

Figure 3.5 Bayesian phylogenetic trees: a) IDU exposure and b) Mucosal exposure

a) IDU exposure (Subjects 11 and 34)





b) Mucosal exposure (Subjects 5 and 14)

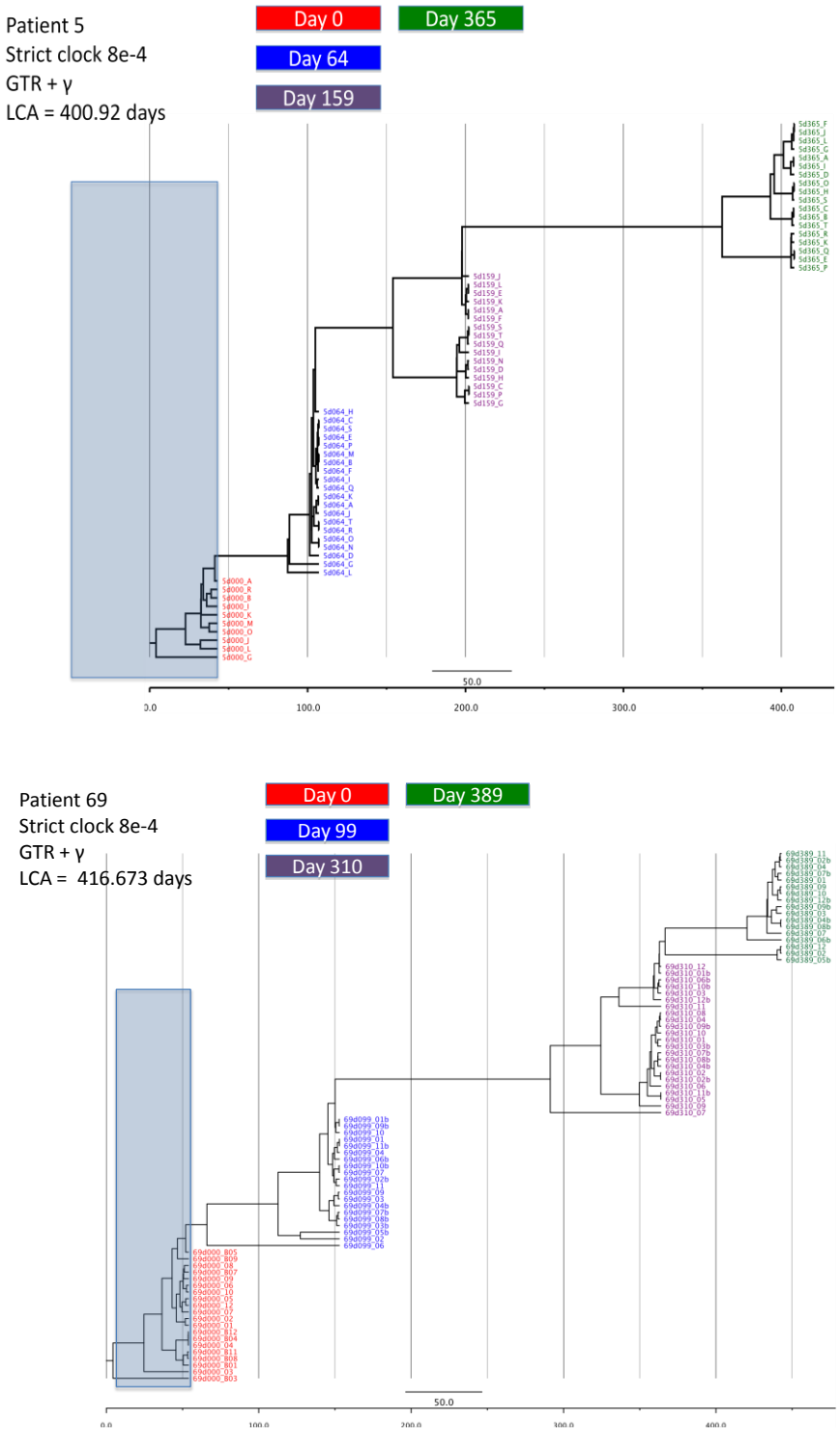


Figure 3.5: 4 Bayesian phylogenetic trees illustrating 2 IDU (Figure 3.5a) and 2 mucosal exposure patients (Figure 3.5b). Trees were constructed using a strict molecular clock with a fixed rate across all branches in BEAST v1.4.8. The blue shading indicates the transmission window (time from last negative PCR to first positive PCR).

Figure 3.6 Bayesian phylogenetic tree analysis of transmitting couples (TC): a) TC1 and b) TC2

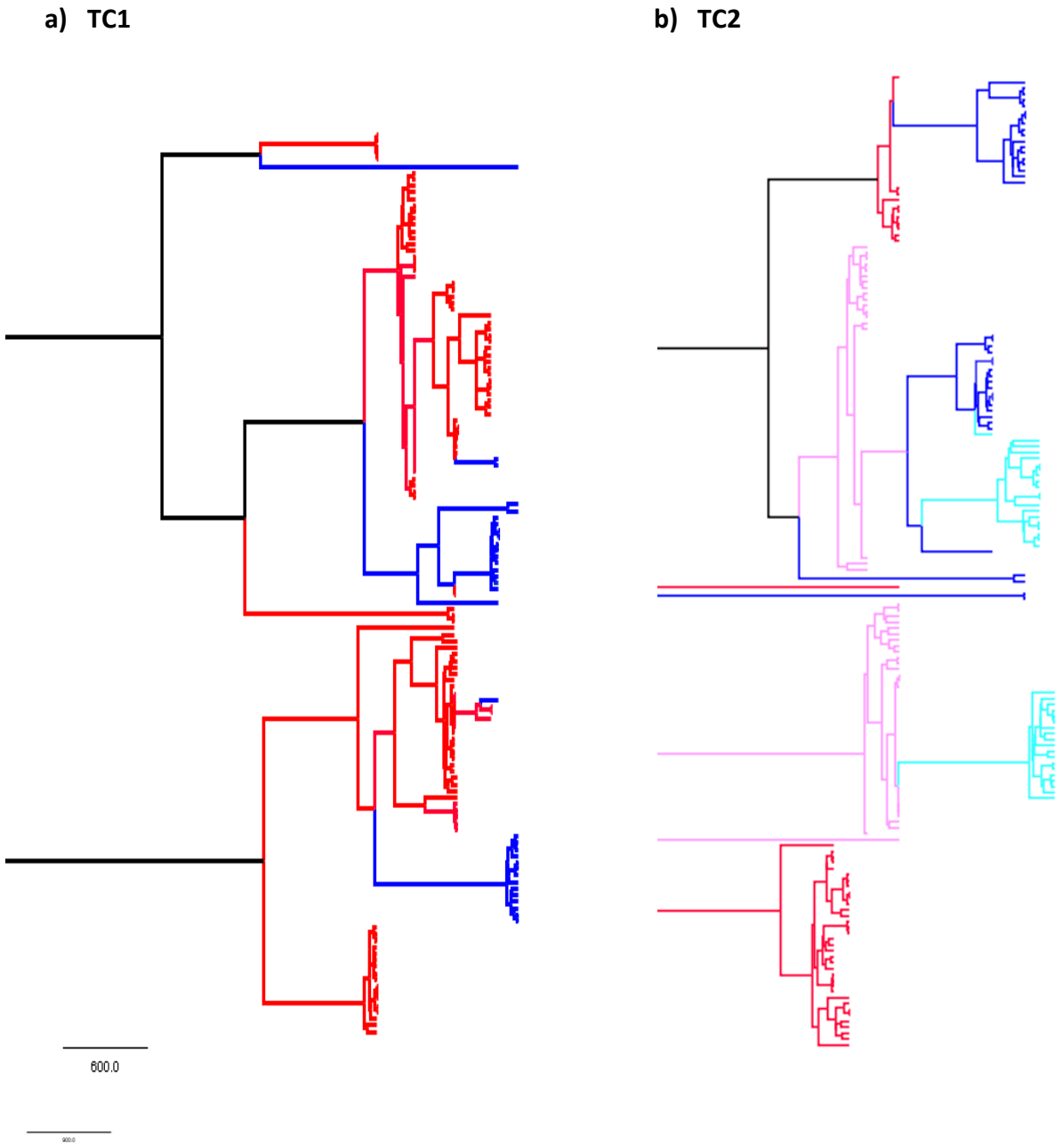


Figure 3.6: Bayesian phylogenetic trees from TC1 and TC2 constructed using a logarithmic relaxed molecular model and $120x10^6$ and $10x10^6$ MCMC respectively in BEAST v1.4.8. Branches derived from each patient are coloured red and blue (Figure 3.6.a TC1: Subjects 13 [red] and 34 [blue]; Figure 3.6b Subjects 9 [red] and 45 [blue]). In addition, in Figure b, PBMC-derived sequences were analysed (light blue; patient 45, pink patient 9). The length of each branch is given in days.

3.7.2 The impact of superinfection and recrudescence of minority strains on viral diversity over time

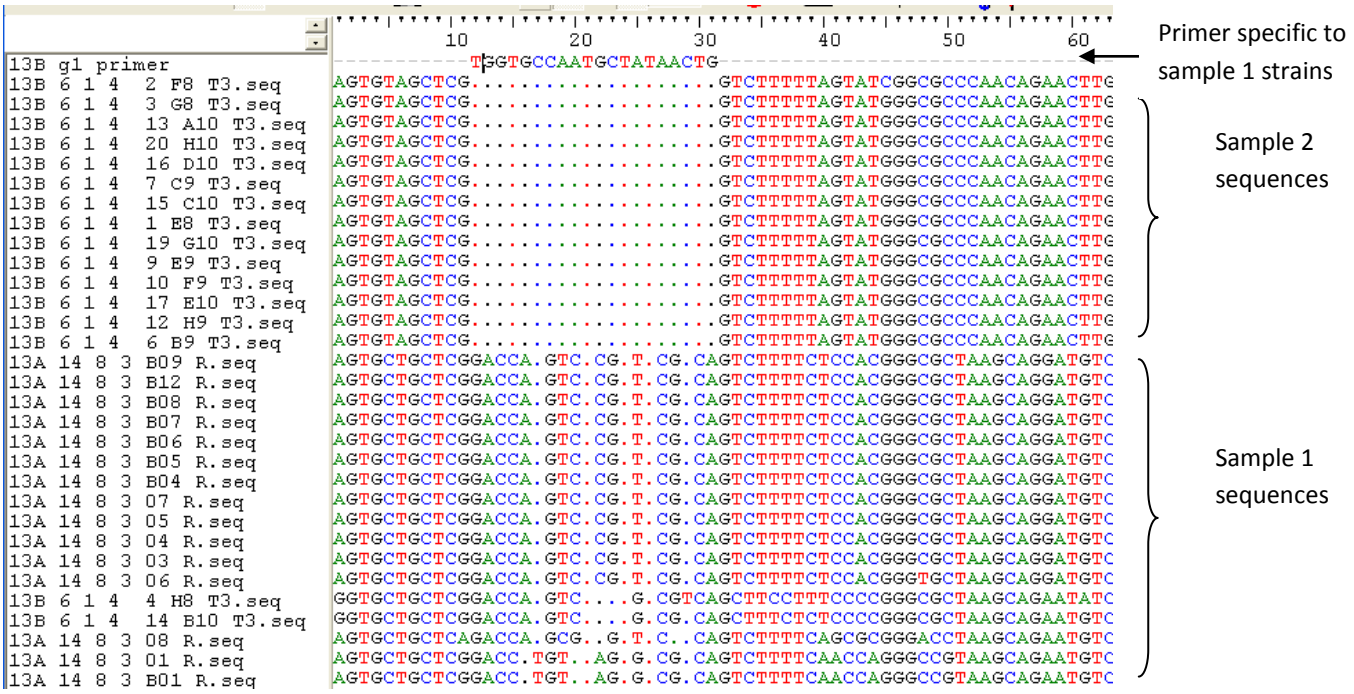
In order to investigate whether new viral strains were being introduced into individual patients as a result of superinfection or as the result of recrudescence of minority variant strains, PCR primers were designed to specifically identify the introduced strains. These were used in a third semi-nested PCR step to amplify the cDNA obtained from the first positive (baseline) sample. 15 patients had sequences sufficiently divergent to design primers to detect the new strain exclusively (Figure 3.7) and PCR product was amplified successfully in 11/15 of these.

Figure 3.7: Strain-specific PCR: a) Primer sequences, b) Alignment of old and new strains detected with strain-specific primer sequences for subject 13, and c) PCR gel electrophoresis image.

a) Primer sequences

Primer name	Patient number	Primer Sequences
ET26643	2	ATGCTTAGTCCGGGCGCC
ET4485	3	GGCCGCGTTTGGTCTC
ET7609	6	CGGGCCGCGTTTGCTC
ET22503	8	CTGCGAATAGCCTTGCC
ET1117	12	CTTCACCTCACTGTTCAA
ET5322_4	13	GACCACGTCTGCTGTCG
ET5322_1	13	TGGTGCCAATGCTATAACTG
ET55108	18	CGCCCGCACTGTGAATAG
ET25196	21	AAGTGCTGCTAAGACCACGTC
ET55427	27	CTGAGACCATGTCTGGTCTCG
ET8438	28	GCCGGGCTTGTCGATCTC
ET21754	29	GGCGCGTTGGGAATTAG
ET5645	31	GGTGCCAATGCTATAACTGG
ET21860	37	GCTATTGCCAAATCCGCATTC
ET27758	39	AAGTGCTGCTCGGACCATG
ET2387	44	CCGCTGGATTTGCCAGCTTCAG

a) Alignment of old and new strains detected with strain-specific primer sequences in patient 13



b) Amplified strain-specific PCR products run on a 2% agarose gel

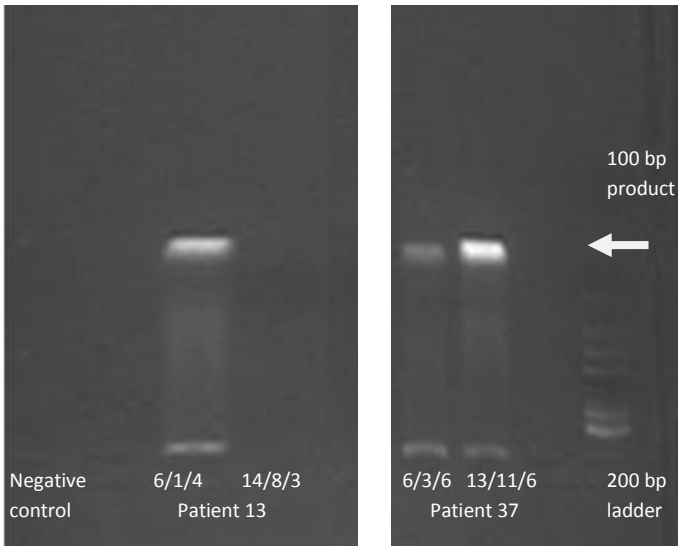


Figure 3.7: Strain-specific PCR: **a)** Primer sequences**, b)** Alignment of old and new strains detected with strain-specific primer sequences for subject 13, and **c)** PCR gel electrophoresis image. HVR-1 amplicons obtained using strain-specific primers in a third semi-nested PCR round. from subjects 13 and 37 at 2 time points. A strain-specific product is present as expected at the second time point in subject 13, but not at the first, while a strain-specific product is detected at both time points in subject 37

Of the 11 patients who had successful amplification of a strain-specific product, sequences characteristic of the new majority strain were identified in baseline samples in 4 patients (36%) by strain-specific PCR. Thus 64% of patients demonstrating evidence of new strains were likely to have been superinfected. The experiment was repeated to exclude contamination and the products were gel extracted and sequenced in order to confirm that non-specific amplification had not occurred.

3.7.3 The impact of compartmentalisation on recrudescence of minority strains over time

3.7.3.1 Compartmentalisation within PBMCs

In order to assess whether sequence strains were archived within PBMCs or the liver, RNA was extracted from PBMCs derived from 20 patients and a paraffin-embedded liver biopsy taken for investigation of deranged liver function in subject 1 during early infection with HCV.

Of 20 patients tested, 11 had a positive PCR result (61%). This was strongly associated with outcome; no SC patients had detectable virus within PBMCs (Table 3.2).

Table 3.2 HCV detection within PBMCs and outcome

	Spontaneous clearance	Plateau progression	Fluctuating viraemia	p value
PBMC PCR positive	0% 0/4	100% 6/6	60% 6/10	0.011*
Median HCV VL (IU/ml)	0 (0, 27)	1260734 (9143, 1731309)	151166 (0, 1002869)	0.02*
PCR positive and VL< 50	0% 0/4	0% 0/6	100% 3/3	0.02*

In order to determine if the presence of HCV within PBMCs had an effect on T cell function, flow cytometry was carried out as described in Chapter 4 in order to determine IFNγ, IL-2, IL-17 and TNFα expression in HCV+ and HCV- PBMCs in response to pooled HCV peptides and PMA/ionomycin. Expression of IL-2, IL-17 and TNFα did not differ between groups. However, a significant difference in IFNγ production was noted between the groups stimulated with HCV peptides (Table 3.3). No difference was noted between PBMC total IFNγ production in response to PMA/ionomycin but CD4 IFNγ production was slightly reduced in PBMCs of the HCV+ group (p=0.05).

Table 3.3: IFNγ expression in HCV+ and HCV- PBMCs following stimulation with pooled HCV peptides

	PBMC HCV+	PBMC HCV-	p value
Total IFNγ	0	1.48	0.002**
	(0, 0.44)	(0.58, 9.48)	
CD4 IFNγ	0	0.47	0.01*
	(0, 0.01)	(0, 1.48)	
CD8 IFNγ	0	0.45	0.02*
	(0, 0.07)	(0, 1.95)	

3.7.3.2 Compartmentalisation within the liver

One patient (subject 1) had a liver biopsy at the time of acute HCV infection. He had had acute HCV infection (genotype 1a) in January 2004. His viral load initially descended to undetectable in February 2004 despite deranged liver function tests, but became positive again within 1 month. He was subsequently treated with pegIFNα and ribavirin for one year. Six months after completion of treatment, the HCV RNA was undetectable by PCR (detection limit <12 IU/ml) and the ALT was normal. In May 2006 his CD4 count dropped to 106 $x10^6$

cells/mm^3 and he required treatment for *Pneumocystis jeroveci* pneumonia. In October 2006, his previously normal ALT rose to 133 IU/l and HCV-RNA was detectable once again. He denied any episodes of unprotected sex or contact with his previous partner, or any other high- risk exposure to HCV. We aimed to investigate whether this was due to reinfection or reactivation of virus, and to see if variation in strains was present in PBMCs obtained in April 2007, the liver biopsy from February 2004 (taken when the plasma HCV VL was negative) and a blood sample taken prior to this, during detectable viraemia. No virus was detected within PBMCs from April 2007. However, PCR for HVR-1 was successful following RNA extraction from a 1mm section of paraffin embedded liver biopsy. A neighbour-joined phylogenetic tree was constructed revealing that sequences derived from all three samples were derived from the same branch

Figure 3.8: Neighbour-joined circular phylogenetic tree of subject 1 and reference sequences

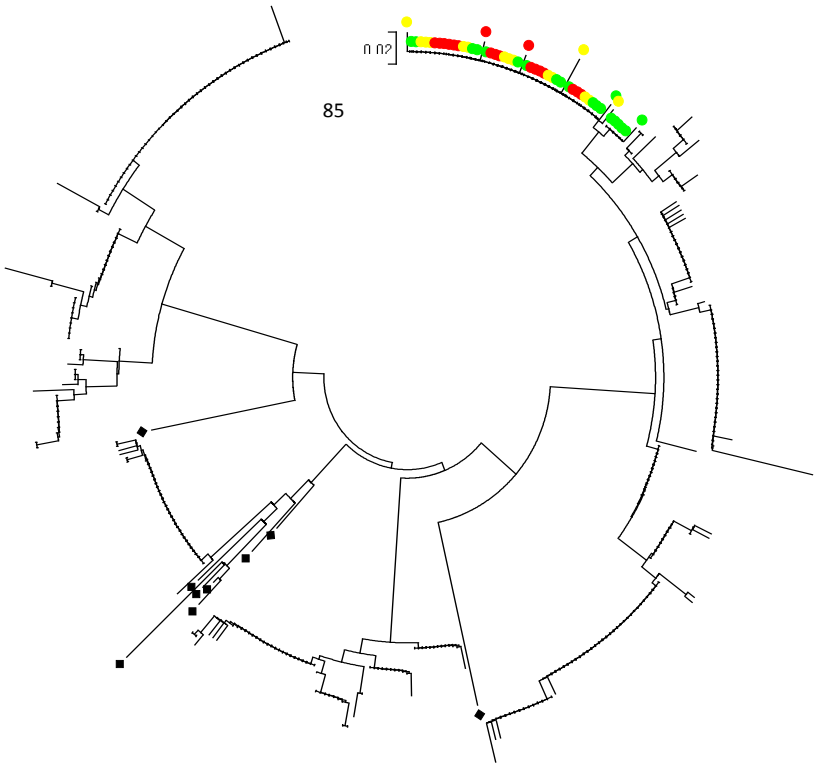


Figure 3.8: Neighbour-joined circular phylogenetic tree of subject 1 and reference sequences. Kimura's two-parameter model was implemented using MEGA 4. An unrooted circular neighbour-joined tree (Kimura two-parameter distance) was constructed using HVR-1 sequences (n=442). Branches derived from patient HVR-1 sequences (n=56) are coloured in yellow (February 2004), green (April 2007) and red (liver biopsy, February 2004) and compared with genotype 1a sequences derived from ten similar HIV and HCV positive patients seen at St Mary's Hospital (n=386) and reference strains (n=6; black diamonds) from the Los Alamos database. Sequences derived from the patient cluster together, indicating they are closely related. The relevant branch was supported by a bootstrap value of 85 (1000 replicates).

3.7.4 The impact of positive or negative selection pressure on viral diversification

A number of techniques were used to investigate the impact of the adaptive immune response on viral evolution. As described in Chapter 2, higher genetic diversity and dN/dS ratios within the HCV E2 HVR-1 region occurred in progressors by 150 days of infection. These increased with time in PV patients, fluctuated in patients with FV and decreased in the SC group. We aimed to identify further

evidence of selection within each individual patient (or transmitting pair) and over the population sampled over multiple time points. This was carried out using the codon-based Z test implemented in MEGA 4.0, neutrality testing using a sliding window analysis and finally using codon-based selection methods (HyPhy and CODEML).

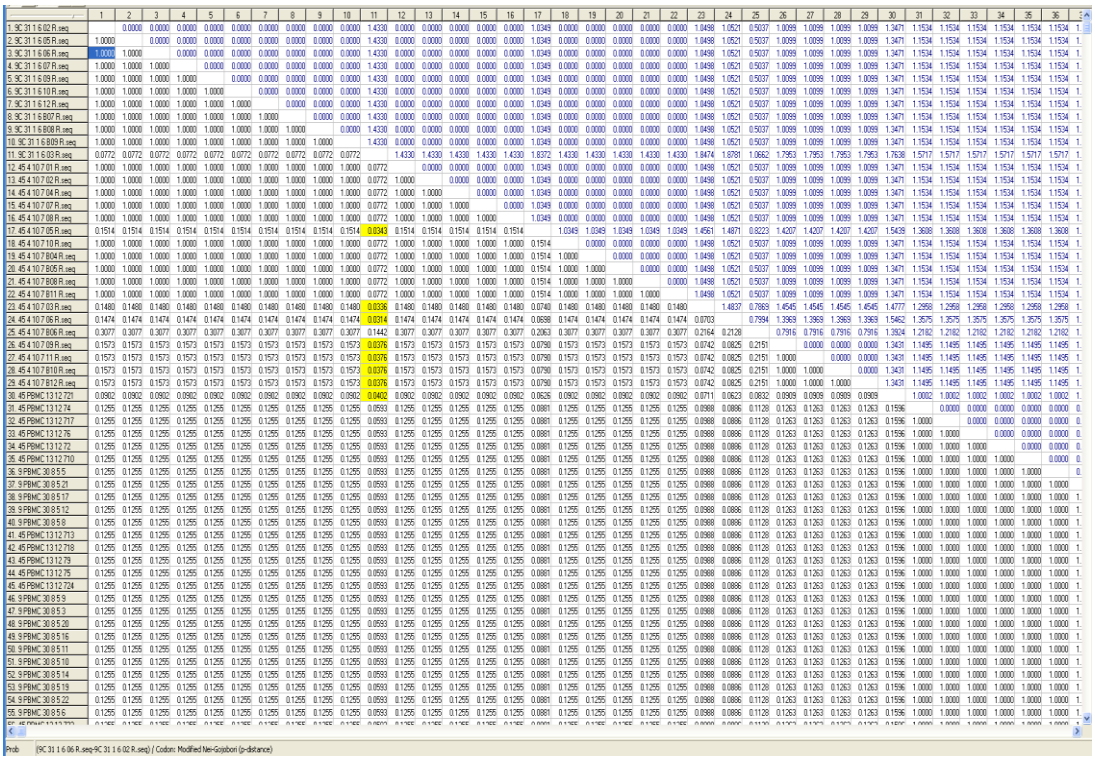
3.7.4.1 **Codon based z test**

The codon based Z test provides evidence of positive or negative selection by comparing multiple paired sequences and providing a model to test for either positive or negative (purifying) selection.

In Figure 3.9, a matrix is displayed showing pairwise comparisons between sequences obtained from transmitting couple 2. The majority of sequences are under purifying selection although a smaller number show evidence of positive selection. This was the most common picture within all progressor patients sampled. No sequences derived from SC patients were subject to positive selection, while the majority showed evidence of negative selection.

Figure 3.9: Evidence of selection using the codon-based Z test: a) Positively selected sequences and b) Negatively selected sequences.

a)



b)

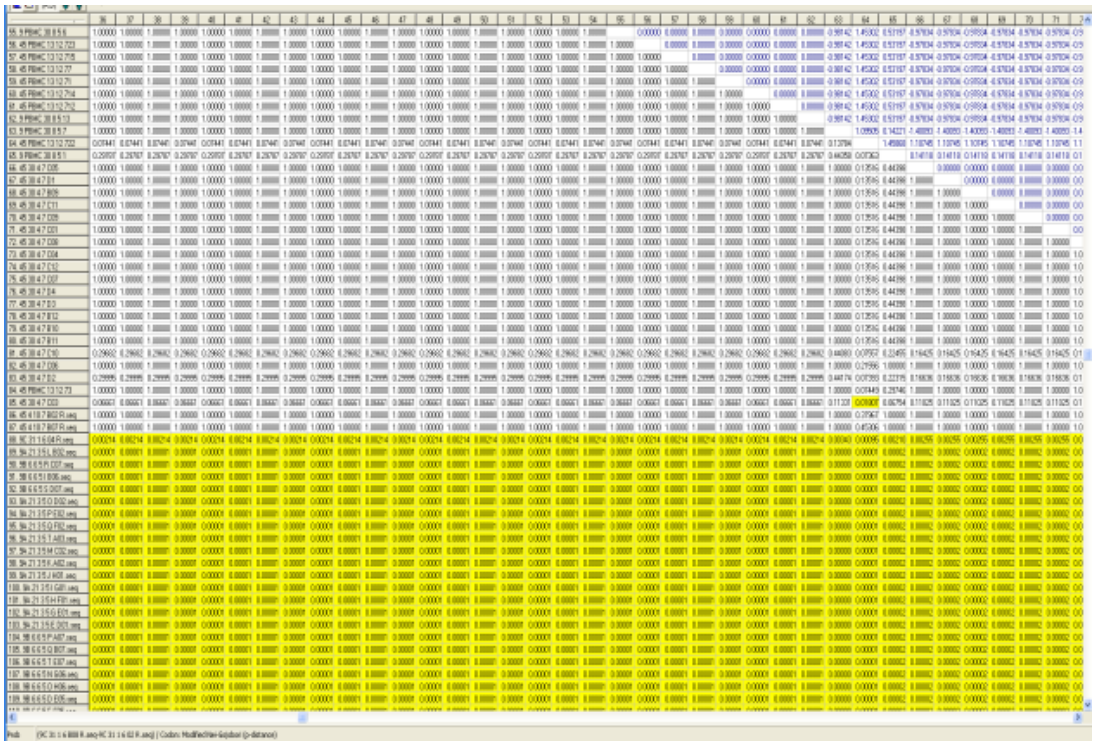


Figure 3.9: Evidence of selection using the codon-based Z test: a) Positively selected sequences and b) Negatively selected sequences. Matrices are displayed showing pairwise comparisons between sequences obtained from a transmitting couple with acute HCV (TC2). The Z score for each pairwise comparison is shown above the diagonal, with values of dN-dS>1 in sequences under positive selection (Figure 3.9a) and values of dS-dN>1 in sequences under negative selection (Figure 3.9b). Sequence pairs with statistical evidence of positive or negative selection ($p<0.05$) are highlighted in yellow.

3.7.4.2 Neutrality testing

In order to identify regions of the E2 HVR-1 under neutral selection pressure, Fu and Li's neutrality test was implemented using DNAsp v5. The null hypothesis in this test is that the sequences are under neutral selection pressure. Thus a negative d test statistic with a significant p value is suggestive of positive selection or population expansion with a large number of minority polymorphisms, while a positive d test statistic is suggestive of balancing selection.

For sequences derived from the whole population (patients 1-10), Fu and Li's D test was negative ($p<0.02$) suggesting positive selection. In order to pinpoint which areas of the E2 HVR-1 were under positive selection, a sliding window analysis was performed in SC and FV/PV patients. Positive selection was evident at several sites at both 5' and 3' ends of the E2 HVR-1 region in progressor patients, while only a small region was targeted in SC patients (Figure 3.10).

Figure 3.10: Fu and Li's D test in a) clearers and b) PV/FV patients

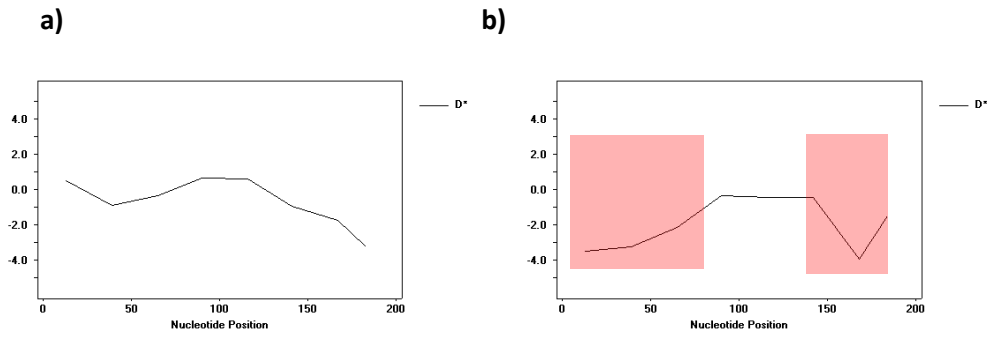


Figure 3.10: Fu and Li's D test in a) clearers and b) PV/FV patients. Fu and Li's neutrality test was carried out using a sliding window analysis of 424 sequences from subjects 1-10. A window length of 25 nucleotides was used. Positive selection was evident at nucleotide positions 1-25 ($p<0.02$), 26-51 ($p<0.02$), 26-77($p<0.02$), 130-180 ($p<0.05$) and 155-180 ($p<0.02$) in chronically infected patients (highlighted in red) but did not reach statistical significance in SC patients.

3.7.4.3 Codon-specific selection

In order to identify evidence of codon-specific selection, 2 methods were employed. Both methods are similar and involve calculating a dN/dS ratio across all branches of a maximum likelihood phylogenetic tree and comparing this ratio with ratios derived from each branch on the tree. Initially each method was compared and found to be broadly similar. As CODEML was found to be the slightly more conservative method, HyPhy was used to identify possible codons under selection. A Bayes factor result of >50 was considered to be statistically significant (equivalent to $p<0.02$).

Table 3.4 Number of codons under positive and negative pressure by group.

	Cleared	PV	FV	p value
Negatively selected	**0 (0, 0.9)**	**0 (0,0)**	**0 (0,0)**	**0.65**
Positively selected	**0 (0,0)**	**0 (0, 4.7)**	**0 (0,1)**	**0.05***

Figure 3.11: Frequency of positively selected codons within the E2 HVR-1 region.

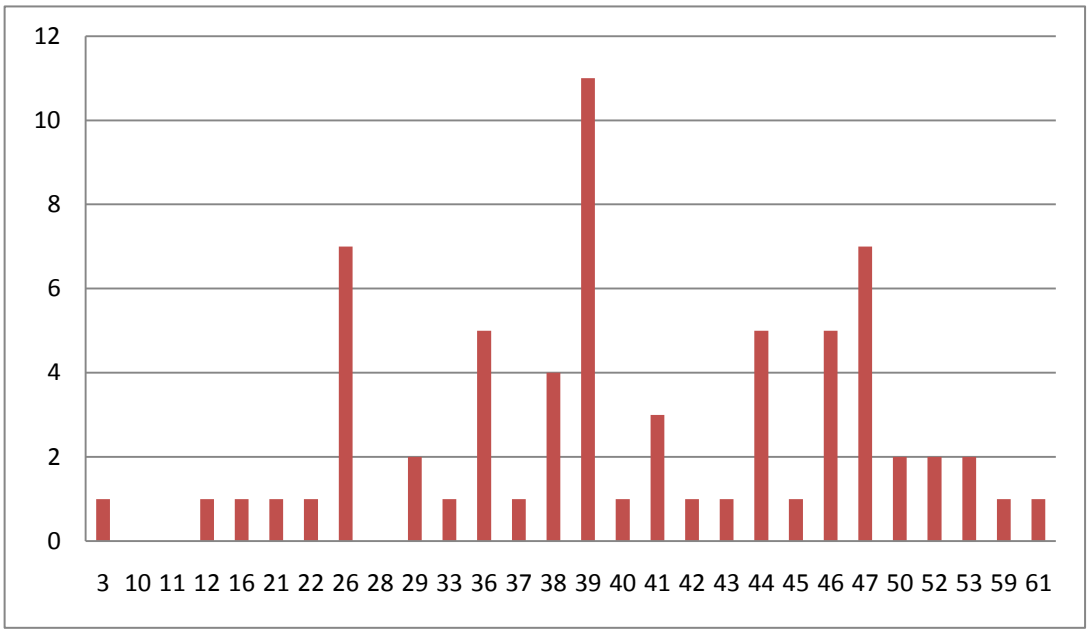


Figure 3.11: Frequency of positively selected codons within the E2 HVR-1. The most frequently recognised codon (39) corresponds to amino acid position 13 of E2 of the H77 reference sequence and lies within a previously defined antibody and DRB1*1101 restricted T helper cell epitope. The arrowed area corresponds to the HVR-1 within E2.

3.8 Discussion

The central aim of this chapter was to investigate in further detail the mechanisms involved in defining viral diversification of early HCV infection in HIV-positive individuals. In particular, the roles of transmission diversity, superinfection and the adaptive immune response were addressed.

3.8.1 Transmission diversity

IDU patients were dispersed throughout the cohort (Figure 3.4) and could therefore represent a high-risk reservoir of infection. It is compelling to hypothesise that the emergence of acute HCV within this cohort may be related to multiple introductions via the

IDU route into the MSM population. Patients using parenteral drugs were found to differ from mucosal exposure patients in a number of significant ways. Firstly, both the baseline and peak HCV VL and the baseline ALT were significantly higher, strongly suggesting a higher inoculating dose. Furthermore, as might be expected, the diversity at baseline was also significantly higher as HCV acquired via the IDU route is not subject to mucosal immunity. The rate of spontaneous clearance in IDUs was lower than that of non-IDU patients, but this observation did not reach statistical significance. Strikingly, however, the SVR rate in IDU patients was markedly lower than that seen in mucosal infection; 20% versus 86%; p=0.002. This could, in part, account for higher SVR rates seen in cohorts of MSM with acute HCV (Gilleece et al, 2005) when compared with studies carried out in chronic HCV and HIV co-infection which included significant number of IDU patients (Torriani et al, 2004) and must be a significant confounding factor. The increased diversity observed at transmission in IDUs was illustrated with the use of Bayesian inference showing that infecting diversity in IDUs is higher. These trees should be regarded with a degree of caution as they were carried out using samples obtained over a relatively short period of time, during acute infection and were constructed using relatively short PCR fragment sequences. They did however, coalesce easily and all had ESS values of >100.

Two transmitting couples, both with dual genotype 1a and 4d infection were analysed in detail using the molecular clock model (Figure 3.6b). Multiple probable transmission events can be seen on the tree with the appearance and disappearance of strains acquired and re-acquired from each partner. TC2 illustrates the same phenomenon, and PBMC samples were also available for analysis from this couple. A large amount of genetic diversity is apparent within both patients, some of which is specific to the PBMC compartment of each patient.

Within the genotype 1a region, it is apparent that PBMC specific strains in subject 9 have resulted in infection of the plasma and subsequently the PBMCs of patient 45. A similar phenomenon is seen within genotype 4 with transfer of infection from the PBMCs of patient 9 to the PBMCs of patient 45. This appearance is strongly suggestive that PBMC-specific strains of HCV exist and may act as a reservoir for future recrudescence of virus; a phenomenon that has been reported previously (Maggi et al, 1997; Roque Alfonso, 1999).

3.8.2 Compartmentalisation

We next aimed to investigate whether compartmentalisation of HCV occurred within PBMCs and the liver. Interestingly, this was the case in 61% of patients and correlated with outcome ($p=0.01$); None of the SC patients had HCV within PBMCs, while plateau progressors exhibited 100% positivity. FV patients had 60% infection of PBMCs. Initially, we considered that this could be secondary to passive adsorption of virus from the plasma and that the presence of HCV within PBMCs could simply reflect higher viral load within each outcome group. As predicted, viral load and the presence of HCV within PBMCs did correlate ($p=0.02$). However, when PBMCs were examined in SC and FV patients following the first VL<50 IU/ml in plasma after infection, 100% of FV patients had HCV within their PBMCs, while 0% of spontaneous clearers had virus present ($p=0.02$). Thus, compartmentalisation of HCV in PBMCs appears to be a significant occurrence in HIV positive MSM and the presence of HCV within PBMCs could help to predict the outcome of acute infection. Furthermore, flow cytometry revealed that IFNγ expression in HCV+ PBMCs was virtually undetectable when compared with HCV- PBMCs ($p=0.002$). This effect was seen most strongly in CD4+

cells but also significantly affected the CD8+ response to HCV. It appeared to be a general effect, as the CD4+IFNγ response to PMA/ionomycin was also low in HCV+ PBMCs.

As well as examining PBMCs, we aimed also to investigate the role of the liver as a compartment. As liver biopsies are not carried out routinely during acute HCV infection, only one patient had a liver biopsy available for retrospective analysis. RNA was retrospectively extracted from a paraffin-embedded section obtained during early HCV infection and compared with sequences derived from blood. At the time of the biopsy, the plasma HCV VL was undetectable (<12IU/ml). However, the liver contained virus which was closely related to the original infection prior to transient aviraemia. Subsequently, the patient developed severe CD4+ cell deficiency secondary to HIV and the HCV infection re-emerged. Thus the quality of the CD4 response is highly likely to be central to the control of HCV.

3.8.3 Superinfection

While it initially appeared likely that the above patient had superinfection with HCV, the likelihood is that he developed a recrudescent viraemia due to loss of CD4 control. Many patients, however, had evidence of the introduction of new strains of HCV after their initial infection. This initial conclusion, described in Chapter 2, has some limitations. Clonal sequencing was limited to the analysis of only 20 variant strains at a time – a tiny fraction of the actual circulating virus population. A strain-specific PCR was therefore designed to improve the sensitivity of detection of minority variants possibly present at the first point of infection. Interestingly, although the majority of patients initially identified as being

superinfected had no evidence of dual infection at the outset, a significant minority (36%) had evidence of a small population which later became dominant in the plasma. Thus what appears to be superinfection following the detection of divergent viral strains may not be, either as a result of lack of detection by clonal analysis or compartmentalisation within the PBMCs or liver.

3.8.4 Adaptive immunity

We originally hypothesised that adaptive immunity would define viral diversification and the final outcome of infection. To investigate this, a number of computational predictive experiments were carried out. Firstly, the codon-based Z test was used to compare sequence pairs. This revealed strong evidence of both purifying and positive selection pressures operating within each patient over time. We next applied Fu and Li's neutrality test using DNAsp v5 software. This revealed that the null hypothesis of neutral evolution was invalid when applied to sequences obtained from 10 representative patients from the cohort (424 sequences from subjects 1-10). A statistically significant negative d score at multiple time points in progressor patients, but not SC patients was highly suggestive that positive selection pressure was operating during the evolution of viral chronicity. A sliding window analysis provided some insight into which parts of the E1/E2 fragment were being targeted. In order to investigate this further, patient sequences from 50 subjects within the cohort were analysed using CODEML and HyPhy in order to detect evidence of codon-specific immune selection. A number of significantly selected epitopes were identified (Figure 3.11), many within the HVR-1, a well-recognised site of immune selection. Codon-

specific positive selection within the HVR-1 was associated with progression to chronicity (p=0.05).

3.9 Conclusions

In summary, viral diversification is significantly related to route of transmission, probably as a result of infecting dose and viral diversity. Compartmentalisation and lack of detection of minority quasispecies can result in overestimation of superinfection although this is also an extremely common event in HIV positive MSM. Based on predictive modelling, the adaptive immune response is likely to play a significant role in limiting viral diversity and is the subject of the next chapter of this thesis.

Chapter 4: Cellular immune responses to HCV in HIV-positive individuals

4.1 Introduction

4.1.1 T cells and their role in the adaptive immune response

Added together, the total mass of lymphocytes in the human body (2×10^{12} cells) make the immune system comparable in size to the liver or brain and their function is as vital in maintaining life. In the adult, between 25 and 100 million distinct naive T cell clones exist (Arstila et al, 1999). The response to foreign antigens is initiated by the innate immune system following the recognition of pathogen-associated molecular patterns (PAMPs) via toll-like receptors (TLRs) and other receptors present on antigen presenting cells such as dendritic cells. Recognition of pathogens by dendritic cells is central to the control of infection and, in part, governs the type of immune response produced by the adaptive immune system; for example, the release of interleukin-12 (IL-12) by antigen-presenting cells polarises CD4+ cells towards a Th1 profile and increases IFNγ production.

Following recognition of foreign material by cells expressing MHC class 1 (most nucleated cells) or class 2 (antigen-presenting cells), the cellular immune response is initiated. Peptides are processed within cells, bound to the MHC in the cytosol and expressed on the cell surface. T cell receptors then recognise the MHC-antigen complex and a signalling cascade is initiated. MHC class I molecules bind antigen in a longitudinal conformation within the peptide-binding cleft and present the complex to CD8+ cells. Both ends of this cleft are closed and therefore only short peptide fragments are usually expressed at the cell surface (8-10 amino acids). Longer fragments may bind, but are cleaved by exopeptidases within the endoplasmic reticulum prior to transport to the cell surface. MHC class II molecules are

different in structure to MHC I and have a longitudinal binding cleft with open ends. Thus longer peptide fragments may bind and be expressed at the cell surface (usually >13 amino acids in length). The interaction is stabilised by CD4 and CD8 which act as co-receptors and enhanced by co-stimulatory molecules such as CD28, which is present on naive T cells. After stimulation, T cells enter a process of maturation, characterised by the expression of a variety of cell surface receptors (Table 4.1).

Table 4.1: Protein expression during T cell maturation in naive, effector and memory T cells (adapted from Janeway, 7th edition, 2008).

Receptor	Naive	Effector	Memory	
CD44	+	+++	+++	Cell-adhesion molecule
CD45RO	+	+++	+++	Involved in TCR signalling
CD45RA	+++	+	+++	Involved in TCR signalling
CD62L	+++	-	+++	Lymph node homing
CCR7	+++	+/-	+++	Lymph node homing
CD69	-	+++	-	Early activation
IFN	-	+++	+++	Effector cytokine
Granzyme B	-	+++	+/-	Effector molecule in cell killing
Fasl	-	+++	+	Effector molecule in cell killing
CD122	+/-	++	++	IL-2/IL15 receptor
CD25	-	++	-	IL-2 receptor
CD127	++	-	+++	IL7 receptor
Ly6C	+	+++	+++	GPI-linked protein
CXCR4	+	+	++	CXCL12 receptor; tissue migration
CCR5	+/-	++	+++	CCL3 and CCL4 receptor; tissue migration

After stimulation, CD4+ T cells mainly secrete cytokines while CD8+ T cells act as cytotoxic killer cells. The elimination of cells infected with virus is mediated by CD8+ cytotoxic T lymphocytes (CTL). Following recognition of peptides derived from intracellular viral proteins bound to class I MHC molecules, CTLs insert perforins into the target-cell

membrane creating pores through which granzymes enter into the target cell with resultant activation of caspase enzymes and apoptosis. CD8+ cytotoxic cells can also bind Fas molecule via Fas ligand (FasL), a process that also activates caspases and results in apoptosis. In addition to killing infected cells directly, CD8+ T cells also produce cytokines, including tumour necrosis factor (TNFα) and IFNγ.

CD4+ cells act as either effector cells (Th1, Th2 and Th17 subsets) or as regulatory cells expressing FoxP3 and CD25 markers. CD4+ effector T cells are characterised by polarisation into 3 main groups based on cytokine expression profile. Th1 CD4+ cells [Mosmann et al, 1986] are associated with IFNγ production, while Th2 CD4+ cells [Romagnani, 1991] produce IL-4, IL-5, IL-9, IL-10 and IL-13. Th1 cells are formed following exposure to IL12 produced by myeloid dendritic cells (DCs) as well as IFNγ produced by NK cells and plasmacytoid DCs. Th1 responses occur in response to a variety of viral infections as well as intracellular bacteria e.g. Mycobacterium and protozoan parasites such as Leishmania. Th2 polarisation occurs in the presence of IL4, and Th2 predominant responses occur in the presence of allergic inflammatory disorders and helminth infections. Some CD4+ cells are able to produce both IFNγ and IL4 and are designated Th0 cells [Mosmann & Sad, 1996]. Another group of CD4+ cells; Th17 cells have recently been described in mice and in humans and are characterised by their ability to secrete IL-17A. IL-17A release may result in massive tissue inflammation as a result of neutrophil activation. Such cells may also secrete IL-17F, IL-21, IL-22, IL6 and TNFα. Th17 cells may share a developmental origin with Th1 cells (Annunziato et al, 2007) as both lineages produce IFNγ and express IL12 and IL-23 receptors and the transcription factor T bet. Th17 cells express the receptor CD161 (Cosmi et al, 2008) which is also expressed on NKT cells. CD161+CD4+Th17 cells may interact with a number of ligands, including lectin-like

transcript 1 (LLT1), C-type lectin domain family 2A (CLEC2A) and proliferation-induced lymphocyte-associated receptor (PILAR; Huarte et al, 2008). Th17 CD161+ cells are concentrated in the liver and the intestine and have been associated with increased evidence of liver damage in chronic hepatitis B infection (Zhang et al, 2009) and alcoholic liver disease (Yasumi et al, 2007; Lemmers et al, 2009). In the mouse model, Th17 cells may upregulate the expression of antiapoptotic molecules and could therefore increase the survival of virus-infected cells (Hou et al, 2009).

4.1.2 The adaptive immune response to HCV

Although most patients infected with HCV develop chronic infection, a significant proportion of individuals spontaneously control virus after acute infection, with the accompanying emergence of adaptive immune responses. Further studies to define these protective immune responses are critical for future vaccine development and immunotherapies. While the phenomenon of resistance to HCV is well recognised, the mechanism is not clear.

Much work on the mechanisms underlying spontaneous control of HCV has been carried out, but this has typically been based on small patient groups. The reason for this is that acute HCV virus infection rarely presents clinically, so the time-course of spontaneous clearance has not been extensively studied. However, we do know from small human (Cox et al 2005; Kantzanou et al 2003; Gruner et al 2001, Thursz et al 1999) and animal (Harcourt, 2001) studies that both CD4+ and CD8+ T cell immune responses are highly active during acute-phase disease. It is thought that such responses are central to acute control of virus, and that failure of such responses is accompanied by progression to chronic infection.

CD8+ T cell responses are of key importance in the control of HCV infection. MHC class 1 associations (particularly HLA B27) with spontaneous clearance provide evidence of this (McKiernan et al, 2004; Neumann-Haefelin et al, 2007). In the chimpanzee model, a reduction in viraemia coincides with the onset of T cell responses at weeks 6 and 8 post-infection. A rapid (10-fold) decrease in VL occurs around week 8, following the emergence of IFNγ-producing HCV-specific CD4+ and CD8+ T cells (Woollard et al, 2003). Escape from such responses by viral mutation in key CD8+ T cell epitopes is accompanied by progression to chronicity, and depletion of CD8+ T cells leads to a prolonged course of viraemia (Cooper et al 1999). In humans, acute CD8+ T cell responses may be substantial (up to 8% of total CD8+ T cells), and are activated during acute infection (Cox et al 2005), targeting a wide range of viral epitopes (Table 4.1). Many of these HCV-specific CD8+ cells express the liver homing markers CXCR6 and CD161 (Northfield et al, 2008). These CD161+ cells are made up largely of CCR7- effector memory T cells that produce IFNγ and TNFα but contain scanty amounts of cytolytic molecules (granzyme B and perforin) and proliferate poorly in vitro.

The emergence of HCV-specific CD8+ IFN+ T cells are associated with viral clearance (Grüner et al, 2000; Cox et al, 2005). A number of epitopes are recognised by CD8+ cells within the HCV structural and NS proteins (Cox et al, 2005) including a number of immunodominant epitopes such as NS3 1073-81 which is commonly recognised in individuals with the HLA A2 allele and is particularly targeted by SC patients. In prospective human studies of acute infection, immune escape has been observed in a subset of targeted epitopes (Cox et al 2005) although this is not the only way in which HCV evades the immune response, as mutations within NS3 and other regions often do not occur, despite resurgence of viraemia (Kantzanou et al, 2003). The necessity of a sustained CD8+ response is key in self-limiting

infection. Many patients who eventually progress to chronicity exhibit an initially robust but non-sustained T cell response in the absence of immune escape with eventual downregulation of markers of activation such as CD38 (Lechner et al, 2000). The mechanism for this is not yet fully understood. T cells extracted from patients with acute HCV evolving towards chronicity commonly express the negative T cell regulator and exhaustion marker PD-1, and blockade of PD-1 or its receptor PD-L1 is associated with the restoration of functional T cell responses both in HIV infection (Day et al, 2006) and in acute HCV infection (Urbani et al, 2008). However, while restoration of T cell responses occurs in HCV-infected patients following blockade of PD-1 and PDL1, high levels of PD-1 expression have been observed to occur early in acute infection during both resolved and chronic stages of infection, irrespective of clinical outcome (Kasprowicz et al, 2008). Thus analysis of PD-1 expression alone is not sufficient to predict infection outcome.

Once chronicity is established, while CD8+ T cell responses are weak or undetectable by standard screening methods, they can still be detected using enhanced tetramer staining and magnetic bead enrichment (Komatsu, 2003; Barnes et al, 2004). Cells present in chronic infection function poorly and are of an immature phenotype, having failed to express markers of T cell maturation, such as perforin (Gruener et al 2001). In contrast, CD8+ T cell responses in those who have spontaneously resolved infection are readily detectable, and may target a broad range of antigens (Cooper et al 1999; Cox et al 2005). Escape from such responses can occur, and may be central to progression to chronicity (Timm et al, 2004; Cox et al, 2005; Guglietta et al , 2005). Polyfunctional CD8+ T cell responses (characterised by both IFNγ and IL2 secretion) have been observed to correlate with an increased likelihood of

spontaneous clearance of HCV and may be restored after early treatment with IFNα and ribavirin (Badr et al, 2008) although this has not yet been confirmed in a large study.

In addition to CD8+ T cell responses, CD4+ T cell responses are important in resolution of infection (Thursz et al 1999; Harcourt et al 2001; Zajac et al 1998). Indeed, strong multispecific CD8+ responses in isolation may not be sufficient to control HCV (Lauer et al, 2005). Longitudinal and cross-sectional studies of immune responses, as well as genetic evidence, support the key role of CD4+ cells in the control of infection. HLA types DRB1*0101, DR1B1*1101 (OR=2.14) and DQB1*0301 (OR=2.22) are associated with spontaneous resolution of acute HCV (Thursz et al, 1999) and an improved immune response (Harcourt, 2001) while HLA-DRB1*0701 is associated with viral persistence (Fanning et al, 2001).

In chimpanzees, depletion of CD4+ T cells in immune animals results in persistent infection following re-exposure to HCV (Grakoui et al, 2003). CD4+ T cell responses in humans predominantly target a variety of regions within core and the non-structural genes (Appendix 3). Targeting of NS proteins has been associated with spontaneous clearance in HIV-negative individuals, while responses to core are commoner in chronically evolving infection (Gerlach et al, 2005; Aberle et al, 2006; Ruys et al, 2008). The same may be the case in HIV-positive patients; in a small study of HIV-positive individuals (n=4), individuals with resolved infection or temporary suppression of HCV-RNA had HCV-specific CD4+ T cell responses directed predominantly against the NS proteins while an individual with high HCV RNA plasma concentrations had CD4+ T cell responses predominantly directed against core (van den Berg, 2009). Thus CD4+ responses to core may not be protective. HCV infection is

associated with an increase in core-binding protein (gC1qR) expression on CD4+ T cells, which may increase the susceptibility to core-mediated immune dysregulation and facilitate the establishment of HCV persistence (Cummings et al, 2009). Further characterisation of the epitopes targeted by CD4+ cells has yet to be carried out. Particular conserved epitopes have been suggested as protective targets (Appendix 3), but the true nature of any single protective epitope has yet to be confirmed in larger studies. This has been in part because CD4+ T cell responses are difficult to detect during chronic infection and cohorts of acutely infected individuals have been limited in size. A recent study investigated the role of specific mutations in core protein in association with CD4+ responses. Viral sequence analysis revealed sequence mutation in 2 of 21 individuals and in both of these cases, the circulating sequence variant was poorly recognised by host CD4+ T cells (Fleming et al, 2010). IFNγ responses were present, however, in the majority of donors, often targeting multiple epitopes, and a new immunodominant peptide in core was identified. Although the NS proteins might be a better region to examine for immune escape, responses to these proteins are often only weakly detectable.

It is likely that CD4+ cells have an effect both directly and indirectly by providing support for CD8+ T cells. In animal models, CD4+ T cell help is critical in maintaining long term protective CD8+ T cell immunity (Zajac et al 1998). This appears to be the case also in humans with maturation of CD8+ responses following on from a protective strong, broad CD4+ response (Urbani et al, 2006). All patients (n=16) in this study were found to have weak and narrow CD8+ responses at the onset of infection while CD4 responses were found to be weak and narrow in patients with a chronic outcome but strong and broad with a Th1 type profile in resolving infection. This was shown to be followed by a progressive expansion

and maturation of CD8+ cells to a CD127+ memory phenotype in SC patients but not those progressing to chronicity. Thus CD4+ responses may contribute to the control of HCV by promoting maturation of protective memory CD8+ responses. The IL7 receptor CD127 is required for the establishment and maintenance of memory CD4+ and CD8+ T lymphocytes, and cells lacking it demonstrate impaired IL-2 secretion and have a short life-span (Golden-Mason et al, 2006). Thus, evolution to chronic HCV is characterized by T cells that are functionally impaired and exhibit an immature phenotype.

Both IFNγ and IL-2 production are associated with self-limiting infection and the priming of CD8+ T cells following the production of IL-2 by CD4+ cells in response to HCV may influence outcome in acute disease (Smyk-Pearson et al, 2008). During chronic infection, some HCV specific CD4+ populations are maintained but that they are low in both IL-2 production and proliferative capacity (Semmo et al, 2005, Semmo et al, 2007). This profile is similar to that seen in CD4+ T cell responses to HIV (Day & Walker, 2003). In one study, the lack of sustained cell mediated immunity which had been observed during early infection (10% of chronically infected individuals compared with 70% of subjects who had cleared HCV) was accounted for by an impaired ability to proliferate that could be rescued in vitro by concomitant exposure to IL-2 (Folgori et al, 2006).

Regulatory CD4+ T cells (FoxP3+CD25+) are present in higher numbers in patients with chronic HCV than those with acute HCV. However, during acute HCV infection, while maintenance of the CD4+ anti-HCV response is key to clearance of infection (Gerlach et al, 1999), there is no evidence of a clear relationship between the presence of HCV-specific regulatory T cells and the evolution of self-limiting or chronic infection (Heeg et al, 2009).

While a Th1 type CD4+ response is associated with spontaneous clearance, it may also be associated with increased levels of fibrosis during chronic infection. HCV-specific Th2 type T cell responses with increased IL-10 expression may play a dual role in HCV infection, dampening effector T-cells to promote viral persistence in acute infection but also protecting against progressive fibrosis in chronic disease (Kaplan et al, 2008). Administration of IL-10 to HCV-infected patients results in improved necroinflammatory and fibrosis scores, but is associated also with an increase in HCV VL (Nelson et al, 2003).

The role of CD4+ T cells is likely to be of particular relevance in patients who are HIV-infected. In a study of acute HCV with and without HIV coinfection, significantly reduced CD4+ IFNγ responses were observed in HIV positive patients (n=14) versus those in HCV-monoinfected individuals (n=8), especially those directed against NS3-5 (1/10 vs. 5/8; p = 0.008; Danta et al, 2008). This could explain in part the reduced rate of spontaneous clearance observed in cohorts of HIV-positive individuals with acute HCV.

While providing important insights into the pathogenesis of HCV infection and how the immune system may successfully counter infection, there are a number of limitations to many of the above studies. Firstly, the cross-sectional design of the majority of these studies mean that the timing of immune responses has not been fully explored, particularly observations in differences in immune response prior to changes in VL. Furthermore, the preclinical phase is under-researched due to lack of presentation. The assessment of T cell responses in blood may not reflect the situation in the liver which is likely to be the main site of immunological attack. These drawbacks do not apply to the animal model of disease, although extensive studies in chimpanzees have not been carried out because of ethical

concerns. We do know however, that in the chimpanzee model, while intrahepatic responses have been observed to correlate most strongly with outcome (Thimme et al, 2001), responses in the blood do reflect those seen in the liver (Shin et al, 2008). Another problem in many studies is that of superinfection which is common in some cohorts (Chapter 2; Herring et al, 2004). If T cell responses are assessed against peptides from a historical infection e.g. against genotype 1 with a dominant genotype 3 superinfection, then the true breadth and vigour of responses may be missed (Harcourt et al, 2003).

In summary, both CD4+ and CD8+ responses are important in resolution of acute HCV infection. CD8+ responses are largely associated with responses directed against a broad range of viral epitopes. CD4+ responses with a Th1 type cytokine profile directed against the NS proteins are protective but must be sustained to achieve clearance of HCV.

The role of innate immune responses in directing an appropriate immune response is still an area of uncertainty. Dendritic cells may direct the course of T cell response towards a Th1 or Th2 profile and it has been suggested that inhibition of IL-12 by HCV may favour a Th2 response with resultant persistence of infection (Bain et al, 2001; Fowler et al, 2003).

The role of antibody responses in the control of HCV is also poorly understood as the neutralising capacity of such responses has been difficult to define. Viral evolution in envelope genes may lead to rapid generation of escape mutants (Farci *et al* 2000) and absence of antibody response may result in a less varied population of HCV quasispecies and persistence of infection (Gaud *et al* 2003). In the chimpanzee model, antibodies limit viraemia (Farci et al, 1996). However, the presence of neutralising antibodies is not likely to

be central to acute HCV resolution; HCV pseudoparticle-specific neutralising antibodies are readily detected *in vitro* in patients with chronic evolution and impaired virus-specific CD4+ T-cell responses but not in patients who clear infection with a robust virus-specific CD4+ T-cell response (Kaplan et al, 2007). Interestingly, after long term clearance of virus, antibody responses to HCV may be lost, but cellular immune responses maintained (Takaki *et al* 2004).

4.1.3 The adaptive immune response to HIV-1 and 2

Untreated, HIV-1 and 2 infections result in a profound depletion of CD4+ cells, leading to severe immunodeficiency and death as a result of opportunistic infection or malignancy. Paradoxically, during this time, strong CD8+ cytotoxic T cell responses evolve and are clearly involved in the control of HIV VL. In monkeys with experimental simian immunodeficiency virus (SIV) infection, depletion of CD8+ cells results in higher levels of HIV VL. In humans, the role of CD8+ cells is clearly implicated by the strong association of MHC class 1 alleles with limited and progressive disease. HLA B57 and B27 are over-represented in elite controller and long-term non-progressor (LTNP) groups (B57 in around 50%; B27 in around 20%; Walker et al, 2007). In particular, HLA type B5701 is strongly associated with long-term non-progression (Migueles et al, 2000). HLA B35, in contrast, is associated with more rapid progression toward AIDS (Jin et al, 2002).

Following initial infection with HIV and a peak in HIV VL, the acquired immune response results in a reduction in VL and plateau viraemia phase. During acute HIV infection, the time at which the peak VL descends correlates with the point of peak CD8+ T cell response

(Wilson et al, 2000). The final “set-point” or VL level at which this plateau phase settles relates directly to the rapidity of progression to AIDS. Some patients are able to control infection for many years with very low or undetectable levels of HIV and are known as LTNPs (HIV VL<2-5000 copies/ml) or “elite controllers” (<50 copies/ml). A number of mechanisms may be responsible in such individuals, but the CD8+ response appears to be particularly important with more than 10% of circulating CD8+ cells in individual patients targeted against HIV epitopes during the acute phase of infection (reviewed in McMichael & Rowland-Jones, 2001). Unlike the situation in HCV infection, it seems that CD8+ responses in HIV remain highly activated and are of a more mature phenotype (although effector function may still be defective). While the breadth and strength of CD8+ responses as measured by ELISpot do not show a clear relationship with viral load or clinical presentation, targeting of certain regions of the virus and the production of multiple cytokines do correlate with outcome.

Many epitopes are recognised during HIV infection. However, preferential targeting of gag is associated with viral control, while decoy targeting of HIV envelope is associated with a higher VL (Kiepiela et al, 2007; Peut & Kent, 2007). HIV-specific CD8+ cells may lyse CD4+ cells by perforin and granzyme release and CD4+ cells expressing gag protein are particularly susceptible to cell lysis. CD4+ responses to gag also correlate with outcome, with higher levels of IFNγ (Lo-Caputo et al, 2003) and IL-2 (Eyeson et al, 2003) expression in LTNPs. Lower levels of IL-10 expression is associated with a protective response (Clerici et al, 1996). Thus Th1 responses appear to be more protective than Th2.

Another correlate of protective immunity in HIV infection is T cell polyfunctionality. Dual production of both IL2 and IFNγ by CD8+ T cells is associated with reduced viral load in elite controllers (Zimmerli et al, 2005). Furthermore, the production of IFNγ, MIP-1β, IL-2 and TNF-α production as well as the expression of CD107a (which is a marker of CD8+ degranulation) correlate even more strongly with viral control (Betts et al, 2006). Production of IL2 by this population of CD8+ cells may result in T cell proliferation in the absence of CD4+ T cell help, likely to be of particular significance in HIV-infected individuals. Polyfunctional CD8+ T cell responses occur with increased antigen sensitivity (measured by calculating the activation threshold in response to defined concentrations of exogenous peptide; Almeida et al, 2009).

4.1.4 Cellular immune responses during co-infection with HCV and HIV

As described above, the role of a persistent CD4+ response to acute HCV infection is strongly linked to resolution of infection. This might be expected to be particularly important in individuals also infected with HIV. In patients with chronic HCV, both the prevalence and breadth of CD4+ and CD8+ responses are lower in co-infected patients (Capa et al, 2007). A recent study of 38 HIV-positive patients with acute HCV found that rare spontaneous clearance (n=5) correlated with weak but significant CD4 T-cell responses (Schnuriger A et al, 2009). Proliferative responses were observed in 35% of cases and were associated with a lower HCV VL ($p = 0.04$) and spontaneous clearance ($p = 0.02$). Anti-NS4 responses were found to be particularly prominent in SC patients ($p = 0.03$). Interestingly, although IFNγ-producing T cells were detectable *ex vivo* in 81% of cases, they were found to be independent of HCV outcome in this study.

CD4+ cell dysfunction may also impact on CD8+ responses during HIV infection, both in chimpanzees and humans. In chimpanzees, depletion of CD4+ cells results in impairment of CD8+ responses and immune escape (Grakoui et al, 2003). In human, a cross-sectional analysis of HCV-specific CD8+ cells responses during chronic HCV and HIV infection revealed that HIV-related CD4 cell depletion was associated with significantly lower HCV-specific CD8+ cells ($p<0.0001$; Kim et al, 2005).

While the immune response to HCV is clearly affected by the presence of HIV infection, the immune response to HIV may also be altered. In one study, HLA A2-restricted responses to the HIV-immunodominant gag epitope SLYNVATL were different in HCV-infected children and adolescents with haemophilia with a shift towards a more immature phenotype, more typical of HCV and a reduction in perforin and the senescent cell marker CD57 characteristic of HIV (Harcourt et al, 2005).

In this study, we aimed to characterise the T cell response in a large cohort of HIV-infected patients with acute HCV infection to see if associations could be made with the type of response and the likelihood of spontaneous clearance.

4.2 Hypotheses

The early detection of strong multispecific CD4+ and CD8+ T cell responses determines outcome in acute HCV in HIV positive individuals

4.2.1 Spontaneous clearance is associated with a strong multispecific (broad) T cell response

4.2.2 Plateau viraemia is associated with a weak and narrow T cell response

4.2.3 Fluctuating viraemia in patients without evidence of superinfection may be due to variation in T cell function over time

4.2.4 Use of intravenous methamphetamine may impair the cell-mediated response to acute HCV

4.3 Aims

4.3.1 To characterise T cell responses in this cohort using IFNγ detection by ELISpot and multichannel flow cytometry in spontaneous clearance, plateau viraemia and fluctuating viraemia

4.3.2 To assess the effect of IV methamphetamine use on T cell function using by ELISpot and multichannel flow cytometry

4.4 Methods

4.4.1 Separation of peripheral blood mononuclear cells (PBMCs)

Blood samples were obtained using vacutainer tubes containing ethylenediamine tetraacetic acid (EDTA). Each sample was added slowly to a 50 ml tube containing 7.5 ml of Ficoll-Paque solution and centrifuged at 800g for 20 minutes with no brake. The white blood cell fraction was transferred to a new 50 ml tube using a sterile Pasteur pipette, made up to 15ml using phosphate buffered saline (PBS) and centrifuged for 5 minutes at 400g. The supernatant was discarded and the pellet resuspended in 5ml of PBS. 100µl trypan blue dye (0.5%) was added to 100µl of solution and the cells were counted by direct microscopy. A further centrifugation step was carried out for 5 minutes at 400g. The cells were then resuspended in heat-inactivated foetal calf serum (FCS) at a concentration of 5 million cells/ml FCS. A 20% solution of dimethyl sulphoxide (DMSO) in Roswell Park Memorial Institute (RPMI) medium was prepared and 1ml added to each ml of resuspended cells. The PBMCs were placed in 2ml NUNC tubes, insulated, frozen overnight at -80^{0}C and then stored in liquid nitrogen.

4.4.2 Thawing of PBMCs

Thawing medium (TM) was made by diluting CTL Wash supplement medium 1:10 in RPMI, with 1:100 L-glutamine and 50U/ml Benzonase. DNAse was added at 1:100. TM and R10 medium [RPMI 1640 (Sigma-Aldrich), 10% foetal calf serum (Sigma-Aldrich), and 10 mM HEPES buffer (Sigma-Aldrich)] were prewarmed in a 37^{o}C (+/- 1^{o}C) waterbath. Cells were thawed quickly with gentle agitation in a 37^{o}C (+/- 1^{o}C) water bath and transferred using a sterile 1 ml pipette to a 50ml tube. TM was added to each tube drop wise in 1-2 minutes of

dispensing/mixing cycles diluting to 10x the original volume. Cells were then centrifuged at 18-20^{o}C at 250g for 10 minutes. The supernatant was aspirated, the cells resuspended in 1ml RC lysis buffer (Gibco) with 4 ml/thawed vial of R10 medium and transferred to a 37°C incubator in a 5% CO_2 atmosphere overnight. Cell viability was assessed using flow cytometry (Guava) and cell numbers included in each assay were determined as the total number of live cells (range 70-96%).

4.4.3 HLA typing

Genomic DNA was extracted from 200µl whole blood or 1x10^{6} PBMCs using the QIAamp DNA Blood Mini Kit (Qiagen) and the concentration calculated using a nanodrop machine or spectrophotometer. HLA typing was then carried out at the Anthony Nolan Institute, Royal Free Hospital.

4.4.4 Ex-vivo IFNγ ELISpot assay

Polyvinylidene plates (96-well; Millipore) were coated with 100µl 1:200 dilution of mouse anti-human gamma interferon (IFNγ) antibody (MAbTech) in sterile phosphate-buffered saline at 4°C overnight. Thawed PBMCs were added at 200,000 live cells/well in 100µl of R10 medium containing 2mM glutamine and 50U of penicillin-streptomycin/ml. Samples obtained from 5 patients were analysed using 100,000 live cells/well due to limited availability of cells. Genotype-specific peptides (genotypes 1a, 1b and 3a) were added directly to the wells at a final concentration of 6µg/ml (Table 4.2). Genotype 1b peptides were used for patients with genotype 4d infection. The plates were incubated for 18 hours at 37°C in 5% CO2. Plates were then washed, labelled with a 1:2000 dilution of biotinylated mouse anti-human IFNγ monoclonal antibody (MAbTech), and developed by incubation with alkaline phosphatase-conjugated anti-biotin monoclonal antibody (Vector Laboratories), followed by incubation with 5-bromo- 4-chloro-indolylphosphate (BCIP)–

nitroblue tetrazolium (NBT) (Pierce) in Tris buffer (pH 9.5). The reaction was stopped by washing with tap water, and the plates were dried overnight, prior to counting. The numbers of spots per well were counted using an automated Elispot plate reader (AID, Germany) and results expressed as spot-forming cells (SFCs)/million PBMCs after subtracting the negative well from test well values. All plates were read using the same calibration settings. If the negative control (DMSO) background was greater than 55 spot-forming cells (SFC)/10^6 PBMC, the sample was retested. Responses were considered positive if the number of spots per well minus the background was at least 25 SFC/10^6 PBMC according to an established laboratory protocol at the University of Oxford ; concavalin A stimulated cells served as a positive control.

Table 4.2: Peptide pools

a) Genotype 1a peptides

Peptide Pool	Abbreviation	a.a. range	Peptide at Start of Pool	Peptide at End of Pool
HCV H77 Peptide Pool A	Core	1-191	MSTNPKPQRKTKRNTNRR	LALLSCLTVPASA
HCV H77 Peptide Pool B	E1	192-383	YQVRNSSGLYHVTNDCPN	WAKVLVVLLLFAGVDA
HCV H77 Peptide Pool C	E2	384-746	ETHVTGGNAGRTTAGLV	CLWMMLLISQAEA
HCV H77 Peptide Pool D	p7 + NS2	747-1026	ALENLVILNAASLAGTH	PADGMVSKGWRLL
HCV H77 Peptide Pool E	*NS3p*	1027-1353	APITAYAQQTRGLLGCII	AETAGARLVVLATATPPG
HCV H77 Peptide Pool F	*NS3h*	1343-1657	LVVLATATPPGSVTVSH	ITKYIMTCMSADLEVVT
HCV H77 Peptide Pool G	*NS4*	1658-1972	STWVLVGGVLAALAAYCL	HQWISSECTTPC
HCV H77 Peptide Pool H	*NS5A*	1973-2420	SGSWLRDIWDWICEVL	VSSGADTEDVVCC
HCV H77 Peptide Pool I	*NS5BI*	2421-2718	SMSYSWTGALVTPCAA	ASGVLTTSCGNTLTCYIK
HCV H77 Peptide Pool J	*NS5BII*	2708-3011	SCGNTLTCYIKARAACRA	LLAAGVGIYLLPNR

b) Genotype 1b peptides

Peptide Pool	Abbreviation	a.a. range of pool	Peptide at Start of Pool	Peptide at End of Pool
HCV J4 Peptide Pool A	Core	1-191	MSTNPKPQRKTKRNTNRR	LALLSCLTIPASA
HCV J4 Peptide Pool B	E1	192-383	YEVRNVSGIYHVTNDCSN	WAKVLIVALLFAGVDG
HCV J4 Peptide Pool C	E2	384-746	ETHTTGRVAGHTTSGF	RVCACLWMMLLIAQAEA
HCV J4 Peptide Pool D	p7 + NS2	747-1026	ALENLVVLNAASVAGAH	ADSLEGQGWRLL
HCV J4 Peptide Pool F	*NS3p*	1027-1353	APITAYSQQTRGVLGCII	AETAGARLVVLATATPPG
HCV J4 Peptide Pool G	*NS3h*	1343-1657	LVVLATATPPGSVTVPH	ITKYIMACMSADLEVVT
HCV J4 Peptide Pool H	*NS4*	1658-1972	STWVLVGGVLAALAAYCL	HQWINEDCSTPC
HCV J4 Peptide Pool I	*NS5A*	1973-2419	SGSWLRDVWDWICTVL	TVSEEASEDVVCC
HCV J4 Peptide Pool L	*NS5BI*	2420-2717	SMSYTWTGALITPCAA	ASGVLTTSCGNTLTCYLK
HCV J4 Peptide Pool M	*NS5BII*	2707-3010	SCGNTLTCYLKATAACRA	LLSVGVGIYLLPNR

c)

d) Genotype 3a peptides

Peptide Pool	Abbreviation	a.a. Range of pool	Peptide at Start of Pool	Peptide at End of Pool
HCV NS3 Peptide Pool A	Core	1-191	MSTLPKPQRKTKRNTIRR	LALFSCLIHPAAS
HCV NS3 Peptide Pool B	E1	192-383	LEWRNTSGLYVLTNDCSN	WAKVAIIMVMFSGVDA
HCV NS3 Peptide Pool C	E2	384-752	STHVTAGQAARNAYGI	ARVCVALWLILTISQAEA
HCV NS3 Peptide Pool D	p7 + NS2	753-1032	ALENLVTLNAVAAAGTH	ADDYREMGWRLL
HCV NS3 Peptide Pool F	*NS3p*	1033-1359	APITAYAQQTRGLLGTIV	AETAGVRLTVLATATPPG
HCV NS3 Peptide Pool G	*NS3h*	1349-1663	LTVLATATPPGSITVPH	ITKYVMACMSADLEVTT
HCV NS4 Peptide Pool H	*NS4*	1664-1978	STWVLLGGVLAAVAAYCL	RRLHQWINEDYPSPC
HCV NS5a Peptide Pool I	*NS5A*	1979-2430	SGDWLRIIWDWVCSVV	TVSDSEEQSVVCC
HCV NS5b Peptide Pool L	*NS5BI*	2431-2726	SMSYSWTGALITPCSA	CRASGVLPTSFGNTITCY
HCV NS5b Peptide Pool M	*NS5BII*	2716-3021	PTSFGNTITCYIKATAAA	LLTVGVGIFLLPAR

4.4.5 Flow cytometry

PBMCs were thawed and 100µl containing $1x10^6$ live cells added to a 96 well plate and left in an incubator for 2-4 hours prior to stimulation. Peptides formed 3 antigen pools (corresponding to combined pools used for ELISpot analysis) as shown in Table 4.3.

Table 4.3: Peptide pools used for flow cytometry

	Stocks	Final conc.
P1	Pools ABCD Core, E1, E2, p7, NS2 (0.3mg/ml)	1ug/ml
P2	Pools EFG NS3HP, NS4AB (0.3mg/ml)	1ug/ml
P3	Pools HIJ NS5A, NS5BI and II (0.3mg/ml)	1ug/ml

Cells to which 1µl DMSO or 1µl PMA and ionomyosin were added instead of peptide pools served as negative and positive controls respectively. The samples were then incubated for 2 hours at 37°C. 2µl of brefeldin A was next added at a concentration of 10ug/ml and the samples were incubated overnight at 37°C. The next day, 100µl PBS was added to each well with a multichannel pipette and the plates were spun at 1500rpm for 5 minutes. The supernatant was discarded and 50 µl of a mastermix containing 1:50 live/dead dye, 1:50 CD19-Pacific Blue (Invitrogen) and 1:100 CD14-Pacific Blue (Invitrogen) antibodies was added and incubated for 20-30min in the dark at room temperature. CD14, CD19 and live dead dye were used to reduce the likelihood of non-specific background staining. 150µl PBS

was next added to wash the cells and the plates spun at 1500rpm for 5minutes. The supernatant was discarded and the cells incubated in 100 µl 1% FACS fix (1ml formaldehyde + 36ml PBS) for 20 minutes. 100µl of Foxp3 Perm buffer was added followed by centrifugation at 1600rpm for 5 minutes and the supernatant discarded. The cells were then incubated in 200µl of a 1:10 dilution of FoxP3 Perm buffer for 20 minutes. The cells were spun at 1600rpm for 5minutes and the supernatant discarded, then resuspended in an antibody cocktail made in FoxP3 Perm buffer (50µl per well; Table 4.4) and incubated for 30 minutes.

Table 4.4: Antibodies used for flow cytometry

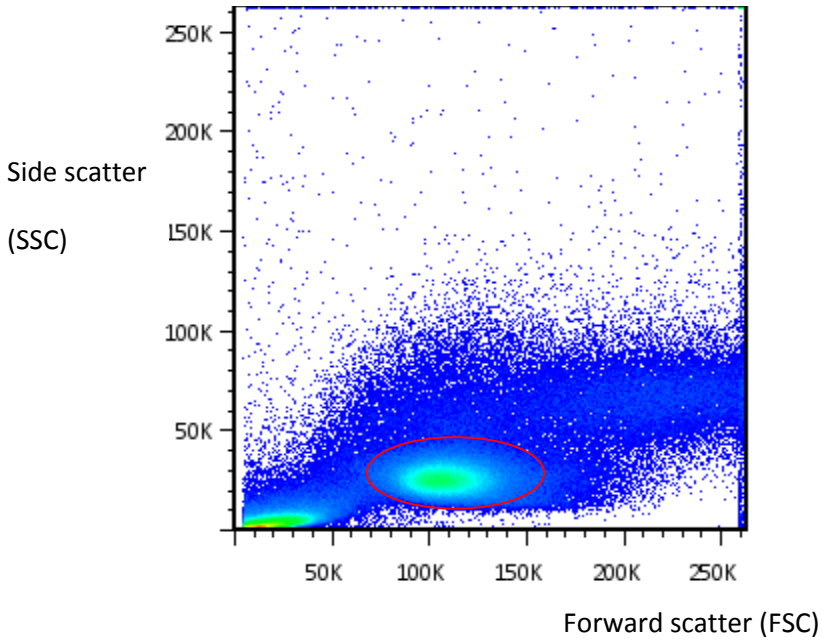
Antibody	Manufacturer	Concentration
Anti-IL-17-PE	eBiosciences	1:25 IL-17-PE
Anti-Human CD8 PerCP-Cy5.5	BD	1:100 CD8-PerCP Cy5.5
Anti-Human IFNγ Alx700	BD	1:50 IFNg-AlexaFluor700
Anti-IL-2-APC	BD	1:25 IL-2-APC
Anti-human TNFα	BD	1:25 TNFa-PE-Cy7
Anti-CD3 Pacific Orange	Invitrogen	1:50 CD3-Pacific Orange
Anti-human CD4 QDot605	Invitrogen	1:200 CD4-Qdot 605

The cells were next washed in 150µl of buffer and 200µl of PBS, centrifuged at 1600rpm for 5 minutes and resuspended in 200µl PBS. Cells were analysed using an LSRII flow cytometer. Prior to analysis of samples, compensation controls were set up on ice with 4 µl of each antibody using compensation beads and a compensation matrix set up using FACS Diva software. A negative control sample was used to adjust voltage thresholds for forward and side scatter and each antibody. Gates were created to select for lymphocytes, single cells,

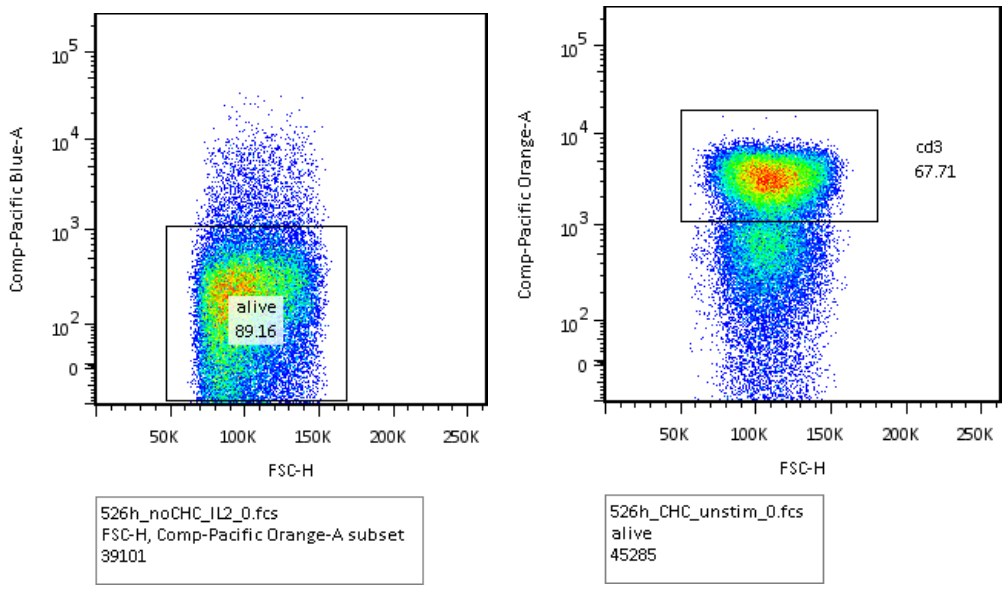
live CD3 positive cells and then CD4/CD8, IFN, TNF, IL-2 and IL-17 (Figure 4.1). Lymphocyte gates were adjusted as necessary to include the appropriate cells for each sample, but all other gates remained unchanged. Data was analysed using FlowJo v7.5 software (Tree Star).

Figure 4.1 Flow cytometry analysis

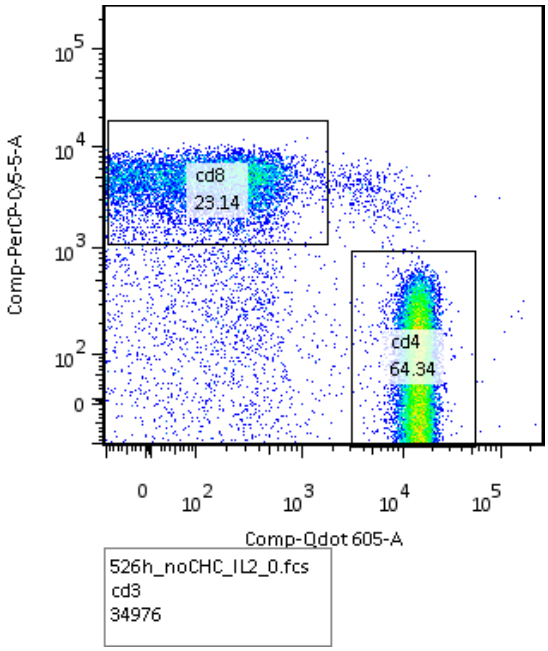
a. FSC vs SSC plot for PBMCs



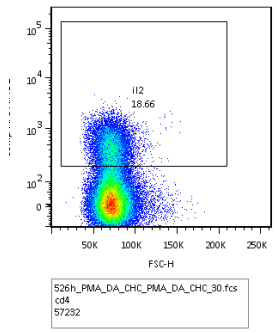
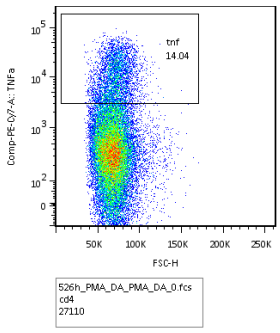
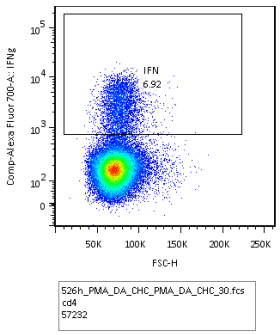
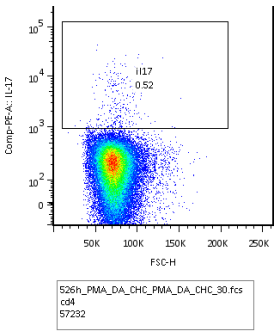
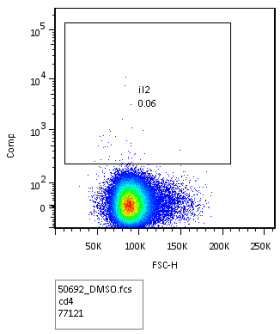
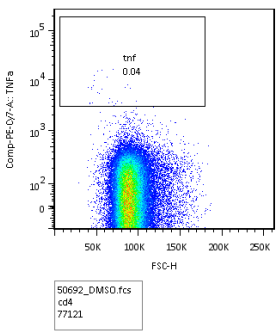
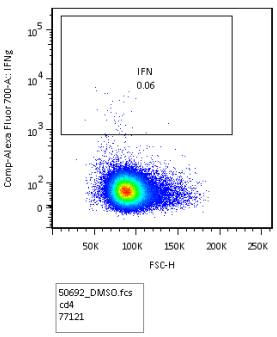
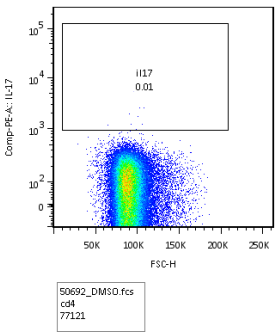
b. Gates used to select for live CD3+ T cells



c. Selection of CD4+ and CD8+ populations

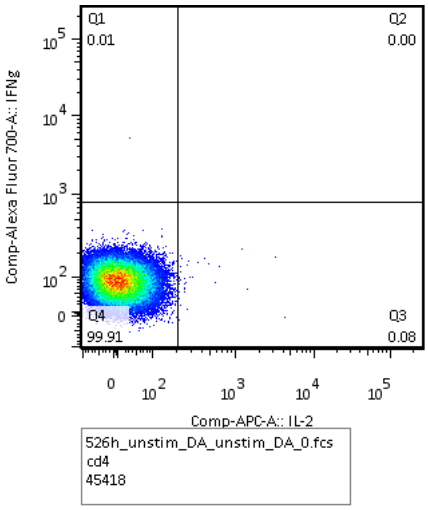


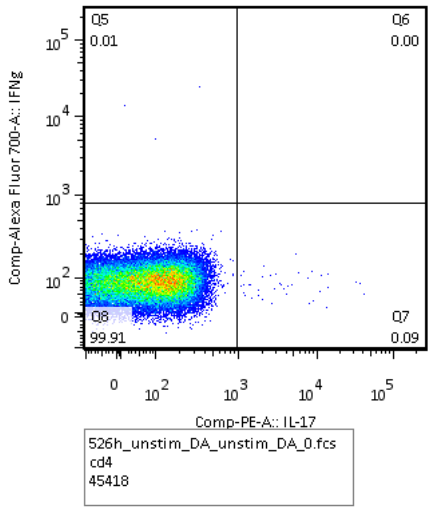
d. Single cytokine stains for IL2, TNFα, IFNγ and IL17 in CD4+ cells

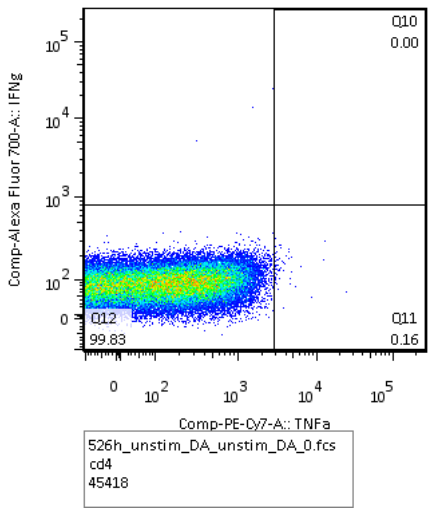
	IL2	TNFα	IFNγ	IL17
Positive control				
Negative control				

e. Dual cytokine gates for IFN, IL2, IL17, and TNF in CD4+ cells

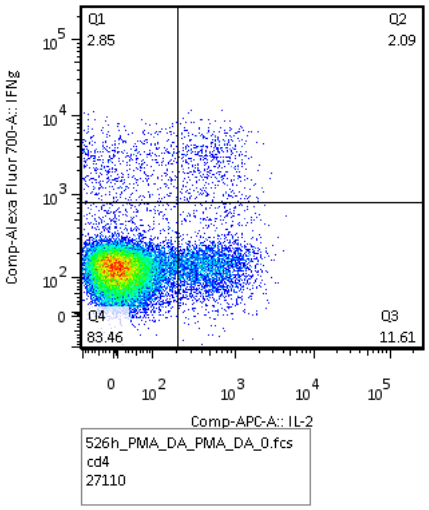
Negative control

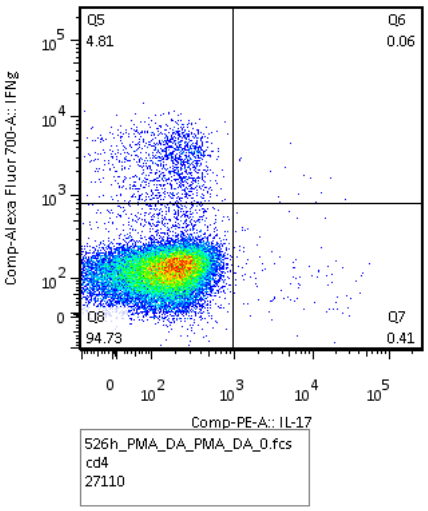






Positive control





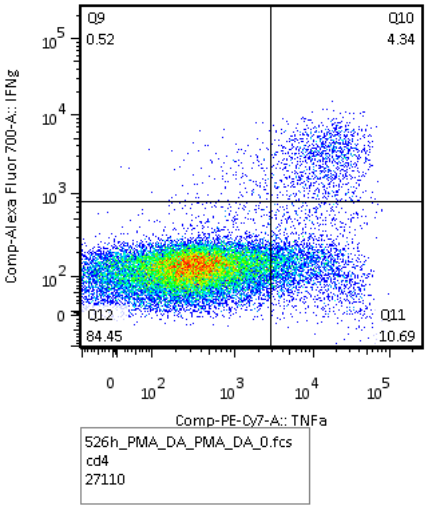


Figure 4.1 Flow cytometry analysis a) FSC vs SSC plot for PBMCs The lymphocyte population was selected as illustrated **b) Gates used to select for live CD3+ T cells c) Selection of CD4+ and CD8+ populations d) Single cytokine stains for IL2, TNFα, IFNγ and IL17 in CD4+** The same gates were used in the analysis of CD8+ cells **e) Dual cytokine gates for IFN, IL2, IL17, and TNF in CD4+ cells** The same gates were used in the analysis of CD8+ cells

4.5 Results

4.5.1 HLA typing

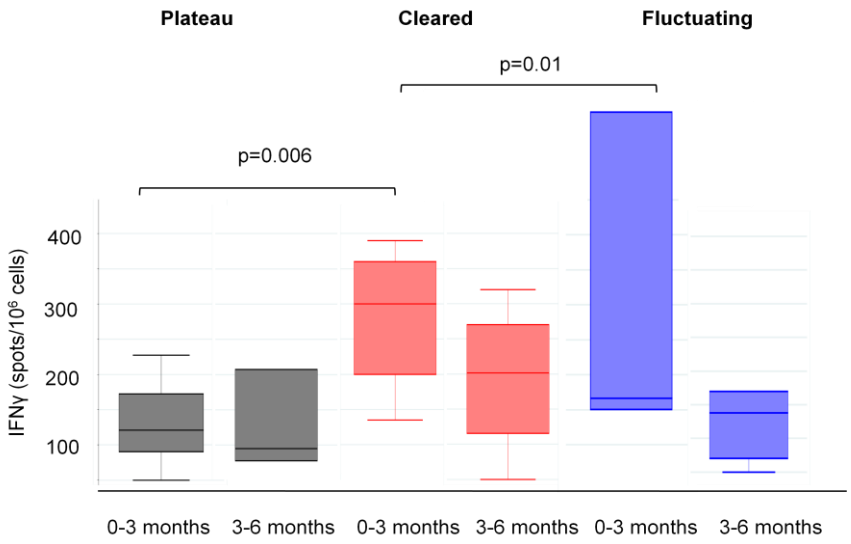
After HLA typing, patients were initially grouped into those with a favourable or unfavourable HLA type, based on previous studies. HLA B27, DRB1*0101, DRB1*1101 and DQB1*0301 were considered to be protective and assigned a score of +1, while DRB1*0701 was considered unfavourable and assigned a score of -1. Other HLA types were assigned a score of 0. Fisher's exact test with the Bonferroni correction for multiple comparisons was used to analyse the data. Initially, MHC class appeared to be weakly associated with outcome ($p=0.07$). However, when the associations were divided into classes I and II, while class I MHC was not associated with outcome ($p=0.54$), class II alleles were strongly associated ($p<0.0001$). Specific new HLA associations were not investigated due to sample size.

4.5.2 ELISpots

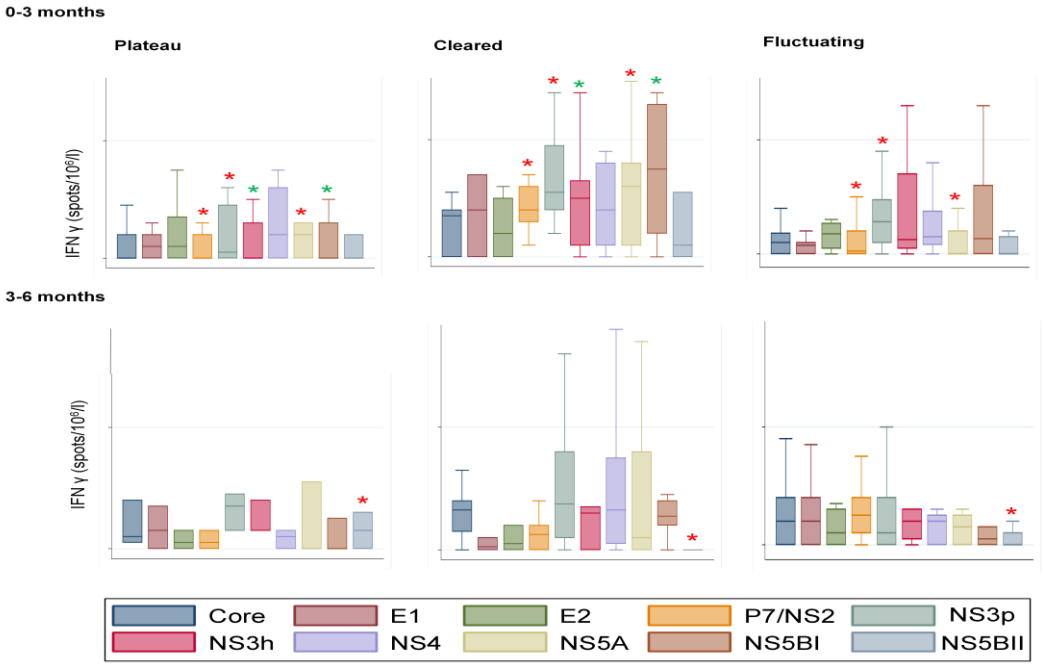
Interferon gamma (IFNγ) production in response to pooled peptides spanning the entire viral genome were measured by ELISpot at 2 time points in 40 patients with genotype 1a infection (Figure 4.2). Total responses at baseline were significantly higher in SC versus PV ($p=0.006$) and FV ($p=0.01$) patients while responses were not significantly different by 3-6 months. Spontaneous clearance was associated with significantly increased early responses to p7/NS2, NS3 protease and NS5A when compared with both PV and FV progressors. SC patients also had significantly increased responses to NS3 helicase and NS5B than PV patients but not FV patients.

Figure 4.2 *Ex vivo* IFNγ ELISpot results by group over time a) Total T cell responses b) T cell responses across the HCV genome c) T cell responses to individual peptide pools

a) Total T cell responses at baseline and 3-6 months



b) T cell responses across the HCV genome at baseline and 3-6 months



c) T cell responses to individual peptide pools at baseline and 3-6 months

Time	Cleared Median spots/10^6 cells (95%CI) [% with positive well]	Plateau Median spots/10^6 cells (95%CI) [% with positive well]	Fluctuating Median spots/10^6 cells (95%CI) [% with positive well]	p value Cleared vs Plateau Cleared vs Fluctuating (Median spots)
Baseline (0-3 months)				
Core	18 (0, 25) [14%]	0 (0, 13) [9%]	5 (0, 8) [10%]	0.36 0.49
E1	20 (0, 35) [43%]	5 (0 11) [9%]	4 (0, 5) [10%]	0.28 0.38
E2	10 (0, 28) [29%]	5 (0 17.5) [9%]	9 (3,13) [15%]	0.45 0.91
p7/NS2	20 (8, 33) [29%]	0 (0, 11) [9%]	1 (0, 10) [10%]	0.006* 0.005*
NS3p	28 (13.14286 62.9) [71%]	3 (0 23) [18%]	14 (5, 22) [25%]	0.007* 0.05*
NS3h	25 (1.571429 58.) [56%]	0 (0 15.9) [9%]	6 (3, 33) [30%]	0.02* 0.42
NS4A and I	20 (1.571429 43.4) [43%]	10 (0 30.7) [27%]	8 (5, 18) [15%]	0.14 0.35
NS5A	30 (1.571429 64) [57%]	10 (0 25.7) [18%]	0 (0, 10) [10%]	0.24 0.03*
NS5BI	38 (3, 68) [71%]	0 (0, 18) [18%]	7 (0, 21) [26%]	0.02* 0.08
NS5BII	5 (0 77) [29%]	0 (0, 10) [9%]	0 (0, 9) [16%]	0.22 0.46

	Cleared Median spots/10^6 cells (95%CI) [% with positive well]	Plateau Median spots/10^6 cells (95%CI) [% with positive well]	Fluctuating Median spots/10^6 cells (95%CI) [% with positive well]	p value Cleared vs Plateau Cleared vs Fluctuati (Median spots)
3-6 months				
Core	16 (1, 31) [17%]	5 (3, 20) [0%]	10 (0, 22) [13%]	0.43 0.53
E1	1 (0, 23) [17%]	8 (0, 18) [0%]	10 (0, 34) [21%]	0.50 0.24
E2	3 (0, 26) [17%]	3 (0, 8) [0%]	5 (0,17) [7%]	0.89 0.53
p7/NS2	6 (0, 19) [0%]	3 (0, 8) [0%]	13 (5, 26) [21%]	0.51 0.21
NS3p	19 (1, 76) [33%]	18 (8, 23) [0%]	5 (0, 23) [27%]	0.90 0.37
NS3h	15 (0, 56) [17%]	8 (8, 20) [0%]	10 (1, 47) [21%]	1.00 0.79
NS4A and E	16 (0, 85) [33%]	5 (0, 8) [0%]	10 (0, 14) [14%]	0.24 0.42
NS5A	5 (0, 81) [33%]	0 (0, 28) [33%]	8 (0, 14) [14%]	0.57 0.93
NS5BI	14 (1, 22) [0%]	0 (0, 13) [0%]	3 (0, 30) [21%]	0.15 0.23
NS5BII	0 (0, 0) [0%]	8 (0, 15) [0%]	0 (0, 8) [14%]	0.03* 0.06

Figure 4.2: T cells responses to pooled peptides spanning the entire HCV genome were assessed by ELISpot in 40 patients with genotype 1a infection at 2 time points. Significant results ($p<0.05$) are highlighted with asterisks. **a) Total T cell responses b) and c) T cell responses to individual peptide pools across the HCV genome**

Within individual patients, T cell responses were variable over time, particularly in the FV group (Figure 4.3).

Figure 4.3 ELISpot responses in representative patients from each group

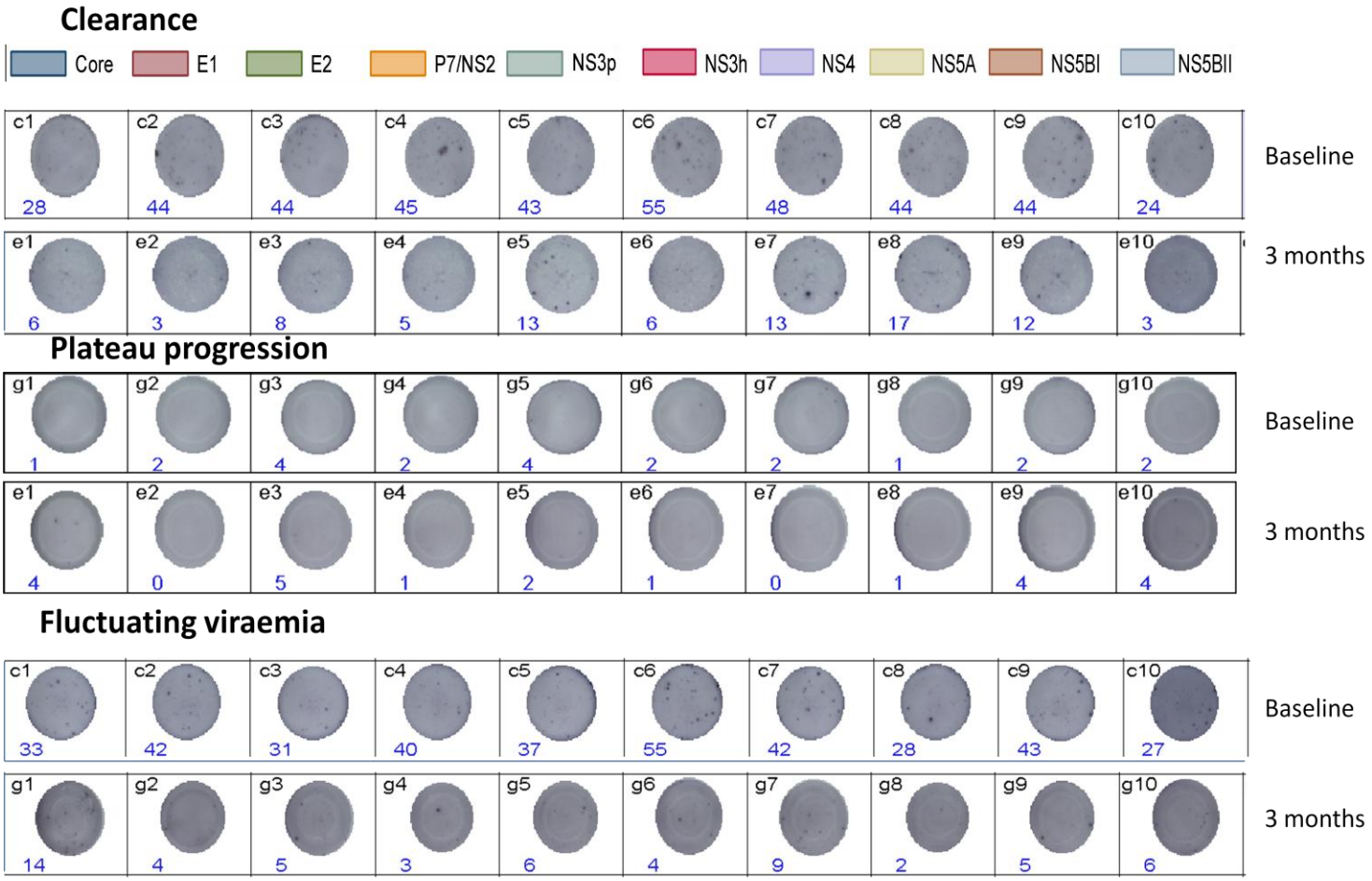


Figure 4.3: T cells responses were assessed by IFNγ ELISpot. Spot forming cells (SFCs) represent cells expressing IFNγ. SC patients had significantly higher responses at baseline than PV patients. FV patients displayed a non-sustained response.

4.5.3 Flow cytometry

4.5.3.1 Flow cytometry results by outcome group

In order to correlate outcome with cytokine expression, 50 patients had PBMC samples available for flow cytometry analysis. Initially, total T cell responses to peptides from across the genome were measured and subsequently, the response to each peptide pool was assessed after gating for CD4 or CD8 expression (Tables 4.5-4.10). All results are shown after subtraction of the DMSO negative control result.

4.5.3.1.1 CD4+ responses by outcome group

Production of IFNγ by CD4+ cells was higher in SC patients, reaching a statistically significant difference following stimulation with peptide pool 3 (containing peptides derived from NS5A and NS5B; p=0.04; Table 4.6). While IFNγ expression was also higher following stimulation with peptide pools 1 and 2, this did not reach statistical significance (Appendix 4).

Table 4.5: Total CD4+ cell responses: all peptide pools

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFNγ	**0.45**	**0.08,1.24**	**0.01**	**0, 0.27**	**0.02**	**0, 0.12**	**0.05***
IL-17	0	0,0.08	0.02	0,0.07	0	0,0.03	0.54
TNFα	0.05	0,0.23	0.21	0,0.89	0	0,0.11	0.83
IL-2	0.04	0,0.19	0	0,0.01	0.02	0,0.04	0.82

Table 4.6: CD4+ cell responses following stimulation with peptide pool 3

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFNγ	**0.58**	**0,2.73**	**0**	**0,0**	**0**	**0,0.05**	**0.04***
IL-17	0.04	0,0.63	0.02	0,0.09	0	0,0.05	0.51
TNFα	0.1	0,3.4	0.02	0,0.94	0	0,0.6	0.65
IL-2	0.02	0,0.22	0	0,0.03	0.02	0,0.1	0.60

Within the CD4+ IFN+ population, dual expression of IFN+TNF+ and IFN+IL17+ were also significantly higher in the SC group than in progressors (p=0.01 and p=0.006 respectively; Table 4.7).

Table 4.7 Dual cytokine expression in CD4+ cells to all peptides

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN+ve TNF+ve	**0.15**	**0.01,0.39**	**0**	**0,0.04**	**0**	**0,0**	**0.01***
IFN+ve IL-17+ve	**0.01**	**0.01,0.03**	**0**	**0,0**	**0**	**0,0**	**0.006****
IFN+ve IL-2+ve	0	0,0.02	0	0,0	0	0,0	0.40

These IFN+TNF+ and IFN+IL17+ populations were evident following stimulation with peptide pool 3 but not 1 or 2 (Table 4.8; Figure 4.4; Appendix 4).

Table 4.8 Dual cytokine expression in CD4+ positive cells stimulated with peptide pool 3

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN+ve TNF+ve	**0.37**	**0,2.67**	**0**	**0,0.04**	**0**	**0,0.03**	**0.03***
IFN+ve IL-17+ve	**0.03**	**0,0.12**	**0**	**0,0**	**0**	**0,0**	**0.0007****
IFN+ve IL-2+ve	0	0,0.03	0	0,0.01	0	0,0.01	0.68

Figure 4.4: IFNγ and IL-17 expression in CD4+ cells stimulated with peptide pool 3.

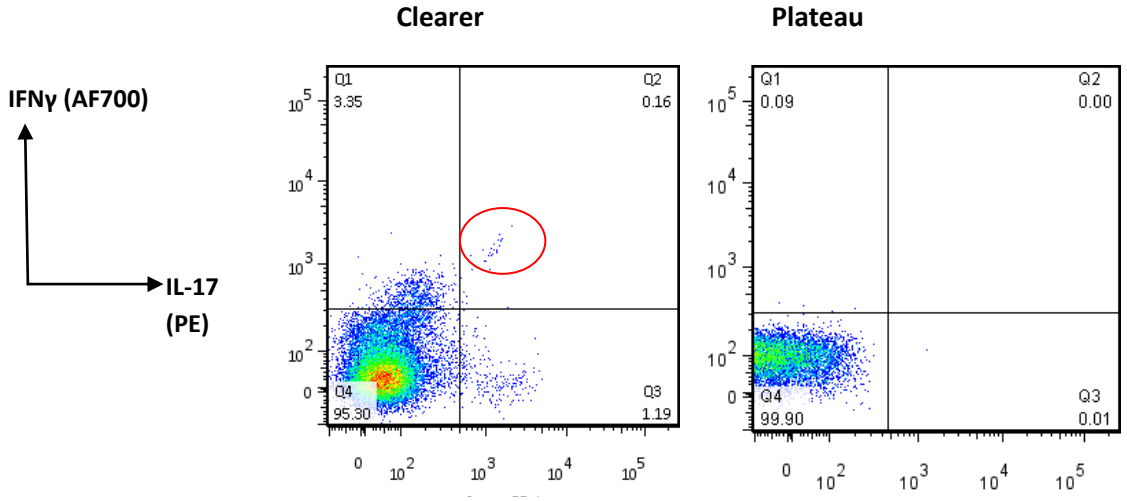


Figure 4.4: IFNγ and IL17 secretion in 2 representative patients with spontaneous clearance and plateau progression. The small population of IFN+IL17+ CD4+ cells highlighted in red in the SC patient is not observed in the PV patient.

4.5.3.1.2 CD8+ responses by outcome group

Production of IFNγ by CD8+ cells was higher in SC patients but this difference was not statistically significant (p=0.75; Appendix 4). However, dual expression of IFNγ and TNFα by CD8+ cells was increased in the SC group (p=0.04; Table 4.9; Appendix 4).

Table 4.9 Dual cytokine expression in CD8+ cells to all peptides

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN+ve TNF+ve	**0.14**	**0.01,0.38**	**0**	**0,0.05**	**0**	**0,0.01**	**0.04***
IFN+ve IL-17+ve	0	0,0.01	0	0,0.01	0.01	0,0.02	0.33
IFN+ve IL-2+ve	0	0,0.02	0	0,0.01	0	0,0.01	0.50

4.5.3.1.3 Fluctuating viraemia

As with ELISpot analyses, patients with fluctuating viraemia were noted to have marked variation in T cell response over time, in association with changes in HCV viral load (Figure 4.5).

Figure 4.5: CD4+ IFNγ and IL-17 expression in a FV patient.

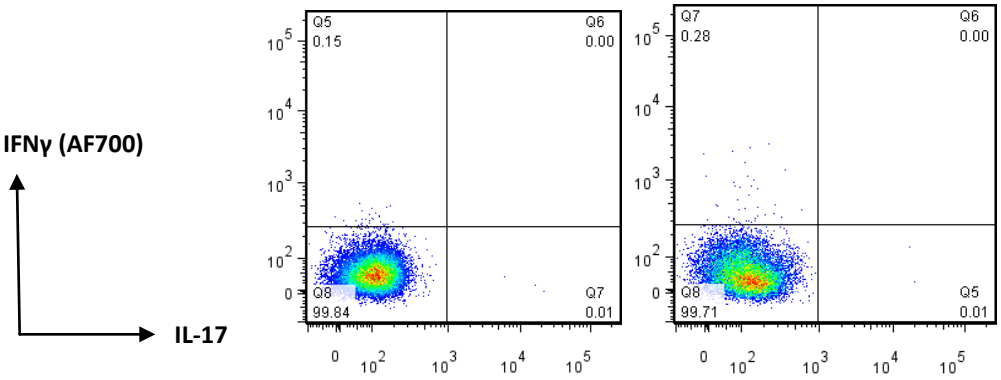


Figure 4.5: This is the same FV patient as illustrated in Figure 4.3 at 2 time points, 3 months apart, revealing variation in IFNγ expression from CD4+ cells. The CD4+IFNγ+IL-17+ expression seen in spontaneous clearers is absent at both time points.

4.5.3.2 Flow cytometry results by route of infection

In order to investigate if T cell responses were significantly different in patients who used intravenous or intramuscular drugs (IDUs), T cell responses were compared between the groups as above (Tables 4.10-4.16).

4.5.3.2.1 CD4+ responses in IDUs versus non-IDUs

IDUs were significantly more likely to have low levels of IL-2 expression by CD4+ cells (p=0.0004; Table 4.10).

Table 4.10: Total responses in CD4+ cells to all pools

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFNγ	0.01	0,0.1	0	0,0.04	0.26
IL-17	0	0,0.02	0	0,0.04	0.90
TNFα	0.01	0,0.14	0	0,0.99	0.67
IL-2	**0.01**	**0,0.03**	**0**	**0,0**	**0.0004****

Lack of IL-2 expression by CD4+ cells in the IDU population was particularly marked in response to stimulation with the NS proteins (pools 2 and 3; Table 4.11).

Table 4.11: CD4+ cell responses by peptide pool a) Peptide pool 2 b) Peptide pool 3

a) Peptide pool 2 (NS3 protease and helicase, NS4A and NS4B)

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFNγ	0.01	0,0.6	0	0.16,	0.37
IL-17	0	0,0	0	0,0.07	0.49
TNFα	0	0,0.19	0	0,2.94	0.86
IL-2	**0.06**	**0.02,0.63**	**0**	**0,0.01**	**0.002****

b) Peptide pool 3 (NS5A, NS5BI and II)

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFNγ	0	0,0.19	0	0,0.49	0.77
IL-17	0	0,0.1	0.01	0,0.1	0.80
TNFα	0.01	0,0.32	0.1	0,4.38	0.38
IL-2	**0.01**	**0,0.03**	**0**	**0,0**	**0.05***

A difference in dual expression of IFNγ and IL-2 in the CD4+ population in IDUs was also evident (p=0.0003; Table 4.12; Figure 4.6).

Table 4.12 Dual cytokine expression in CD4+ cells to all peptides

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN+ve TNF+ve	0.01	0,0.04	0	0,0.04	0.83
IFN+ve IL-17+ve	0	0,0.01	0	0,0.01	0.71
IFN+ve IL-2+ve	**0**	**0,0.01**	**0**	**0,0**	**0.0003****

This loss if IFN+ IL2+ population was most evident following stimulation with the NS proteins (pools 2 and 3; Table 4.13).

Table 4.13 Dual cytokine expression in CD4+ positive cells by peptide pool a) Peptide pool 1 b) Peptide pool 2 c) Peptide pool 3

a) Peptide pool 2

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN+ve TNF+ve	0	0,0.14	0	0,0.38	0.86
IFN+ve IL-17+ve	0	0,0.01	0	0,0.02	0.92
IFN+ve IL-2+ve	**0.01**	**0,0.02**	**0**	**0,0**	**0.01***

b) Peptide pool 3

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN+ve TNF+ve	0	0,0.05	0	0,0.42	1.00
IFN+ve IL-17+ve	0	0,0	0	0,0.03	0.70
IFN+ve IL-2+ve	**0**	**0,0.01**	**0**	**0,0**	**0.04***

Figure 4.6: Dual secretion of IL2 and IFNγ from CD4+ cells in HIV-positive IDUs versus non-IDUs.

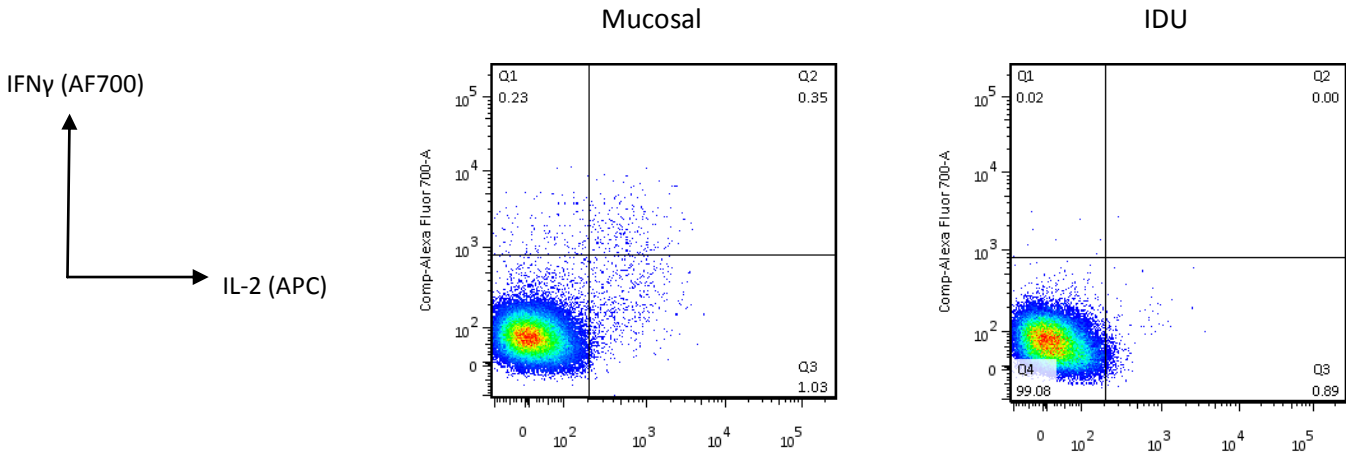


Figure 4.6: Expression of CD3+CD4+ cells secreting IL-2 and IFNγ in a patient with acute HCV following mucosal exposure versus a patient with both mucosal (intranasal drug use/sexual exposure) and IDU exposures.

4.5.3.2.2 CD8+ responses in IDUs versus non-IDUs

IFNγ release from CD8+ cells was significantly lower in IDUs than non-IDUs (p=0.05; Table 4.14). IL2 secretion was also lower.

Table 4.14: Total CD8+ cell responses to all peptide pools

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFNγ	**0.06**	**0,0.34**	**0**	**0,0.06**	**0.05***
IL-17	0	0,0	0	0,0.06	0.65
TNFα	0.05	0,0.19	0	0,0.79	0.88
IL-2	**0.01**	**0,0.07**	**0**	**0,0**	**0.01***

This was most apparent in response to stimulation with peptide pools 2 and 3 although only IL2 secretion reached statistical significance when individual peptide pools were examined (Table 4.15).

Table 4.15: CD8+ cell responses by peptide pool a) Peptide pool 2 b) Peptide pool 3

a) Peptide pool 2 (NS3 protease and helicase, NS4A and NS4B).

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFNγ	0.09	0,0.89	0.01	0,0.36	0.28
IL-17	0	0,0.03	0.03	0,0.14	0.72
TNFα	0.11	0,0.68	0	0,4.6	0.88
IL-2	**0.12**	**0.04,1.25**	**0**	**0,0.25**	**0.01***

b) Peptide pool 3 (NS5A, NS5BI and II).

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFNγ	0.08	0,0.55	0	0,0.54	0.22
IL-17	0	0,0.1	0	0,0.09	0.79
TNFα	0.01	0,0.21	0.15	0,6.18	0.31
IL-2	0.01	0,0.11	0	0,0	0.08

Dual secretion of IFNγ and IL-2 from CD8+ positive cells was significantly lower in IDU patients despite the fact that the cell populations examined were a small percentage of total CD8+ cells (Table 4.16).

Table 4.16 Dual cytokine expression in CD8+ positive cells by peptide pool a) Peptide pool 1 b) Peptide pool 2 c) Peptide pool 3

a) Peptide pool 2

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN+ve TNF+ve	0	0,0.14	0	0,0.38	0.86
IFN+ve IL-17+ve	0	0,0.01	0	0,0.02	0.92
IFN+ve IL-2+ve	**0.01**	**0,0.02**	**0**	**0,0**	**0.01***

b) Peptide pool 3

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN+ve TNF+ve	0	0,0.05	0	0,0.42	1.00
IFN+ve IL-17+ve	0	0,0	0	0,0.03	0.70
IFN+ve IL-2+ve	**0**	**0,0.01**	**0**	**0,0**	**0.04***

4.6 Discussion

4.6.1 The evolution of cellular immune responses in spontaneous clearance and progression to chronicity

This study is currently the largest longitudinal study of cellular immune responses during acute HCV infection in HIV-positive patients. The results indicate that CD4+ responses are central to the control of acute HCV in HIV-positive individuals and reveal the role of newly described HCV antigen-specific immune responses which are associated with clinical outcome.

Protective immunity in HIV-negative individuals is known to be associated with broad and multi-specific CD4+ and CD8+ T-cell responses (Cooper et al, 1999; Lechner et al, 2000; Harcourt et al, 2001; Cox et al, 2005). We therefore aimed to ascertain if the decreased dN/dS ratios and viral diversity observed within HCV E2 and NS5B in SC and FV patients in this HIV-positive cohort (Chapters 2-3) were related to a protective T-cell response, using ELISpot and flow cytometry to measure responses to overlapping pooled peptides spanning the entire HCV genome. While variation within the HCV E2 protein is largely antibody driven (Farci et al, 2000; Gaud et al, 2003), it does contain T cell epitopes (Appendices 3 and 4) and also indirectly reflects the T-cell responses to other parts of the genome such as the non-structural proteins through a genetic hitchhiking effect (Klenerman et al, 2000).

We found, as in other studies in HIV-negative populations, that SC patients exhibited significantly stronger and broader (multispecific) IFNγ responses than either progressor group (PV p=0.006; FV p=0.01, Figure 4.2). In particular, significant differences in response to NS2/p7, NS3 protease and NS5A were detected (SC versus FV and PV progressor groups). SC patients also had significantly increased responses to NS3 helicase and NS5B than PV

progressors. These responses were significantly different within the first 3 months of infection. The possibility of overestimation of statistical significance due to multiple comparisons is a limitation of the identification of specific regions in this study although the significance of the total breadth and strength of the response is robust. Further work to identify immunodominant regions at the epitope level is indicated.

Using flow cytometry, the phenotype of the IFNγ-producing cells observed with ELISpot analysis

was found to be mainly CD4+ and predominantly directed against the non-structural proteins (especially against NS5A and NS5B). As in other studies, although responses to core protein were detected, they were not significantly associated with a beneficial outcome (Gerlach et al, 2005; Aberle et al, 2006; Ruys et al, 2008). The impact of the CD4+ response in controlling HCV is highly likely to be prominent in this cohort, at least in part because of co-infection with HIV. While only a trend was observed when comparing absolute CD4 count with outcome (Chapter 2), CD4 cell function was clearly different between patient groups. This was found to be a main determining factor in another recent study of HIV-positive patients with acute HCV (Schnuriger et al, 2009). In this study, although CD4+ T cell responses were generally weak (<150 SFC)/10^6 PBMCs) as in the Schnuriger study, they were a major determining factor of self resolving or chronically progressing infection. Interestingly, of the IFNγ-secreting CD4 cells, a significant larger proportion dually expressed IL-17 or TNFα also ($p<0.006$; $p=0.03$ respectively) in response to stimulation with NS5A and NS5B peptides in SC patients versus progressors. While the percentage of these cells was small, a distinct CD4+IL-17+ population could be visualised in SC patients with a small increase in CD4+IL-17+IFNγ+ cells (Figure 4.4). Such cells are known to belong to the CD161+

population (Cosmi et al, 2008). As HIV-1 infection is associated with a marked depletion of CD161+ Th17 cells, occurring before the onset of clinically apparent HIV-related disease (Prendergast et al, 2010), this group might be expected to impact on outcome in this group of HIV-infected individuals. CD161 is associated with liver homing and therefore recognition of NS5A and NS5B within the liver may be central to mounting a successful immune response. Th17+CD161+ cells have been associated with a higher histological activity index after liver biopsy in patients with chronic hepatitis B virus (HBV) infection, possibly as a consequence of the release of proinflammatory cytokines (Zhang et al, 2009). Whether the situation is similar in HCV infected patients is not yet known.

While CD4+ phenotypic and functional differences between patient groups were most marked, CD8+ responses were also significantly different. Of note, although total CD8+ IFNγ+ responses were not markedly different between groups, dual expression of IFNγ and TNFα in response to the NS5A/NS5B pool was significantly higher in SC patients. It is likely that these CD8+ responses are governed by the CD4+ response.

While most studies indicate that cellular immunity is the main determinant of acute HCV outcome, spontaneous clearance may also occur in the absence of strong multispecific responses. This has been reported in chimpanzees (Thomson et al, 2003) and in humans during enhanced screening of high-risk individuals, for example in the prison setting (Meyer et al, 2007). One patient in this human cohort developed a transient low-level viraemia and rapidly cleared HCV in the absence of elevation in LFTs with a weakly detectable ELISA response which later disappeared. Innate immune mechanisms are likely to have played a role in eliminating infection in this case and more research is indicated in this area. Another area warranting further exploration is the role of polyfunctional HCV-specific T cells. These

are better correlates of clinical outcome in HIV infection and their role in HCV is as yet unexplored. The advent of sophisticated computer programmes such as PESTLE and SPICE designed to analyse such responses make this a realistic prospect.

4.6.2 Fluctuating viraemia

As expected from the variation in dN/dS ratios over time in the FV group (Chapter 2), T cell responses were noted to vary significantly over time (Figures 4.3, 4.5). Despite early strong and multispecific IFNγ responses in many FV patients, this was often not sustained. This could reflect the increased expression of inhibitory molecules such as the exhaustion marker PD-1 (Day et al, 2006; Urbani et al, 2008). As the majority of patients with FV were found to be superinfected with new HCV strains, the immune response to the circulating strain may be weak while the eliminated strain remains strong. Further experiments using autologous peptides could help to delineate this problem. More work is also required to characterise the inhibitory and activation marker expression in this group. Another possibility is that although FV patients are able to eliminate virus in the periphery, the appropriate cells may not home efficiently to the liver. This would be in keeping with the significantly lower NS5A/NS5B CD4+ IL-17 responses observed in FV patients when compared with SC patients at all time points. This could indicate that although virus is targeted in the periphery, intrahepatic responses may be lower in this group. Compartmentalisation of virus in the liver appears to occur in some patients in the absence of viraemia (Chapter 3).

4.6.3 Cellular immune responses in intravenous methamphetamine users

Unexpectedly, route of transmission resulted in a different profile in HCV-specific CD4+ and CD8+ responses. Patients were divided into those who had a possible intramuscular or intravenous injection (IDU) exposure to HCV versus those whose only risk factors were mucosal (unprotected sexual intercourse and/or intranasal use of cocaine or ketamine). The most commonly injected drug was crystal methamphetamine (Chapter 2). Patients using intravenous or intramuscular drugs are dispersed throughout the cohort, have higher viral diversity at baseline and lower SVR rates (Chapter 3). Thus although sexual transmission is the most frequent route of transmission within this patient population (Danta et al, 2007), a significant minority may also have been exposed to infection via the parenteral route. We aimed to investigate if T cell responses were different in these groups.

Patients who injected methamphetamine had significantly different immune responses to those who did not. CD4+ IL-2 expression was significantly reduced in the IDU group and dual expression of IL2 and IFNγ by CD4+ cells was also significantly reduced. CD8+ responses were also affected with a reduction both in IL2 and IFNγ expression including a reduction in dual expression of IFN+ IL2+ CD8+ cells. Thus proliferative responses are highly likely to be reduced in the IDU population. These CD8+ and CD4+ responses were directed primarily against the NS proteins. Thus the T cell response in patients using parenteral recreational drugs (mainly crystal methamphetamine) are suppressed in this cohort. Methamphetamine is associated with cellular immune dysfunction in mice and in humans (Yu et al, 2002; Martinez et al, 2009; Toussi et al, 2009). The mechanism for this is unclear and warrants further investigation. Another explanation for the reduction in T cell function could be exposure to a higher viral load and more diverse infecting inoculum in IDU patients

resulting in T cell exhaustion. More work is warranted to investigate these processes further.

4.7 Conclusions

In conclusion, the CD4+ response in HIV-positive individuals is central to the resolution of infection with HCV. The evidence of this comes from class II MHC association and functional and phenotypic analyses. In particular, IFNγ+ CD4+ responses directed against the NS proteins are significantly stronger during self-limiting disease. Further work to map protective, cross-specific epitopes is urgently required. Patients with fluctuating viraemia have wavering T cell responses and further work on activation and inhibitory receptor expression over time in this group would provide insights into the mechanisms behind this phenomenon. Intravenous drug users have dysfunctional CD4+ and CD8+ T cell responses and this affects the specific response to HCV. More work to identify the mechanism for this is warranted.

Chapter 5: Final Discussion

The natural history of acute HCV in HIV-positive MSM

In this cohort of HIV positive MSM with acute HCV infection, three clinical courses of infection were observed; spontaneous clearance (14%), fluctuating viraemia (38%) and plateau progression (48%). These outcomes were strongly associated with viral diversification at transmission, superinfection and the evolution of the adaptive cellular immune response.

5.1 Spontaneous clearance

Patients with spontaneous clearance had an initial rise and rapid decline in HCV VL within 100 days of infection. This coincided with a decline in viral diversity and a decreasing dN/dS ratio (<1), highly suggestive of purifying selection. Purifying selection was confirmed to be the main mechanism of viral evolution in these patients as defined by the codon-based z test, Fu and Li's neutrality test and by identification of codons under selection pressure using HyPhy and CODEML-based analyses. An early CD4+ IFNγ+ response was associated with spontaneous clearance and was directed predominantly against the NS proteins. Interestingly, a slight but significant increase in the Th17 population of CD4+IFN+ cells responding to peptide pool 3 containing NS5A and B peptides was noted in resolving infection and may represent a liver-homing population of cells. Dual secretion of TNFα and IFNγ was also associated with clearance (p=0.006; p=0.03 respectively). Importantly, PBMCs infected with HCV had very low levels of IFNγ expression. It is not clear if this is a direct

effect of HCV and HIV within the cell and it would be extremely interesting to investigate this further.

5.2 Plateau viraemia

Patients with plateau viraemia had a peak and then plateau HCV VL during acute HCV infection. In these patients, evidence of positive selection as a result of viral evolution was clearly evident. A high dN/dS ratio was characteristic and multiple positively selected codons were detected in this population within the HCV E2 HVR-1. The number of positively selected codons within this region in the presence of weak or absent CD4+ and CD8+ responses to the NS proteins was significantly associated with final outcome, suggesting that the HCV E2 HVR-1 region may act as an immunological decoy. In the absence of clear CD4+ and CD8+ responses directed against these epitopes, it is likely that they were selected by the antibody-mediated response. Further work to clarify this would be worthwhile.

5.3 Fluctuating viraemia

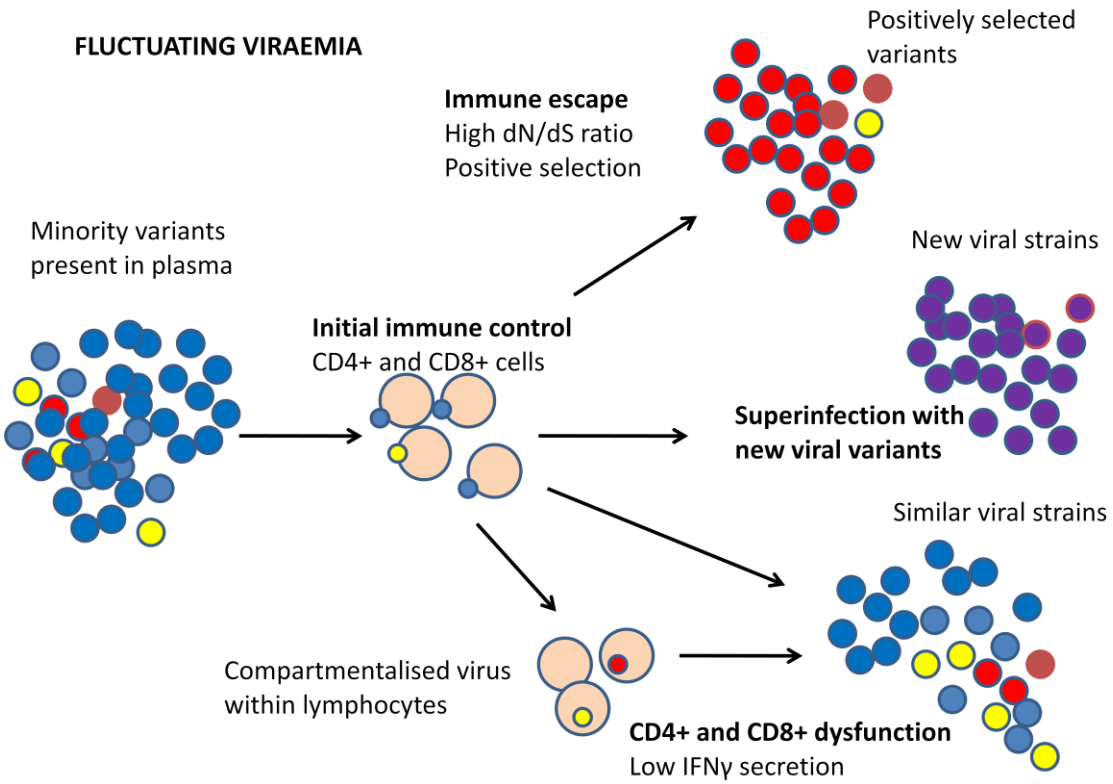
A peak and subsequent slow decline in HCV VL occurred in FV patients who do did not clear the infection in the long term (p=0.001; OR 2.76). FV patients who became transiently PCR negative took a median of 154 days to reach negativity, while SC patients took a median of only 108 days (p=0.04). Furthermore, progressor patients were noted to take an average of 129 days versus 27 days to reach a 1.7$\log_{10}$ drop in viraemia (p=0.009). The mechanism for this appears to be multi-factorial and includes superinfection with new viral strains, re-

emergence of minority or compartmentalised variants and delayed onset or loss of CD4+ and CD8+ HCV specific responses.

In order to ascertain whether the introduction of new viral strains (superinfection) defined this group, a phylogenetic analysis of 2295 sequences obtained from 50 patients within the cohort at a median of 3 time points was carried out. A large number of patients had evidence of the introduction of new viral strains both within the same strain (30%) and with new genotypes (10%). This was significantly more common in FV patients than SC or PV patients; 63% of FV patients had evidence of superinfection by clonal sequencing. A strain-specific PCR later revealed however, that the new dominant quasispecies in 36% of these patients had in fact emerged from minority strains present at the onset of infection. Thus approximately 41% of FV patients had clear evidence of new superinfecting HCV infection. Some of the remainder may have been re-infected with the same strain of HCV, as previously described in the chimpanzee model, but the remainder developed recrudescent viraemia in the absence of reinfection. We hypothesised on the basis of clinical and virological data that partial immune control was responsible for this phenomenon. A common theme in studies investigating the T cell response to HCV in HIV-negative individuals is that protective CD4+ and CD8+ responses must be sustained over time. This is likely to be a particularly important factor in HIV-infected individuals, due to CD4 cell dysfunction. Loss of immune control during the first 150 days of infection in FV patients was predicted by initially low but increasing dN/dS ratios, rising from <1 (suggestive of purifying selection) to a median of 1.78 (highly suggestive of positive selection). The role of cellular immunity in defining this population was later established using flow cytometry and ELISpot analyses. Interestingly, all patients who became PCR negative in this group had evidence of

HCV within PBMCs (100% of FV versus 0% of SC patients with a negative HCV VL). Furthermore, PBMCs contained sequences not seen at the same time points in plasma, suggesting that this compartment may support independent replication of the virus at low level and therefore may act as a reservoir of infection. One patient with acute HCV had a liver biopsy carried out during early HCV infection (in the absence of detectable plasma HCV VL). This revealed HCV sequences from the initial infection. Following successful treatment, the patient developed a recrudescent VL following a period of severe immunosupression associated with a low CD4 count. Lack of CD4+ control of HCV present in the liver but not the blood is likely to have resulted in this phenomenon. In summary, FV occurs due to superinfection events, compartmentalisation of virus in PBMCs and the liver and due to loss of immune regulation by CD4+ and CD8+ T cells (Figure 5.1).

Figure 5.1 Model of viral evolution in FV patients

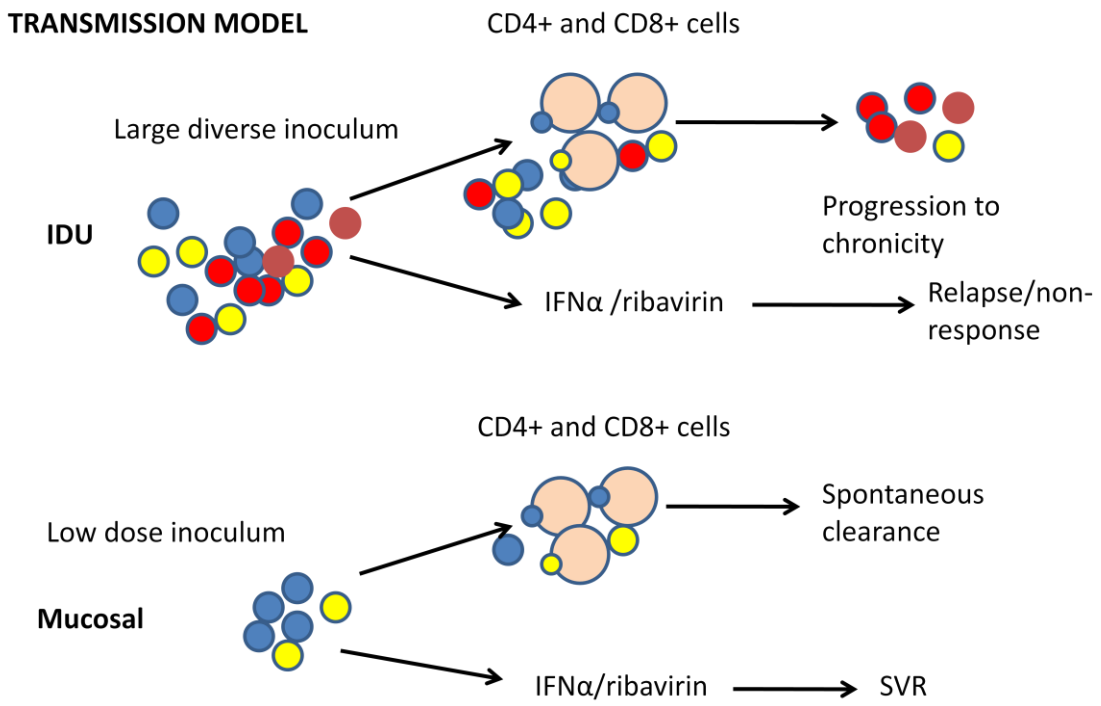


5.4 Route of transmission and final outcome

We found that route of transmission significantly impacted on the likelihood of response to treatment although not significantly on spontaneous clearance. Strikingly low SVR rates were observed in IDUs versus patients with mucosal exposure to HCV (20% versus 86%; p=0.002). This was significantly associated with higher viral diversity at baseline and pre-treatment. Interestingly, both IL2 and IFNγ production was lower in IDUs, affecting both the CD4+ and CD8+ response. This could be due to higher diversity of the infecting inoculum and subsequent T cell exhaustion (Figure 5.2) or a direct effect of methamphetamine which has

been associated with immune toxicity in mouse models. Further work to explore the role of methamphetamine on control PBMCs would help to clarify this.

Figure 5.2 Transmission diversity and outcome; route of transmission hypothesis



5.5 Treatment outcome and viral evolution

The response to treatment with pegIFNα and ribavirin was clearly related to viral diversity within the baseline and subsequent samples (p=0.008 and 0.004 respectively). An increasing dN/dS ratio was also strongly associated with outcome (p=0.007) and a higher number of mutations within the putative ISDR region of NS5A when compared with HCV-1 reference sequence. Although the p values comparing the number of amino acid changes suggested

that this is a real effect (p=0.00001), the overlapping confidence intervals and low number of actual deviations from the reference sequence were so small that it would be impractical to use this test as part of standard practice at the current time.

5.6 Conclusions

In this thesis, we aimed to investigate the following hypotheses:

1. HCV diversification and evolution predicts the outcome of acute infection in a cohort of HIV-infected individuals
2. Diversification occurs early during the course of infection and defines the later viral population and response to treatment
3. The evolution of acute HCV in this cohort is defined by the adaptive cellular immune response

Using a number of experimental techniques, including extensive longitudinal sequence analysis, computational prediction of positive or negative selection pressures and functional and phenotypic analyses, it is apparent that all of the above hypotheses are valid. The outcome of acute HCV infection in HIV-positive patients is defined by the rate of viral evolution within the first 150 days of infection. This is influenced by transmission diversity (and the route of infection), superinfection events, compartmentalisation of virus in the PBMCs and the liver and is governed by the T cell response. The CD4+ response is key in limiting infection as might be expected in an HIV-positive population and delayed or non-sustained responses are associated with progression to chronicity. Further work is urgently required to identify specific and cross-protective epitopes (most likely within the non-

structural proteins) which are recognized during acute HCV infection to progress the search for an effective vaccine against the virus.

Chapter 6: Future work

Following on from this work, a number of further experiments are planned and in progress.

1. Pyrosequencing analysis of cDNA amplicons using a nested PCR step with fusion primers in 20 patients in order to look for evidence of minority strains present within baseline samples. This will allow a depth of several thousand sequences and will add weight to superinfection being a major mechanism of diversification in progression to chronicity.

2. Microarray analysis. A number of patients within the cohort belong to an HIV seroconverter study (SPARTAC) and have had PBMC samples taken both before and after acute HCV infection. A study has been designed to carry out a microarray analysis from these PBMCs, looking at mRNA expression across the whole genome. Interferon pathways will be of particular interest when analysing this data.

3. In order to further prediction of spontaneous clearance and SVR, ideally a large multicentre cohort should be created. This would increase the statistical power of the study and create the possibility of a large scale multivariate analysis. A large cohort of such patients would also pave the way for new interventional treatment studies.

Bibliography

Aberle, J. H., Formann, E., Steindl-Munda, P., Weseslindtner, L., Gurguta, C., Perstinger, G., Grilnberger, E., Laferl, H., Dienes, H. P., Popow-Kraupp, T., Ferenci, P. & Holzmann, H. (2006) Prospective study of viral clearance and CD4(+) T-cell response in acute hepatitis C primary infection and reinfection. *Journal of Clinical Virology : The Official Publication of the Pan American Society for Clinical Virology,* 36 (1), 24-31.

Adams, N. J., Prescott, L. E., Jarvis, L. M., Lewis, J. C., McClure, M. O., Smith, D. B. & Simmonds, P. (1998) Detection in chimpanzees of a novel flavivirus related to GB virus-C/hepatitis G virus. *The Journal of General Virology,* 79 (Pt 8) (Pt 8), 1871-1877.

Agnello, V., Abel, G., Elfahal, M., Knight, G. B. & Zhang, Q. X. (1999) Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. *Proceedings of the National Academy of Sciences of the United States of America,* 96 (22), 12766-12771.

Agwale, S. M., Tanimoto, L., Womack, C., Odama, L., Leung, K., Duey, D., Negedu-Momoh, R., Audu, I., Mohammed, S. B., Inyang, U., Graham, B. & Ziermann, R. (2004) Prevalence of HCV coinfection in HIV-infected individuals in Nigeria and characterization of HCV genotypes. *Journal of Clinical Virology : The Official Publication of the Pan American Society for Clinical Virology,* 31 Suppl 1, S3-6.

Almeida JR, Sauce D, Price DA, Papagno L, Shin SY, Moris A, Larsen M, Pancino G, Douek DC, Autran B, Sáez-Cirión A & Appay V. Antigen sensitivity is a major determinant of CD8+ T-cell polyfunctionality and HIV-suppressive activity. (2009) *Blood*. 113(25), 6351-60.

Alter, M. J. (2006) Epidemiology of viral hepatitis and HIV co-infection. *Journal of Hepatology,* 44 (1 Suppl), S6-9.

Amorosa, V. K., Slim, J., Mounzer, K., Bruno, C., Hoffman-Terry, M., Dorey-Stein, Z., Ferrara, T., Kostman, J. R. & Lo Re, V.,3rd. (2010) The influence of abacavir and other antiretroviral agents on virological response to HCV therapy among antiretroviral-treated HIV-infected patients. *Antiviral Therapy,* 15 (1), 91-99.

Annunziato, F., Cosmi, L., Santarlasci, V., Maggi, L., Liotta, F., Mazzinghi, B., Parente, E., Fili, L., Ferri, S., Frosali, F., Giudici, F., Romagnani, P., Parronchi, P., Tonelli, F., Maggi, E. & Romagnani, S. (2007) Phenotypic and functional features of human Th17 cells. *The Journal of Experimental Medicine,* 204 (8), 1849-1861.

Arnold, D. M., Julian, J. A., Walker, I. R. & Association of Hemophilia Clinic Directors of Canada. (2006) Mortality rates and causes of death among all HIV-positive individuals with hemophilia in Canada over 21 years of follow-up. *Blood,* 108 (2), 460-464.

Arstila, T. P., Casrouge, A., Baron, V., Even, J., Kanellopoulos, J. & Kourilsky, P. (1999) A direct estimate of the human alphabeta T cell receptor diversity. *Science (New York, N.Y.),* 286 (5441), 958-961.

Ayele, W., Nokes, D. J., Abebe, A., Messele, T., Dejene, A., Enquselassie, F., Rinke de Wit, T. F. & Fontanet, A. L. (2002) Higher prevalence of anti-HCV antibodies among HIV-positive compared to HIV-negative inhabitants of Addis Ababa, Ethiopia. *Journal of Medical Virology,* 68 (1), 12-17.

Badr G, Bédard N, Abdel-Hakeem MS, Trautmann L,Willems B,Villeneuve JP,Haddad EK, Sékaly RP, Bruneau J & Shoukry NH (2008) Early Interferon Therapy for Hepatitis C Virus Infection Rescues Polyfunctional, Long-Lived CD8$^+$ Memory T Cells *Journal of Virology,* 82(20), 10017–10031

Bain, C., Fatmi, A., Zoulim, F., Zarski, J. P., Trepo, C. & Inchauspe, G. (2001) Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. *Gastroenterology,* 120 (2), 512-524.

Bani-Sadr, F., Carrat, F., Rosenthal, E., Piroth, L., Morand, P., Lunel-Fabiani, F., Bonarek, M., Colin de Verdiere, N., Pialoux, G., Cacoub, P., Pol, S., Perronne, C. & ANRS HC02-Ribavic Study Team. (2005) Spontaneous hepatic decompensation in patients coinfected with HIV and hepatitis C virus during interferon-ribavirin combination treatment. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America,* 41 (12), 1806-1809.

Bani-Sadr, F., Goderel, I., Morand, P., Payan, C., Lunel, F., Pol, S., Perronne, C., Carrat, F. & Cacoub, P. (2007) High hepatitis C virus viral load in HIV/hepatitis C virus-co-infected patients: a different influence of protease inhibitor and non-protease inhibitor-based HAART? *AIDS (London, England),* 21 (12), 1645-1648.

Bani-Sadr, F., Lapidus, N., Bedossa, P., De Boever, C. M., Perronne, C., Halfon, P., Pol, S., Carrat, F., Cacoub, P., French National Agency for Research on AIDS & Viral Hepatitis-HC02-Ribavic Study Team. (2008) Progression of fibrosis in HIV and hepatitis C virus-coinfected patients treated with interferon plus ribavirin-based therapy: analysis of risk factors. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America,* 46 (5), 768-774.

Barba, G., Harper, F., Harada, T., Kohara, M., Goulinet, S., Matsuura, Y., Eder, G., Schaff, Z., Chapman, M. J., Miyamura, T. & Brechot, C. (1997) Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. *Proceedings of the National Academy of Sciences of the United States of America,* 94 (4), 1200-1205.

Barin, F. (2000) Controlled viral risks. *Transfusion Clinique Et Biologique : Journal De La Societe Francaise De Transfusion Sanguine,* 7 (1), 24-29.

Barin, F., M'Boup, S., Denis, F., Kanki, P., Allan, J. S., Lee, T. H. & Essex, M. (1985) Serological evidence for virus related to simian T-lymphotropic retrovirus III in residents of west Africa. *Lancet,* 2 (8469-70), 1387-1389.

Barnes, E., Ward, S. M., Kasprowicz, V. O., Dusheiko, G., Klenerman, P. & Lucas, M. (2004) Ultra-sensitive class I tetramer analysis reveals previously undetectable populations of antiviral CD8+ T cells. *European Journal of Immunology,* 34 (6), 1570-1577.

Barreiro, P., Rodriguez-Novoa, S., Labarga, P., Ruiz, A., Jimenez-Nacher, I., Martin-Carbonero, L., Gonzalez-Lahoz, J. & Soriano, V. (2007) Influence of liver fibrosis stage on plasma levels of antiretroviral drugs in HIV-infected patients with chronic hepatitis C. *The Journal of Infectious Diseases,* 195 (7), 973-979.

Barre-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W. & Montagnier, L. (1983) Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS) *Science (New York, N.Y.),* 220 (4599), 868-871.

Barrett, S., Ryan, E. & Crowe, J. (1999) Association of the HLA-DRB1*01 allele with spontaneous viral clearance in an Irish cohort infected with hepatitis C virus via contaminated anti-D immunoglobulin. *Journal of Hepatology,* 30 (6), 979-983.

Bartenschlager, R., Ahlborn-Laake, L., Mous, J. & Jacobsen, H. (1994) Kinetic and structural analyses of hepatitis C virus polyprotein processing *Journal of Virology,* 68 (8), 5045-5055.

Bartenschlager, R., Kaul, A. & Sparacio, S. (2003) Replication of the hepatitis C virus in cell culture. *Antiviral Research,* 60 (2), 91-102.

Barth, H., Schafer, C., Adah, M. I., Zhang, F., Linhardt, R. J., Toyoda, H., Kinoshita-Toyoda, A., Toida, T., Van Kuppevelt, T. H., Depla, E., Von Weizsacker, F., Blum, H. E. & Baumert, T. F. (2003) Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. *The Journal of Biological Chemistry,* 278 (42), 41003-41012.

Bartosch, B., Vitelli, A., Granier, C., Goujon, C., Dubuisson, J., Pascale, S., Scarselli, E., Cortese, R., Nicosia, A. & Cosset, F. L. (2003) Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. *The Journal of Biological Chemistry,* 278 (43), 41624-41630.

Belzile, J. P., Richard, J., Rougeau, N., Xiao, Y. & Cohen, E. A. (2010) HIV-1 Vpr induces the K48-linked polyubiquitination and proteasomal degradation of target cellular proteins to activate ATR and promote G2 arrest. *Journal of Virology,* 84 (7), 3320-3330.

Benga, W. J., Krieger, S. E., Dimitrova, M., Zeisel, M. B., Parnot, M., Lupberger, J., Hildt, E., Luo, G., McLauchlan, J., Baumert, T. F. & Schuster, C. (2010) Apolipoprotein E interacts with hepatitis C virus nonstructural protein 5A and determines assembly of infectious particles. *Hepatology (Baltimore, Md.),* 51 (1), 43-53.

Benhamou, Y., Bochet, M., Di Martino, V., Charlotte, F., Azria, F., Coutellier, A., Vidaud, M., Bricaire, F., Opolon, P., Katlama, C. & Poynard, T. (1999) Liver fibrosis progression in human immunodeficiency virus and hepatitis C virus coinfected patients. The Multivirc Group. *Hepatology (Baltimore, Md.),* 30 (4), 1054-1058.

Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J, Lederman MM, Benito JM, Goepfert PA, Connors M, Roederer M, Koup RA. (2006) HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* 107(12),4781-9.

Bica, I., McGovern, B., Dhar, R., Stone, D., McGowan, K., Scheib, R. & Snydman, D. R. (2001) Increasing mortality due to end-stage liver disease in patients with human immunodeficiency virus infection. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America,* 32 (3), 492-497.

Bishop, P. E., McMillan, A. & Gilmour, H. M. (1987) Immunological study of the rectal mucosa of men with and without human immunodeficiency virus infection. *Gut,* 28 (12), 1619-1624.

Blackard, J. T., Hiasa, Y., Smeaton, L., Jamieson, D. J., Rodriguez, I., Mayer, K. H. & Chung, R. T. (2007) Compartmentalization of hepatitis C virus (HCV) during HCV/HIV coinfection. *The Journal of Infectious Diseases,* 195 (12), 1765-1773.

Blackard, J. T., Smeaton, L., Hiasa, Y., Horiike, N., Onji, M., Jamieson, D. J., Rodriguez, I., Mayer, K. H. & Chung, R. T. (2005) Detection of hepatitis C virus (HCV) in serum and peripheral-blood mononuclear cells from HCV-monoinfected and HIV/HCV-coinfected persons. *The Journal of Infectious Diseases,* 192 (2), 258-265.

Brau, N., Salvatore, M., Rios-Bedoya, C. F., Fernandez-Carbia, A., Paronetto, F., Rodriguez-Orengo, J. F. & Rodriguez-Torres, M. (2006) Slower fibrosis progression in HIV/HCV-coinfected patients with successful HIV suppression using antiretroviral therapy. *Journal of Hepatology,* 44 (1), 47-55.

Brohm, C., Steinmann, E., Friesland, M., Lorenz, I. C., Patel, A., Penin, F., Bartenschlager, R. & Pietschmann, T. (2009) Characterization of determinants important for hepatitis C virus p7 function in morphogenesis by using trans-complementation. *Journal of Virology,* 83 (22), 11682-11693.

Brook, G., Main, J., Nelson, M., Bhagani, S., Wilkins, E., Leen, C., Fisher, M., Gilleece, Y., Gilson, R., Freedman, A., Kulasegaram, R., Agarwal, K., Sabin, C., Deacon-Adams, C. & BHIVA Viral Hepatitis Working Group. (2010) British HIV Association guidelines for the management of coinfection with HIV-1 and hepatitis B or C virus 2010 *HIV Medicine,* 11 (1), 1-30.

Bukh, J. (2004) A critical role for the chimpanzee model in the study of hepatitis C. *Hepatology (Baltimore, Md.),* 39 (6), 1469-1475.

Bukh, J., Thimme, R., Meunier, J. C., Faulk, K., Spangenberg, H. C., Chang, K. M., Satterfield, W., Chisari, F. V. & Purcell, R. H. (2008) Previously infected chimpanzees are not consistently protected against reinfection or persistent infection after reexposure to the identical hepatitis C virus strain. *Journal of Virology,* 82 (16), 8183-8195.

Callens, N., Ciczora, Y., Bartosch, B., Vu-Dac, N., Cosset, F. L., Pawlotsky, J. M., Penin, F. & Dubuisson, J. (2005) Basic residues in hypervariable region 1 of hepatitis C virus envelope glycoprotein e2 contribute to virus entry. *Journal of Virology,* 79 (24), 15331-15341.

Capa, L., Soriano, V., Garcia-Samaniego, J., Nunez, M., Romero, M., Cascajero, A., Munoz, F., Gonzalez-Lahoz, J. & Benito, J. M. (2007) Influence of HCV genotype and co-infection with human immunodeficiency virus on CD4(+) and CD8(+) T-cell responses to hepatitis C virus. *Journal of Medical Virology,* 79 (5), 503-510.

Carrat, F., Bani-Sadr, F., Pol, S., Rosenthal, E., Lunel-Fabiani, F., Benzekri, A., Morand, P., Goujard, C., Pialoux, G., Piroth, L., Salmon-Ceron, D., Degott, C., Cacoub, P., Perronne, C. & ANRS HCO2 RIBAVIC Study Team. (2004) Pegylated interferon alfa-2b vs standard interferon alfa-2b, plus ribavirin, for chronic hepatitis C in HIV-infected patients: a randomized controlled trial. *JAMA : The Journal of the American Medical Association,* 292 (23), 2839-2848.

Chamie, G., Bonacini, M., Bangsberg, D. R., Stapleton, J. T., Hall, C., Overton, E. T., Scherzer, R. & Tien, P. C. (2007) Factors associated with seronegative chronic hepatitis C virus infection in HIV infection. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America,* 44 (4), 577-583.

Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W. & Houghton, M. (1989) Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science (New York, N.Y.),* 244 (4902), 359-362.

Chung, R. T., Andersen, J., Volberding, P., Robbins, G. K., Liu, T., Sherman, K. E., Peters, M. G., Koziel, M. J., Bhan, A. K., Alston, B., Colquhoun, D., Nevin, T., Harb, G., van der Horst, C. & AIDS Clinical Trials Group A5071 Study Team. (2004) Peginterferon Alfa-2a plus ribavirin versus interferon alfa-2a plus ribavirin for chronic hepatitis C in HIV-coinfected persons. *The New England Journal of Medicine,* 351 (5), 451-459.

Clerici, M., Balotta, C., Salvaggio, A., Riva, C., Trabattoni, D., Papagno, L., Berlusconi, A., Rusconi, S., Villa, M. L., Moroni, M. & Galli, M. (1996) Human immunodeficiency virus (HIV) phenotype and interleukin-2/ interleukin-10 ratio are associated markers of protection and progression in HIV infection. *Blood,* 88 (2), 574-579.

Cline, J., Braman, J. C. & Hogrefe, H. H. (1996) PCR fidelity of pfu DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Research,* 24 (18), 3546-3551.

Cohen, D. E., Russell, C. J., Golub, S. A. & Mayer, K. H. (2006) Prevalence of hepatitis C virus infection among men who have sex with men at a Boston community health center and its association with markers of high-risk behavior. *AIDS Patient Care and STDs,* 20 (8), 557-564.

Collins, K. L., Chen, B. K., Kalams, S. A., Walker, B. D. & Baltimore, D. (1998) HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature,* 391 (6665), 397-401.

Cooper, S., Erickson, A. L., Adams, E. J., Kansopon, J., Weiner, A. J., Chien, D. Y., Houghton, M., Parham, P. & Walker, C. M. (1999) Analysis of a successful immune response against hepatitis C virus. *Immunity,* 10 (4), 439-449.

Cormier, E. G., Tsamis, F., Kajumo, F., Durso, R. J., Gardner, J. P. & Dragic, T. (2004) CD81 is an entry coreceptor for hepatitis C virus. *Proceedings of the National Academy of Sciences of the United States of America,* 101 (19), 7270-7274.

Cosmi, L., De Palma, R., Santarlasci, V., Maggi, L., Capone, M., Frosali, F., Rodolico, G., Querci, V., Abbate, G., Angeli, R., Berrino, L., Fambrini, M., Caproni, M., Tonelli, F., Lazzeri, E., Parronchi, P., Liotta, F., Maggi, E., Romagnani, S. & Annunziato, F. (2008) Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor. *The Journal of Experimental Medicine,* 205 (8), 1903-1916.

Cox, A. L., Mosbruger, T., Lauer, G. M., Pardoll, D., Thomas, D. L. & Ray, S. C. (2005) Comprehensive analyses of CD8+ T cell responses during longitudinal study of acute human hepatitis C. *Hepatology (Baltimore, Md.),* 42 (1), 104-112.

Cribier, B., Rey, D., Schmitt, C., Lang, J. M., Kirn, A. & Stoll-Keller, F. (1995) High hepatitis C viraemia and impaired antibody response in patients coinfected with HIV. *AIDS (London, England),* 9 (10), 1131-1136.

Cummings, K. L., Rosen, H. R. & Hahn, Y. S. (2009) Frequency of gC1qR+CD4+ T cells increases during acute hepatitis C virus infection and remains elevated in patients with chronic infection. *Clinical Immunology (Orlando, Fla.),* 132 (3), 401-411.

Danta, M., Brown, D., Bhagani, S., Pybus, O. G., Sabin, C. A., Nelson, M., Fisher, M., Johnson, A. M., Dusheiko, G. M. & HIV and Acute HCV (HAAC) group. (2007) Recent epidemic of acute hepatitis C

virus in HIV-positive men who have sex with men linked to high-risk sexual behaviours. *AIDS (London, England),* 21 (8), 983-991.

Danta, M., Semmo, N., Fabris, P., Brown, D., Pybus, O. G., Sabin, C. A., Bhagani, S., Emery, V. C., Dusheiko, G. M. & Klenerman, P. (2008) Impact of HIV on host-virus interactions during early hepatitis C virus infection. *The Journal of Infectious Diseases,* 197 (11), 1558-1566.

Darwin, Charles & Beer, Gillian. *The origin of species* , Oxford : Oxford University Press, 1996 (1998 printing).

David-Neto, E., Abdallah, K. A., Bassit, L., Pereira, A. N., Alquezar, A. S., Fonseca, J. A., Ianhez, L. E. & Sabbaga, E. (1997) Anti-HCV antibody is sensitive but not sufficient to detect HCV active infection in renal transplanted patients: the role of PCR for HCV-RNA. *Transplantation Proceedings,* 29 (1-2), 781-782.

Day, C. L., Kaufmann, D. E., Kiepiela, P., Brown, J. A., Moodley, E. S., Reddy, S., Mackey, E. W., Miller, J. D., Leslie, A. J., DePierres, C., Mncube, Z., Duraiswamy, J., Zhu, B., Eichbaum, Q., Altfeld, M., Wherry, E. J., Coovadia, H. M., Goulder, P. J., Klenerman, P., Ahmed, R., Freeman, G. J. & Walker, B. D. (2006) PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature,* 443 (7109), 350-354.

Day, C. L. & Walker, B. D. (2003) Progress in defining CD4 helper cell responses in chronic viral infections. *The Journal of Experimental Medicine,* 198 (12), 1773-1777.

de Oliveira, T., Pybus, O. G., Rambaut, A., Salemi, M., Cassol, S., Ciccozzi, M., Rezza, G., Gattinara, G. C., D'Arrigo, R., Amicosante, M., Perrin, L., Colizzi, V., Perno, C. F. & Benghazi Study Group. (2006) Molecular epidemiology: HIV-1 and HCV sequences from Libyan outbreak. *Nature,* 444 (7121), 836-837.

Deforges, S., Evlashev, A., Perret, M., Sodoyer, M., Pouzol, S., Scoazec, J. Y., Bonnaud, B., Diaz, O., Paranhos-Baccala, G., Lotteau, V. & Andre, P. (2004) Expression of hepatitis C virus proteins in epithelial intestinal cells in vivo. *The Journal of General Virology,* 85 (Pt 9), 2515-2523.

D'Oliveira, A.,Jr, Voirin, N., Allard, R., Peyramond, D., Chidiac, C., Touraine, J. L., Fabry, J., Trepo, C. & Vanhems, P. (2005) Prevalence and sexual risk of hepatitis C virus infection when human immunodeficiency virus was acquired through sexual intercourse among patients of the Lyon University Hospitals, France, 1992-2002. *Journal of Viral Hepatitis,* 12 (3), 330-332.

Dominguez, S., Ghosn, J., Valantin, M. A., Schruniger, A., Simon, A., Bonnard, P., Caumes, E., Pialoux, G., Benhamou, Y., Thibault, V. & Katlama, C. (2006) Efficacy of early treatment of acute hepatitis C infection with pegylated interferon and ribavirin in HIV-infected patients. *AIDS (London, England),* 20 (8), 1157-1161.

Durante Mangoni, E., Forton, D. M., Ruggiero, G. & Karayiannis, P. (2003) Hepatitis C virus E2 and NS5A region variability during sequential treatment with two interferon-alpha preparations. *Journal of Medical Virology,* 70 (1), 62-73.

Egger, D., Wolk, B., Gosert, R., Bianchi, L., Blum, H. E., Moradpour, D. & Bienz, K. (2002) Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *Journal of Virology,* 76 (12), 5974-5984.

Enomoto, N., Takada, A., Nakao, T. & Date, T. (1990) There are two major types of hepatitis C virus in Japan. *Biochemical and Biophysical Research Communications,* 170 (3), 1021-1025.

Enomoto, N., Sakuma, I., Asahina, Y., Kurosaki, M., Murakami, T., Yamamoto, C., Izumi, N., Marumo, F. & Sato, C. (1995) Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. *The Journal of Clinical Investigation,* 96 (1), 224-230.

Enomoto, N., Sakuma, I., Asahina, Y., Kurosaki, M., Murakami, T., Yamamoto, C., Ogura, Y., Izumi, N., Marumo, F. & Sato, C. (1996) Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *The New England Journal of Medicine,* 334 (2), 77-81.

Eyeson, J., King, D., Boaz, M. J., Sefia, E., Tomkins, S., Waters, A., Easterbrook, P. J. & Vyakarnam, A. (2003) Evidence for Gag p24-specific CD4 T cells with reduced susceptibility to R5 HIV-1 infection in a UK cohort of HIV-exposed-seronegative subjects. *AIDS (London, England),* 17 (16), 2299-2311.

Eyster, M. E., Alter, H. J., Aledort, L. M., Quan, S., Hatzakis, A. & Goedert, J. J. (1991) Heterosexual co-transmission of hepatitis C virus (HCV) and human immunodeficiency virus (HIV). *Annals of Internal Medicine,* 115 (10), 764-768.

Failla, C., Tomei, L. & De Francesco, R. (1994) Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins *Journal of Virology,* 68 (6), 3753-3760.

Fanning, L. J., Levis, J., Kenny-Walsh, E., Whelton, M., O'Sullivan, K. & Shanahan, F. (2001) HLA class II genes determine the natural variance of hepatitis C viral load. *Hepatology (Baltimore, Md.),* 33 (1), 224-230.

Farci, P., Shimoda, A., Coiana, A., Diaz, G., Peddis, G., Melpolder, J. C., Strazzera, A., Chien, D. Y., Munoz, S. J., Balestrieri, A., Purcell, R. H. & Alter, H. J. (2000) The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. *Science (New York, N.Y.),* 288 (5464), 339-344.

Farci, P., Shimoda, A., Wong, D., Cabezon, T., De Gioannis, D., Strazzera, A., Shimizu, Y., Shapiro, M., Alter, H. J. & Purcell, R. H. (1996) Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. *Proceedings of the National Academy of Sciences of the United States of America,* 93 (26), 15394-15399.

Felber, B. K., Hadzopoulou-Cladaras, M., Cladaras, C., Copeland, T. & Pavlakis, G. N. (1989) rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA. *Proceedings of the National Academy of Sciences of the United States of America,* 86 (5), 1495-1499.

Felsenstein, Joseph & Felenstein, Joseph. (2002) *Inferring Phylogenies* 1st edition. , Sinauer Associates.

Fierer, D. S., Uriel, A. J., Carriero, D. C., Klepper, A., Dieterich, D. T., Mullen, M. P., Thung, S. N., Fiel, M. I. & Branch, A. D. (2008) Liver Fibrosis during an Outbreak of Acute Hepatitis C Virus Infection in HIV-Infected Men: A Prospective Cohort Study. *The Journal of Infectious Diseases,* , .

Fleming, C. A., Craven, D. E., Thornton, D., Tumilty, S. & Nunes, D. (2003) Hepatitis C virus and human immunodeficiency virus coinfection in an urban population: low eligibility for interferon

treatment. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America,* 36 (1), 97-100.

Fleming, V. M., Harcourt, G., Barnes, E. & Klenerman, P. (2010) Virologic footprint of CD4+ T cell responses during chronic HCV infection. *The Journal of General Virology,* 91, 1396-406.

Folgori, A., Spada, E., Pezzanera, M., Ruggeri, L., Mele, A., Garbuglia, A. R., Perrone, M. P., Del Porto, P., Piccolella, E., Cortese, R., Nicosia, A., Vitelli, A. & Acute Hepatitis C Italian Study Group. (2006) Early impairment of hepatitis C virus specific T cell proliferation during acute infection leads to failure of viral clearance. *Gut,* 55 (7), 1012-1019.

Forton, D. M., Karayiannis, P., Mahmud, N., Taylor-Robinson, S. D. & Thomas, H. C. (2004) Identification of unique hepatitis C virus quasispecies in the central nervous system and comparative analysis of internal translational efficiency of brain, liver, and serum variants. *Journal of Virology,* 78 (10), 5170-5183.

Fowler, N. L., Torresi, J., Jackson, D. C., Brown, L. E. & Gowans, E. J. (2003) Immune responses in hepatitis C virus infection: the role of dendritic cells. *Immunology and Cell Biology,* 81 (1), 63-66.

Frank, C., Mohamed, M. K., Strickland, G. T., Lavanchy, D., Arthur, R. R., Magder, L. S., El Khoby, T., Abdel-Wahab, Y., Aly Ohn, E. S., Anwar, W. & Sallam, I. (2000) The role of parenteral antischistosomal therapy in the spread of hepatitis C virus in Egypt. *Lancet,* 355 (9207), 887-891.

Fried, M. W., Shiffman, M. L., Reddy, K. R., Smith, C., Marinos, G., Goncales, F. L.,Jr, Haussinger, D., Diago, M., Carosi, G., Dhumeaux, D., Craxi, A., Lin, A., Hoffman, J. & Yu, J. (2002) Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *The New England Journal of Medicine,* 347 (13), 975-982.

Frolov, I., Agapov, E., Hoffman, T. A.,Jr, Pragai, B. M., Lippa, M., Schlesinger, S. & Rice, C. M. (1999) Selection of RNA replicons capable of persistent noncytopathic replication in mammalian cells. *Journal of Virology,* 73 (5), 3854-3865.

Fu and Li (1993) Statistical Tests of Neutrality of Mutations *Genetics* 133 (3): 693

Gale, M. J.,Jr, Korth, M. J. & Katze, M. G. (1998) Repression of the PKR protein kinase by the hepatitis C virus NS5A protein: a potential mechanism of interferon resistance. *Clinical and Diagnostic Virology,* 10 (2-3), 157-162.

Garcia, J. V. & Miller, A. D. (1991) Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. *Nature,* 350 (6318), 508-511.

Gardner, J. P., Durso, R. J., Arrigale, R. R., Donovan, G. P., Maddon, P. J., Dragic, T. & Olson, W. C. (2003) L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus. *Proceedings of the National Academy of Sciences of the United States of America,* 100 (8), 4498-4503.

Gatignol, A., Buckler-White, A., Berkhout, B. & Jeang, K. T. (1991) Characterization of a human TAR RNA-binding protein that activates the HIV-1 LTR. *Science (New York, N.Y.),* 251 (5001), 1597-1600.

Gaud, U., Langer, B., Petropoulou, T., Thomas, H. C. & Karayiannis, P. (2003) Changes in hypervariable region 1 of the envelope 2 glycoprotein of hepatitis C virus in children and adults with humoral immune defects. *Journal of Medical Virology,* 69 (3), 350-356.

Gerlach, J. T., Diepolder, H. M., Jung, M. C., Gruener, N. H., Schraut, W. W., Zachoval, R., Hoffmann, R., Schirren, C. A., Santantonio, T. & Pape, G. R. (1999) Recurrence of hepatitis C virus after loss of virus-specific CD4(+) T-cell response in acute hepatitis C. *Gastroenterology,* 117 (4), 933-941.

Gerlach, J. T., Ulsenheimer, A., Gruner, N. H., Jung, M. C., Schraut, W., Schirren, C. A., Heeg, M., Scholz, S., Witter, K., Zahn, R., Vogler, A., Zachoval, R., Pape, G. R. & Diepolder, H. M. (2005) Minimal T-cell-stimulatory sequences and spectrum of HLA restriction of immunodominant CD4+ T-cell epitopes within hepatitis C virus NS3 and NS4 proteins. *Journal of Virology,* 79 (19), 12425-12433.

Germi, R., Crance, J. M., Garin, D., Guimet, J., Lortat-Jacob, H., Ruigrok, R. W., Zarski, J. P. & Drouet, E. (2002) Cellular glycosaminoglycans and low density lipoprotein receptor are involved in hepatitis C virus adsorption. *Journal of Medical Virology,* 68 (2), 206-215.

Ghosn, J., Pierre-Francois, S., Thibault, V., Duvivier, C., Tubiana, R., Simon, A., Valantin, M. A., Dominguez, S., Caumes, E. & Katlama, C. (2004) Acute hepatitis C in HIV-infected men who have sex with men. *HIV Medicine,* 5 (4), 303-306.

Gilleece, Y. C., Browne, R. E., Asboe, D., Atkins, M., Mandalia, S., Bower, M., Gazzard, B. G. & Nelson, M. R. (2005) Transmission of hepatitis C virus among HIV-positive homosexual men and response to a 24-week course of pegylated interferon and ribavirin. *Journal of Acquired Immune Deficiency Syndromes (1999),* 40 (1), 41-46.

Giordano, T. P., Kramer, J. R., Souchek, J., Richardson, P. & El-Serag, H. B. (2004) Cirrhosis and hepatocellular carcinoma in HIV-infected veterans with and without the hepatitis C virus: a cohort study, 1992-2001. *Archives of Internal Medicine,* 164 (21), 2349-2354.

Golden-Mason, L., Burton, J. R.,Jr, Castelblanco, N., Klarquist, J., Benlloch, S., Wang, C. & Rosen, H. R. (2006) Loss of IL-7 receptor alpha-chain (CD127) expression in acute HCV infection associated with viral persistence. *Hepatology (Baltimore, Md.),* 44 (5), 1098-1109.

Graham, C. S., Baden, L. R., Yu, E., Mrus, J. M., Carnie, J., Heeren, T. & Koziel, M. J. (2001) Influence of human immunodeficiency virus infection on the course of hepatitis C virus infection: a meta-analysis. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America,* 33 (4), 562-569.

Grakoui, A., Shoukry, N. H., Woollard, D. J., Han, J. H., Hanson, H. L., Ghrayeb, J., Murthy, K. K., Rice, C. M. & Walker, C. M. (2003) HCV persistence and immune evasion in the absence of memory T cell help. *Science (New York, N.Y.),* 302 (5645), 659-662.

Greub, G., Ledergerber, B., Battegay, M., Grob, P., Perrin, L., Furrer, H., Burgisser, P., Erb, P., Boggian, K., Piffaretti, J. C., Hirschel, B., Janin, P., Francioli, P., Flepp, M. & Telenti, A. (2000) Clinical progression, survival, and immune recovery during antiretroviral therapy in patients with HIV-1 and hepatitis C virus coinfection: the Swiss HIV Cohort Study. *Lancet,* 356 (9244), 1800-1805.

Greub, G., Ledergerber, B., Battegay, M., Grob, P., Perrin, L., Furrer, H., Burgisser, P., Erb, P., Boggian, K., Piffaretti, J. C., Hirschel, B., Janin, P., Francioli, P., Flepp, M. & Telenti, A. (2000) Clinical progression, survival, and immune recovery during antiretroviral therapy in patients with HIV-1 and hepatitis C virus coinfection: the Swiss HIV Cohort Study. *Lancet,* 356 (9244), 1800-1805.

Gruener, N. H., Lechner, F., Jung, M. C., Diepolder, H., Gerlach, T., Lauer, G., Walker, B., Sullivan, J., Phillips, R., Pape, G. R. & Klenerman, P. (2001) Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. *Journal of Virology,* 75 (12), 5550-5558.

Gruner, N. H., Gerlach, T. J., Jung, M. C., Diepolder, H. M., Schirren, C. A., Schraut, W. W., Hoffmann, R., Zachoval, R., Santantonio, T., Cucchiarini, M., Cerny, A. & Pape, G. R. (2000) Association of hepatitis C virus-specific CD8+ T cells with viral clearance in acute hepatitis C. *The Journal of Infectious Diseases,* 181 (5), 1528-1536.

Guglietta, S., Garbuglia, A. R., Pacciani, V., Scotta, C., Perrone, M. P., Laurenti, L., Spada, E., Mele, A., Capobianchi, M. R., Taliani, G., Folgori, A., Vitelli, A., Ruggeri, L., Nicosia, A., Piccolella, E. & Del Porto, P. (2005) Positive selection of cytotoxic T lymphocyte escape variants during acute hepatitis C virus infection. *European Journal of Immunology,* 35 (9), 2627-2637.

Habersetzer, F., Fournillier, A., Dubuisson, J., Rosa, D., Abrignani, S., Wychowski, C., Nakano, I., Trepo, C., Desgranges, C. & Inchauspe, G. (1998) Characterization of human monoclonal antibodies specific to the hepatitis C virus glycoprotein E2 with in vitro binding neutralization properties. *Virology,* 249 (1), 32-41.

Hadziyannis, S. J., Sette, H.,Jr, Morgan, T. R., Balan, V., Diago, M., Marcellin, P., Ramadori, G., Bodenheimer, H.,Jr, Bernstein, D., Rizzetto, M., Zeuzem, S., Pockros, P. J., Lin, A., Ackrill, A. M. & PEGASYS International Study Group. (2004) Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Annals of Internal Medicine,* 140 (5), 346-355.

Hall B.G. (2008) “Phylogenetic Trees Made Easy: A How-To Manual” Third Edition Sunderland, MA Sinauer Associates, Inc.

Hallam, N. F., Fletcher, M. L., Read, S. J., Majid, A. M., Kurtz, J. B. & Rizza, C. R. (1993) Low risk of sexual transmission of hepatitis C virus. *Journal of Medical Virology,* 40 (3), 251-253.

Harcourt, G., Hellier, S., Bunce, M., Satsangi, J., Collier, J., Chapman, R., Phillips, R. & Klenerman, P. (2001) Effect of HLA class II genotype on T helper lymphocyte responses and viral control in hepatitis C virus infection. *Journal of Viral Hepatitis,* 8 (3), 174-179.

Harcourt, G. C., Donfield, S., Gomperts, E., Daar, E. S., Goulder, P.,Jr, Phillips, R. E., Klenerman, P. & Hemophilia Growth and Development Study (HGDS). (2005) Longitudinal analysis of CD8 T-cell responses to HIV and hepatitis C virus in a cohort of co-infected haemophiliacs. *AIDS (London, England),* 19 (11), 1135-1143.

Harcourt, G. C., Lucas, M., Godkin, A. J., Kantzanou, M., Phillips, R. E. & Klenerman, P. (2003) Evidence for lack of cross-genotype protection of CD4+ T cell responses during chronic hepatitis C virus infection. *Clinical and Experimental Immunology,* 131 (1), 122-129.

Heeg, M. H., Ulsenheimer, A., Gruner, N. H., Zachoval, R., Jung, M. C., Gerlach, J. T., Raziorrouh, B., Schraut, W., Horster, S., Kauke, T., Spannagl, M. & Diepolder, H. M. (2009) FOXP3 expression in hepatitis C virus-specific CD4+ T cells during acute hepatitis C. *Gastroenterology,* 137 (4), 1280-8.e1-6.

Herring, B. L., Page-Shafer, K., Tobler, L. H. & Delwart, E. L. (2004) Frequent hepatitis C virus superinfection in injection drug users. *The Journal of Infectious Diseases,* 190 (8), 1396-1403.

Hou, W., Kang, H. S. & Kim, B. S. (2009) Th17 cells enhance viral persistence and inhibit T cell cytotoxicity in a model of chronic virus infection. *The Journal of Experimental Medicine,* 206 (2), 313-328.

Hsu, M., Zhang, J., Flint, M., Logvinoff, C., Cheng-Mayer, C., Rice, C. M. & McKeating, J. A. (2003) Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proceedings of the National Academy of Sciences of the United States of America,* 100 (12), 7271-7276.

Huarte, E., Cubillos-Ruiz, J. R., Nesbeth, Y. C., Scarlett, U. K., Martinez, D. G., Engle, X. A., Rigby, W. F., Pioli, P. A., Guyre, P. M. & Conejo-Garcia, J. R. (2008) PILAR is a novel modulator of human T-cell expansion. *Blood,* 112 (4), 1259-1268.

Jaeckel, E., Cornberg, M., Wedemeyer, H., Santantonio, T., Mayer, J., Zankel, M., Pastore, G., Dietrich, M., Trautwein, C., Manns, M. P. & German Acute Hepatitis C Therapy Group. (2001) Treatment of acute hepatitis C with interferon alfa-2b. *The New England Journal of Medicine,* 345 (20), 1452-1457.

Janeway (2008)" Immunobiology" New York Garland Science

Janssen, H. L., Brouwer, J. T., van der Mast, R. C. & Schalm, S. W. (1994) Suicide associated with alfa-interferon therapy for chronic viral hepatitis. *Journal of Hepatology,* 21 (2), 241-243.

Jenkins, G. M., Worobey, M., Woelk, C. H. & Holmes, E. C. (2001) Evidence for the non-quasispecies evolution of RNA viruses [corrected]. *Molecular Biology and Evolution,* 18 (6), 987-994.

Jin, X., Gao, X., Ramanathan, M.,Jr, Deschenes, G. R., Nelson, G. W., O'Brien, S. J., Goedert, J. J., Ho, D. D., O'Brien, T. R. & Carrington, M. (2002) Human immunodeficiency virus type 1 (HIV-1)-specific CD8+-T-cell responses for groups of HIV-1-infected individuals with different HLA-B*35 genotypes. *Journal of Virology,* 76 (24), 12603-12610.

John, M., Flexman, J. & French, M. A. (1998) Hepatitis C virus-associated hepatitis following treatment of HIV-infected patients with HIV protease inhibitors: an immune restoration disease? *AIDS (London, England),* 12 (17), 2289-2293.

Jopling, C. L., Yi, M., Lancaster, A. M., Lemon, S. M. & Sarnow, P. (2005) Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science (New York, N.Y.),* 309 (5740), 1577-1581.

Jukes, T. H. and Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, *Vol. 3* (H. N. Munro, ed.) 21–132. Academic Press, New York.

Kane, A., Lloyd, J., Zaffran, M., Simonsen, L. & Kane, M. (1999) Transmission of hepatitis B, hepatitis C and human immunodeficiency viruses through unsafe injections in the developing world: model-based regional estimates. *Bulletin of the World Health Organization,* 77 (10), 801-807.

Kantzanou, M., Lucas, M., Barnes, E., Komatsu, H., Dusheiko, G., Ward, S., Harcourt, G. & Klenerman, P. (2003) Viral escape and T cell exhaustion in hepatitis C virus infection analysed using Class I peptide tetramers. *Immunology Letters,* 85 (2), 165-171.

Kaplan, D. E., Ikeda, F., Li, Y., Nakamoto, N., Ganesan, S., Valiga, M. E., Nunes, F. A., Rajender Reddy, K. & Chang, K. M. (2008) Peripheral virus-specific T-cell interleukin-10 responses develop early in

acute hepatitis C infection and become dominant in chronic hepatitis. *Journal of Hepatology,* 48 (6), 903-913.

Kaplan, D. E., Sugimoto, K., Newton, K., Valiga, M. E., Ikeda, F., Aytaman, A., Nunes, F. A., Lucey, M. R., Vance, B. A., Vonderheide, R. H., Reddy, K. R., McKeating, J. A. & Chang, K. M. (2007) Discordant role of CD4 T-cell response relative to neutralizing antibody and CD8 T-cell responses in acute hepatitis C. *Gastroenterology,* 132 (2), 654-666.

Karayiannis, P. & McGarvey, M. J. (1995) The GB hepatitis viruses. *Journal of Viral Hepatitis,* 2 (5), 221-226.

Karuru, J. W., Lule, G. N., Joshi, M. & Anzala, O. (2005) Prevalence of HCV and HCV/HIV co-infection among in-patients at the Kenyatta National Hospital. *East African Medical Journal,* 82 (4), 170-172.

Kasprowicz, V., Schulze Zur Wiesch, J., Kuntzen, T., Nolan, B. E., Longworth, S., Berical, A., Blum, J., McMahon, C., Reyor, L. L., Elias, N., Kwok, W. W., McGovern, B. G., Freeman, G., Chung, R. T., Klenerman, P., Lewis-Ximenez, L., Walker, B. D., Allen, T. M., Kim, A. Y. & Lauer, G. M. (2008) High level of PD-1 expression on hepatitis C virus (HCV)-specific CD8+ and CD4+ T cells during acute HCV infection, irrespective of clinical outcome. *Journal of Virology,* 82 (6), 3154-3160.

Khakoo, S. I., Thio, C. L., Martin, M. P., Brooks, C. R., Gao, X., Astemborski, J., Cheng, J., Goedert, J. J., Vlahov, D., Hilgartner, M., Cox, S., Little, A. M., Alexander, G. J., Cramp, M. E., O'Brien, S. J., Rosenberg, W. M., Thomas, D. L. & Carrington, M. (2004) HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science (New York, N.Y.),* 305 (5685), 872-874.

Kiepiela, P., Ngumbela, K., Thobakgale, C., Ramduth, D., Honeyborne, I., Moodley, E., Reddy, S., de Pierres, C., Mncube, Z., Mkhwanazi, N., Bishop, K., van der Stok, M., Nair, K., Khan, N., Crawford, H., Payne, R., Leslie, A., Prado, J., Prendergast, A., Frater, J., McCarthy, N., Brander, C., Learn, G. H., Nickle, D., Rousseau, C., Coovadia, H., Mullins, J. I., Heckerman, D., Walker, B. D. & Goulder, P. (2007) CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nature Medicine,* 13 (1), 46-53.

Kim, A. Y., Lauer, G. M., Ouchi, K., Addo, M. M., Lucas, M., Schulze Zur Wiesch, J., Timm, J., Boczanowski, M., Duncan, J. E., Wurcel, A. G., Casson, D., Chung, R. T., Draenert, R., Klenerman, P. & Walker, B. D. (2005) The magnitude and breadth of hepatitis C virus-specific CD8+ T cells depend on absolute CD4+ T-cell count in individuals coinfected with HIV-1. *Blood,* 105 (3), 1170-1178.

Kim, A. Y., Schulze zur Wiesch, J., Kuntzen, T., Timm, J., Kaufmann, D. E., Duncan, J. E., Jones, A. M., Wurcel, A. G., Davis, B. T., Gandhi, R. T., Robbins, G. K., Allen, T. M., Chung, R. T., Lauer, G. M. & Walker, B. D. (2006) Impaired hepatitis C virus-specific T cell responses and recurrent hepatitis C virus in HIV coinfection. *PLoS Medicine,* 3 (12), e492.

Kimura, M. (1968) Evolutionary rate at the molecular level. *Nature,* 217 (5129), 624-626.

Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution,* 16 (2), 111-120.

Klenerman, P., Lechner, F., Kantzanou, M., Ciurea, A., Hengartner, H. & Zinkernagel, R. (2000) Viral escape and the failure of cellular immune responses. *Science (New York, N.Y.),* 289 (5487), 2003.

Klenerman, P. & Kim, A. (2007) HCV-HIV coinfection: simple messages from a complex disease. *PLoS Medicine,* 4 (10), e240.

Komatsu, H., Sierro, S., V Cuero, A. & Klenerman, P. (2003) Population analysis of antiviral T cell responses using MHC class I-peptide tetramers. *Clinical and Experimental Immunology,* 134 (1), 9-12.

Korber, B., Muldoon, M., Theiler, J., Gao, F., Gupta, R., Lapedes, A., Hahn, B. H., Wolinsky, S. & Bhattacharya, T. (2000) Timing the ancestor of the HIV-1 pandemic strains. *Science (New York, N.Y.),* 288 (5472), 1789-1796.

Kosakovsky Pond, S. L., Posada, D., Stawiski, E., Chappey, C., Poon, A. F., Hughes, G., Fearnhill, E., Gravenor, M. B., Leigh Brown, A. J. & Frost, S. D. (2009) An evolutionary model-based algorithm for accurate phylogenetic breakpoint mapping and subtype prediction in HIV-1. *PLoS Computational Biology,* 5 (11), e1000581.

Labarga, P., Soriano, V., Vispo, M. E., Pinilla, J., Martin-Carbonero, L., Castellares, C., Casado, R., Maida, I., Garcia-Gasco, P. & Barreiro, P. (2007) Hepatotoxicity of Antiretroviral Drugs Is Reduced after Successful Treatment of Chronic Hepatitis C in HIV-Infected Patients. *The Journal of Infectious Diseases,* 196 (5), 670-676.

Laskus, T., Operskalski, E. A., Radkowski, M., Wilkinson, J., Mack, W. J., deGiacomo, M., Al-Harthi, L., Chen, Z., Xu, J. & Kovacs, A. (2007) Negative-strand hepatitis C virus (HCV) RNA in peripheral blood mononuclear cells from anti-HCV-positive/HIV-infected women. *The Journal of Infectious Diseases,* 195 (1), 124-133.

Lauer, G. M., Lucas, M., Timm, J., Ouchi, K., Kim, A. Y., Day, C. L., Schulze Zur Wiesch, J., Paranhos-Baccala, G., Sheridan, I., Casson, D. R., Reiser, M., Gandhi, R. T., Li, B., Allen, T. M., Chung, R. T., Klenerman, P. & Walker, B. D. (2005) Full-breadth analysis of CD8+ T-cell responses in acute hepatitis C virus infection and early therapy. *Journal of Virology,* 79 (20), 12979-12988.

Laufer, N., Laguno, M., Perez, I., Cifuentes, C., Murillas, J., Vidal, F., Bonet, L., Veloso, S., Gatell, J. M. & Mallolas, J. (2008) Abacavir does not influence the rate of virological response in HIV-HCV-coinfected patients treated with pegylated interferon and weight-adjusted ribavirin. *Antiviral Therapy,* 13 (7), 953-957.

Lechner, F., Gruener, N. H., Urbani, S., Uggeri, J., Santantonio, T., Kammer, A. R., Cerny, A., Phillips, R., Ferrari, C., Pape, G. R. & Klenerman, P. (2000) CD8+ T lymphocyte responses are induced during acute hepatitis C virus infection but are not sustained. *European Journal of Immunology,* 30 (9), 2479-2487.

Lemmers, A., Moreno, C., Gustot, T., Marechal, R., Degre, D., Demetter, P., de Nadai, P., Geerts, A., Quertinmont, E., Vercruysse, V., Le Moine, O. & Deviere, J. (2009) The interleukin-17 pathway is involved in human alcoholic liver disease. *Hepatology (Baltimore, Md.),* 49 (2), 646-657.

Lemey P., Salemi M. & Vandamme A.M. (2009) “The Phylogenetic Handbook” Cambridge Blackwell

Leruez-Ville, M., Kunstmann, J. M., De Almeida, M., Rouzioux, C. & Chaix, M. L. (2000) Detection of hepatitis C virus in the semen of infected men. *Lancet,* 356 (9223), 42-43.

Levin, M. K., Gurjar, M. & Patel, S. S. (2005) A Brownian motor mechanism of translocation and strand separation by hepatitis C virus helicase. *Nature Structural & Molecular Biology,* 12 (5), 429-435.

Li, X. D., Sun, L., Seth, R. B., Pineda, G. & Chen, Z. J. (2005) Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proceedings of the National Academy of Sciences of the United States of America,* 102 (49), 17717-17722.

Lin, C. & Rice, C. M. (1995) The hepatitis C virus NS3 serine proteinase and NS4A cofactor: establishment of a cell-free trans-processing assay. *Proceedings of the National Academy of Sciences of the United States of America,* 92 (17), 7622-7626.

Lindenbach, B. D., Evans, M. J., Syder, A. J., Wolk, B., Tellinghuisen, T. L., Liu, C. C., Maruyama, T., Hynes, R. O., Burton, D. R., McKeating, J. A. & Rice, C. M. (2005) Complete replication of hepatitis C virus in cell culture. *Science (New York, N.Y.),* 309 (5734), 623-626.

Lindenbach, B. D. & Rice, C. M. (2005) Unravelling hepatitis C virus replication from genome to function *Nature,* 436 (7053), 933-938.

Lissen, E., Clumeck, N., Sola, R., Mendes-Correa, M., Montaner, J., Nelson, M., DePamphilis, J., Pessoa, M., Buggisch, P., Main, J. & Dieterich, D. (2006) Histological response to pegIFNalpha-2a (40KD) plus ribavirin in HIV-hepatitis C virus co-infection. *AIDS (London, England),* 20 (17), 2175-2181.

Lo Caputo, S., Trabattoni, D., Vichi, F., Piconi, S., Lopalco, L., Villa, M. L., Mazzotta, F. & Clerici, M. (2003) Mucosal and systemic HIV-1-specific immunity in HIV-1-exposed but uninfected heterosexual men. *AIDS (London, England),* 17 (4), 531-539.

Lohmann, V., Hoffmann, S., Herian, U., Penin, F. & Bartenschlager, R. (2003) Viral and cellular determinants of hepatitis C virus RNA replication in cell culture. *Journal of Virology,* 77 (5), 3007-3019.

Lozach, P. Y., Lortat-Jacob, H., de Lacroix de Lavalette, A., Staropoli, I., Foung, S., Amara, A., Houles, C., Fieschi, F., Schwartz, O., Virelizier, J. L., Arenzana-Seisdedos, F. & Altmeyer, R. (2003) DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. *The Journal of Biological Chemistry,* 278 (22), 20358-20366.

Macquillan, G. C., Niu, X., Speers, D., English, S., Garas, G., Harnett, G. B., Reed, W. D., Allan, J. E. & Jeffrey, G. P. (2004) Does sequencing the PKRBD of hepatitis C virus NS5A predict therapeutic response to combination therapy in an Australian population? *Journal of Gastroenterology and Hepatology,* 19 (5), 551-557.

Maggi, F., Fornai, C., Vatteroni, M. L., Giorgi, M., Morrica, A., Pistello, M., Cammarota, G., Marchi, S., Ciccorossi, P., Bionda, A. & Bendinelli, M. (1997) Differences in hepatitis C virus quasispecies composition between liver, peripheral blood mononuclear cells and plasma. *The Journal of General Virology,* 78 (Pt 7) (Pt 7), 1521-1525.

Major, M. E., Dahari, H., Mihalik, K., Puig, M., Rice, C. M., Neumann, A. U. & Feinstone, S. M. (2004) Hepatitis C virus kinetics and host responses associated with disease and outcome of infection in chimpanzees. *Hepatology (Baltimore, Md.),* 39 (6), 1709-1720.

Malik, A. N., John, L., Bruceson, A. D. & Lockwood, D. N. (2006) Changing pattern of visceral leishmaniasis, United Kingdom, 1985-2004. *Emerging Infectious Diseases,* 12 (8), 1257-1259.

Manns, M. P., McHutchison, J. G., Gordon, S. C., Rustgi, V. K., Shiffman, M., Reindollar, R., Goodman, Z. D., Koury, K., Ling, M. & Albrecht, J. K. (2001) Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet,* 358 (9286), 958-965.

Martin, P., Di Bisceglie, A. M., Kassianides, C., Lisker-Melman, M. & Hoofnagle, J. H. (1989) Rapidly progressive non-A, non-B hepatitis in patients with human immunodeficiency virus infection. *Gastroenterology,* 97 (6), 1559-1561.

Martinez, L. R., Mihu, M. R., Gacser, A., Santambrogio, L. & Nosanchuk, J. D. (2009) Methamphetamine enhances histoplasmosis by immunosuppression of the host. *The Journal of Infectious Diseases,* 200 (1), 131-141.

Matthews, G. V., Hellard, M., Haber, P., Yeung, B., Marks, P., Baker, D., McCaughan, G., Sasadeusz, J., White, P., Rawlinson, W., Lloyd, A., Kaldor, J., Dore, G. J. & Australian Trial in Acute Hepatitis C Study Group. (2009) Characteristics and treatment outcomes among HIV-infected individuals in the Australian Trial in Acute Hepatitis C. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America,* 48 (5), 650-658.

Mauss, S., Valenti, W., DePamphilis, J., Duff, F., Cupelli, L., Passe, S., Solsky, J., Torriani, F. J., Dieterich, D. & Larrey, D. (2004) Risk factors for hepatic decompensation in patients with HIV/HCV coinfection and liver cirrhosis during interferon-based therapy. *AIDS (London, England),* 18 (13), F21-5.

McKiernan, S. M., Hagan, R., Curry, M., McDonald, G. S., Kelly, A., Nolan, N., Walsh, A., Hegarty, J., Lawlor, E. & Kelleher, D. (2004) Distinct MHC class I and II alleles are associated with hepatitis C viral clearance, originating from a single source. *Hepatology (Baltimore, Md.),* 40 (1), 108-114.

McMichael, A. J. & Rowland-Jones, S. L. (2001) Cellular immune responses to HIV. *Nature,* 410 (6831), 980-987.

Mehta, S. H., Thomas, D. L., Torbenson, M., Brinkley, S., Mirel, L., Chaisson, R. E., Moore, R. D. & Sulkowski, M. S. (2005) The effect of antiretroviral therapy on liver disease among adults with HIV and hepatitis C coinfection. *Hepatology (Baltimore, Md.),* 41 (1), 123-131.

Mendel, I., Clotteau, L., Lambert, S. & Buffet-Janvresse, C. (1995) Hepatitis C virus infection in an HIV-positive population in Normandy: antibodies, HCV RNA and genotype prevalence. *Journal of Medical Virology,* 47 (3), 231-236.

Mercenne, G., Bernacchi, S., Richer, D., Bec, G., Henriet, S., Paillart, J. C. & Marquet, R. (2010) HIV-1 Vif binds to APOBEC3G mRNA and inhibits its translation. *Nucleic Acids Research,* 38 (2), 633-646.

Meyer, M. F., Lehmann, M., Cornberg, M., Wiegand, J., Manns, M. P., Klade, C. & Wedemeyer, H. (2007) Clearance of low levels of HCV viremia in the absence of a strong adaptive immune response. *Virology Journal,* 4, 58.

Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Bartenschlager, R. & Tschopp, J. (2005) Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature,* 437 (7062), 1167-1172.

Micallef, J. M., Kaldor, J. M. & Dore, G. J. (2006) Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies. *Journal of Viral Hepatitis,* 13 (1), 34-41.

Migueles, S. A., Sabbaghian, M. S., Shupert, W. L., Bettinotti, M. P., Marincola, F. M., Martino, L., Hallahan, C. W., Selig, S. M., Schwartz, D., Sullivan, J. & Connors, M. (2000) HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proceedings of the National Academy of Sciences of the United States of America,* 97 (6), 2709-2714.

Monazahian, M., Bohme, I., Bonk, S., Koch, A., Scholz, C., Grethe, S. & Thomssen, R. (1999) Low density lipoprotein receptor as a candidate receptor for hepatitis C virus. *Journal of Medical Virology,* 57 (3), 223-229.

Montagnier, L., Chermann, J. C., Barre-Sinoussi, F., Klatzmann, D., Wain-Hobson, S., Alizon, M., Clavel, F., Brun-Vezinet, F., Vilmer, E. & Rouzioux, C. (1984) Lymphadenopathy associated virus and its etiological role in AIDS. *Princess Takamatsu Symposia,* 15, 319-331.

Montal, M. (2003) Structure-function correlates of Vpu, a membrane protein of HIV-1. *FEBS Letters,* 552 (1), 47-53.

Moreau, I., Levis, J., Crosbie, O., Kenny-Walsh, E. & Fanning, L. J. (2008) Correlation between pre-treatment quasispecies complexity and treatment outcome in chronic HCV genotype 3a. *Virology Journal,* 5, 78.

Morikawa, K., Zhao, Z., Date, T., Miyamoto, M., Murayama, A., Akazawa, D., Tanabe, J., Sone, S. & Wakita, T. (2007) The roles of CD81 and glycosaminoglycans in the adsorption and uptake of infectious HCV particles. *Journal of Medical Virology,* 79 (6), 714-723.

Nakagawa, M., Sakamoto, N., Ueyama, M., Mogushi, K., Nagaie, S., Itsui, Y., Azuma, S., Kakinuma, S., Tanaka, H., Enomoto, N. & Watanabe, M. (2010) Mutations in the interferon sensitivity determining region and virological response to combination therapy with pegylated-interferon alpha 2b plus ribavirin in patients with chronic hepatitis C-1b infection. *Journal of Gastroenterology,* , .

Nastouli, E., Thomson, E. C., Karayiannis, P., Main, J., McClure, M. & Muir, D. (2009) Diagnosing acute hepatitis C in HIV-infected patients: nucleic acid testing compared with antibody and antigen-antibody detecting methods. *Journal of Clinical Virology : The Official Publication of the Pan American Society for Clinical Virology,* 44 (1), 78-80.

Nelson, D. R., Tu, Z., Soldevila-Pico, C., Abdelmalek, M., Zhu, H., Xu, Y. L., Cabrera, R., Liu, C. & Davis, G. L. (2003) Long-term interleukin 10 therapy in chronic hepatitis C patients has a proviral and anti-inflammatory effect. *Hepatology (Baltimore, Md.),* 38 (4), 859-868.

Netski, D. M., Mosbruger, T., Astemborski, J., Mehta, S. H., Thomas, D. L. & Cox, A. L. (2007) CD4+ T cell-dependent reduction in hepatitis C virus-specific humoral immune responses after HIV infection. *The Journal of Infectious Diseases,* 195 (6), 857-863.

Netski, D. M., Mosbruger, T., Depla, E., Maertens, G., Ray, S. C., Hamilton, R. G., Roundtree, S., Thomas, D. L., McKeating, J. & Cox, A. (2005) Humoral immune response in acute hepatitis C virus infection. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America,* 41 (5), 667-675.

Neumann, A. U., Lam, N. P., Dahari, H., Gretch, D. R., Wiley, T. E., Layden, T. J. & Perelson, A. S. (1998) Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science (New York, N.Y.),* 282 (5386), 103-107.

Northfield, J. W., Kasprowicz, V., Lucas, M., Kersting, N., Bengsch, B., Kim, A., Phillips, R. E., Walker, B. D., Thimme, R., Lauer, G. & Klenerman, P. (2008) CD161 expression on hepatitis C virus-specific CD8+ T cells suggests a distinct pathway of T cell differentiation. *Hepatology (Baltimore, Md.),* 47 (2), 396-406.

Ohno, T., Mizokami, M., Saleh, M. G., Orito, E., Ohba, K. I., Wu, R. R., Koide, T., Tibbs, C. J., Nouri-Aria, K. T., Tokudome, S. & Williams, R. (1996) Usefulness and limitation of phylogenetic analysis for hepatitis C virus core region: application to isolates from Egyptian and Yemeni patients. *Archives of Virology,* 141 (6), 1101-1113.

Pascu, M., Martus, P., Hohne, M., Wiedenmann, B., Hopf, U., Schreier, E. & Berg, T. (2004) Sustained virological response in hepatitis C virus type 1b infected patients is predicted by the number of mutations within the NS5A-ISDR: a meta-analysis focused on geographical differences. *Gut,* 53 (9), 1345-1351.

Patargias, G., Zitzmann, N., Dwek, R. & Fischer, W. B. (2006) Protein-protein interactions: modeling the hepatitis C virus ion channel p7. *Journal of Medicinal Chemistry,* 49 (2), 648-655.

Patel, A. H., Wood, J., Penin, F., Dubuisson, J. & McKeating, J. A. (2000) Construction and characterization of chimeric hepatitis C virus E2 glycoproteins: analysis of regions critical for glycoprotein aggregation and CD81 binding. *The Journal of General Virology,* 81 (Pt 12), 2873-2883.

Perez-Olmeda, M., Soriano, V., Asensi, V., Morales, D., Romero, M., Ochoa, A., Sanchez-Montero, F., Santin, M., Guardiola, J., Blanch, J., Nunez, M., Jimenez-Nacher, I., Garcia-Samaniego, J. & The HCV/HIV Spanish Study Group. (2003) Treatment of chronic hepatitis C in HIV-infected patients with interferon alpha-2b plus ribavirin. *AIDS Research and Human Retroviruses,* 19 (12), 1083-1089.

Pesce, A., Taillan, B., Rosenthal, E., Garnier, G., Vinti, H., Dujardin, P. & Cassuto, J. P. (1993) Opportunistic infections and CD4 lymphocytopenia with interferon treatment in HIV-1 infected patients. *Lancet,* 341 (8860), 1597.

Peut, V. & Kent, S. J. (2007) Utility of human immunodeficiency virus type 1 envelope as a T-cell immunogen. *Journal of Virology,* 81 (23), 13125-13134.

Pietschmann, T., Lohmann, V., Kaul, A., Krieger, N., Rinck, G., Rutter, G., Strand, D. & Bartenschlager, R. (2002) Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. *Journal of Virology,* 76 (8), 4008-4021.

Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R., Weiner, A. J., Houghton, M., Rosa, D., Grandi, G. & Abrignani, S. (1998) Binding of hepatitis C virus to CD81. *Science (New York, N.Y.),* 282 (5390), 938-941.

Pohlmann, S., Zhang, J., Baribaud, F., Chen, Z., Leslie, G. J., Lin, G., Granelli-Piperno, A., Doms, R. W., Rice, C. M. & McKeating, J. A. (2003) Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. *Journal of Virology,* 77 (7), 4070-4080.

Polyak, S. J., Khabar, K. S., Paschal, D. M., Ezelle, H. J., Duverlie, G., Barber, G. N., Levy, D. E., Mukaida, N. & Gretch, D. R. (2001) Hepatitis C virus nonstructural 5A protein induces interleukin-8, leading to partial inhibition of the interferon-induced antiviral response. *Journal of Virology,* 75 (13), 6095-6106.

Power, J. P., Lawlor, E., Davidson, F., Holmes, E. C., Yap, P. L. & Simmonds, P. (1995) Molecular epidemiology of an outbreak of infection with hepatitis C virus in recipients of anti-D immunoglobulin. *Lancet,* 345 (8959), 1211-1213.

Prati, D., Taioli, E., Zanella, A., Della Torre, E., Butelli, S., Del Vecchio, E., Vianello, L., Zanuso, F., Mozzi, F., Milani, S., Conte, D., Colombo, M. & Sirchia, G. (2002) Updated definitions of healthy ranges for serum alanine aminotransferase levels. *Annals of Internal Medicine,* 137 (1), 1-10.

Preiksaitis, J. K., Cockfield, S. M., Fenton, J. M., Burton, N. I. & Chui, L. W. (1997) Serologic responses to hepatitis C virus in solid organ transplant recipients. *Transplantation,* 64 (12), 1775-1780.

Prendergast, A., Prado, J. G., Kang, Y. H., Chen, F., Riddell, L. A., Luzzi, G., Goulder, P. & Klenerman, P. (2010) HIV-1 infection is characterized by profound depletion of CD161+ Th17 cells and gradual decline in regulatory T cells. *AIDS (London, England),* 24 (4), 491-502.

Pybus, O. G. (2006) Model selection and the molecular clock. *PLoS Biology,* 4 (5), e151.

Quaglio, G. L., Lugoboni, F., Pajusco, B., Sarti, M., Talamini, G., Mezzelani, P., Des Jarlais, D. C. & GICS. (2003) Hepatitis C virus infection: prevalence, predictor variables and prevention opportunities among drug users in Italy. *Journal of Viral Hepatitis,* 10 (5), 394-400.

Quaranta, J. F., Delaney, S. R., Alleman, S., Cassuto, J. P., Dellamonica, P. & Allain, J. P. (1994) Prevalence of antibody to hepatitis C virus (HCV) in HIV-1-infected patients (nice SEROCO cohort). *Journal of Medical Virology,* 42 (1), 29-32.

Qurishi, N., Kreuzberg, C., Luchters, G., Effenberger, W., Kupfer, B., Sauerbruch, T., Rockstroh, J. K. & Spengler, U. (2003) Effect of antiretroviral therapy on liver-related mortality in patients with HIV and hepatitis C virus coinfection. *Lancet,* 362 (9397), 1708-1713.

Ramos, B., Nunez, M., Rendon, A., Berdun, M. A., Losada, E., Santos, I., Echevarria, S., Ocampo, A., Miralles, C., Arazo, P., Barreiro, P., Romero, M., Labarga, P., Guardiola, J. M., Garcia-Samaniego, J. & Soriano, V. (2007) Critical role of ribavirin for the achievement of early virological response to HCV therapy in HCV/HIV-coinfected patients. *Journal of Viral Hepatitis,* 14 (6), 387-391.

Rauch, A., Rickenbach, M., Weber, R., Hirschel, B., Tarr, P. E., Bucher, H. C., Vernazza, P., Bernasconi, E., Zinkernagel, A. S., Evison, J., Furrer, H. & Swiss HIV Cohort Study. (2005) Unsafe sex and increased incidence of hepatitis C virus infection among HIV-infected men who have sex with men: the Swiss HIV Cohort Study. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America,* 41 (3), 395-402.

Roque Afonso, A. M., Jiang, J., Penin, F., Tareau, C., Samuel, D., Petit, M. A., Bismuth, H., Dussaix, E. & Feray, C. (1999) Nonrandom distribution of hepatitis C virus quasispecies in plasma and peripheral blood mononuclear cell subsets. *Journal of Virology,* 73 (11), 9213-9221.

Ruys, T. A., den Hollander, J. G., Beld, M. G., van der Ende, M. E. & van der Meer, J. T. (2004) Sexual transmission of hepatitis C in homosexual men]. *Nederlands Tijdschrift Voor Geneeskunde,* 148 (47), 2309-2312.

Ruys, T. A., Nanlohy, N. M., van den Berg, C. H., Hassink, E., Beld, M., van de Laar, T., Bruisten, S., Wit, F., Krol, A., Prins, M., Lange, J. & van Baarle, D. (2008) HCV-specific T-cell responses in injecting drug users: evidence for previous exposure to HCV and a role for CD4+ T cells focussing on nonstructural proteins in viral clearance. *Journal of Viral Hepatitis,* 15 (6), 409-420.

Ryan, E. L., Morgello, S., Isaacs, K., Naseer, M., Gerits, P. & The Manhattan HIV Brain Bank. (2004) Neuropsychiatric impact of hepatitis C on advanced HIV. *Neurology,* 62 (6), 957-962.

Sanchez-Quijano, A., Andreu, J., Gavilan, F., Luque, F., Abad, M. A., Soto, B., Munoz, J., Aznar, J. M., Leal, M. & Lissen, E. (1995) Influence of human immunodeficiency virus type 1 infection on the natural course of chronic parenterally acquired hepatitis C. *European Journal of Clinical Microbiology & Infectious Diseases : Official Publication of the European Society of Clinical Microbiology,* 14 (11), 949-953.

Sarobe, P., Lasarte, J. J., Garcia, N., Civeira, M. P., Borras-Cuesta, F. & Prieto, J. (2006) Characterization of T-cell responses against immunodominant epitopes from hepatitis C virus E2 and NS4a proteins. *Journal of Viral Hepatitis,* 13 (1), 47-55.

Scarselli, E., Ansuini, H., Cerino, R., Roccasecca, R. M., Acali, S., Filocamo, G., Traboni, C., Nicosia, A., Cortese, R. & Vitelli, A. (2002) The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *The EMBO Journal,* 21 (19), 5017-5025.

Schnuriger, A., Dominguez, S., Guiguet, M., Harfouch, S., Samri, A., Ouazene, Z., Slama, L., Simon, A., Valantin, M. A., Thibault, V., Autran, B. & ANRS HC EP21 study group. (2009) Acute hepatitis C in HIV-infected patients: rare spontaneous clearance correlates with weak memory CD4 T-cell responses to hepatitis C virus. *AIDS (London, England),* 23 (16), 2079-2089.

Schroeter, M., Zoellner, B., Polywka, S., Laufs, R. & Feucht, H. H. (2005) Prolonged time until seroconversion among hemodialysis patients: the need for HCV PCR. *Intervirology,* 48 (4), 213-215.

Schwarz, M., Wells, T. N. & Proudfoot, A. E. (2001) Chemokine receptors--the next therapeutic target for HIV? *Receptors & Channels,* 7 (6), 417-428.

Scott, C., Day, S., Low, E., Sullivan, A., Atkins, M. & Asboe, D. (2010) Unselected hepatitis C screening of men who have sex with men attending sexual health clinics. *The Journal of Infection,* 60(5), 351-3.

Semmo, N., Day, C. L., Ward, S. M., Lucas, M., Harcourt, G., Loughry, A. & Klenerman, P. (2005) Preferential loss of IL-2-secreting CD4+ T helper cells in chronic HCV infection. *Hepatology (Baltimore, Md.),* 41 (5), 1019-1028.

Semmo, N. & Klenerman, P. (2007) CD4+ T cell responses in hepatitis C virus infection. *World Journal of Gastroenterology : WJG,* 13 (36), 4831-4838.

Serpaggi, J., Chaix, M. L., Batisse, D., Dupont, C., Vallet-Pichard, A., Fontaine, H., Viard, J. P., Piketty, C., Rouveix, E., Rouzioux, C., Weiss, L. & Pol, S. (2006) Sexually transmitted acute infection with a clustered genotype 4 hepatitis C virus in HIV-1-infected men and inefficacy of early antiviral therapy. *AIDS (London, England),* 20 (2), 233-240.

Sheridan, I., Pybus, O. G., Holmes, E. C. & Klenerman, P. (2004) High-resolution phylogenetic analysis of hepatitis C virus adaptation and its relationship to disease progression. *Journal of Virology,* 78 (7), 3447-3454.

Sherman, K. E., Rouster, S. D., Chung, R. T. & Rajicic, N. (2002) Hepatitis C Virus prevalence among patients infected with Human Immunodeficiency Virus: a cross-sectional analysis of the US adult AIDS Clinical Trials Group. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America,* 34 (6), 831-837.

Shimizu, Y. K., Igarashi, H., Kanematu, T., Fujiwara, K., Wong, D. C., Purcell, R. H. & Yoshikura, H. (1997) Sequence analysis of the hepatitis C virus genome recovered from serum, liver, and peripheral blood mononuclear cells of infected chimpanzees. *Journal of Virology,* 71 (8), 5769-5773.

Shimizu, Y. K., Igarashi, H., Kiyohara, T., Cabezon, T., Farci, P., Purcell, R. H. & Yoshikura, H. (1996) A hyperimmune serum against a synthetic peptide corresponding to the hypervariable region 1 of hepatitis C virus can prevent viral infection in cell cultures. *Virology,* 223 (2), 409-412.

Shin, E. C., Capone, S., Cortese, R., Colloca, S., Nicosia, A., Folgori, A. & Rehermann, B. (2008) The kinetics of hepatitis C virus-specific CD8 T-cell responses in the blood mirror those in the liver in acute hepatitis C virus infection. *Journal of Virology,* 82 (19), 9782-9788.

Shire, N. J., Horn, P. S., Rouster, S. D., Stanford, S., Eyster, M. E., Sherman, K. E. & Multicenter Hemophilia Cohort HCV Study Group. (2006) HCV kinetics, quasispecies, and clearance in treated HCV-infected and HCV/HIV-1-coinfected patients with hemophilia. *Hepatology (Baltimore, Md.),* 44 (5), 1146-1157.

Shores, N. J., Maida, I., Soriano, V. & Nunez, M. (2008) Sexual transmission is associated with spontaneous HCV clearance in HIV-infected patients. *Journal of Hepatology,* 49 (3), 323-328.

Shoukry, N. H., Cawthon, A. G. & Walker, C. M. (2004) Cell-mediated immunity and the outcome of hepatitis C virus infection. *Annual Review of Microbiology,* 58, 391-424.

Simmonds, P. (2001) Reconstructing the origins of human hepatitis viruses. *Philosophical Transactions of the Royal Society of London.Series B, Biological Sciences,* 356 (1411), 1013-1026.

Simmonds, P., Smith, D. B., McOmish, F., Yap, P. L., Kolberg, J., Urdea, M. S. & Holmes, E. C. (1994) Identification of genotypes of hepatitis C virus by sequence comparisons in the core, E1 and NS-5 regions. *The Journal of General Virology,* 75 (Pt 5) (Pt 5), 1053-1061.

Singal, A. K. & Anand, B. S. (2009) Management of hepatitis C virus infection in HIV/HCV co-infected patients: clinical review. *World Journal of Gastroenterology : WJG,* 15 (30), 3713-3724.

Smith, B. C., Chapman, C. E., Burt, A. D., Toms, G. L. & Bassendine, M. F. (1997) Outcome of post-transfusion hepatitis C: disease severity in blood-component recipients and their implicated donors. *QJM : Monthly Journal of the Association of Physicians,* 90 (9), 587-592.

Smith, D. B., Pathirana, S., Davidson, F., Lawlor, E., Power, J., Yap, P. L. & Simmonds, P. (1997) The origin of hepatitis C virus genotypes. *The Journal of General Virology,* 78 (Pt 2) (Pt 2), 321-328.

Smith, J. A., Aberle, J. H., Fleming, V. M., Ferenci, P., Thomson, E. C., Karayiannis, P., McLean, A. R., Holzmann, H. & Klenerman, P. (2010) Dynamic coinfection with multiple viral subtypes in acute hepatitis C. *The Journal of Infectious Diseases,* 202 (12), 1770-1779.

Smyk-Pearson, S., Tester, I. A., Klarquist, J., Palmer, B. E., Pawlotsky, J. M., Golden-Mason, L. & Rosen, H. R. (2008) Spontaneous recovery in acute human hepatitis C virus infection: functional T-cell thresholds and relative importance of CD4 help. *Journal of Virology,* 82 (4), 1827-1837.

Sobesky, R., Feray, C., Rimlinger, F., Derian, N., Dos Santos, A., Roque-Afonso, A. M., Samuel, D., Brechot, C. & Thiers, V. (2007) Distinct hepatitis C virus core and F protein quasispecies in tumoral and nontumoral hepatocytes isolated via microdissection. *Hepatology (Baltimore, Md.),* 46 (6), 1704-1712.

Soriano, V., Garcia-Samaniego, J., Bravo, R., Castro, A., Odriozola, P. M., Gonzalez, J., Colmenero, M., Carballo, E., Suarez, D. & Llibre, J. M. (1995) Efficacy and safety of alpha-interferon treatment for chronic hepatitis C in HIV-infected patients. HIV-Hepatitis Spanish Study Group. *The Journal of Infection,* 31 (1), 9-13.

Strasfeld, L., Lo, Y., Netski, D., Thomas, D. L. & Klein, R. S. (2003) The association of hepatitis C prevalence, activity, and genotype with HIV infection in a cohort of New York City drug users. *Journal of Acquired Immune Deficiency Syndromes (1999),* 33 (3), 356-364.

Stumptner-Cuvelette, P., Morchoisne, S., Dugast, M., Le Gall, S., Raposo, G., Schwartz, O. & Benaroch, P. (2001) HIV-1 Nef impairs MHC class II antigen presentation and surface expression. *Proceedings of the National Academy of Sciences of the United States of America,* 98 (21), 12144-12149.

Sulkowski, M. S., Thomas, D. L., Mehta, S. H., Chaisson, R. E. & Moore, R. D. (2002) Hepatotoxicity associated with nevirapine or efavirenz-containing antiretroviral therapy: role of hepatitis C and B infections. *Hepatology (Baltimore, Md.),* 35 (1), 182-189.

Suzich, J. A., Tamura, J. K., Palmer-Hill, F., Warrener, P., Grakoui, A., Rice, C. M., Feinstone, S. M. & Collett, M. S. (1993) Hepatitis C virus NS3 protein polynucleotide-stimulated nucleoside triphosphatase and comparison with the related pestivirus and flavivirus enzymes. *Journal of Virology,* 67 (10), 6152-6158.

Suzuki, H., Kaneko, H., Tamai, N., Miyano-Kurosaki, N., Hashimoto, K., Shimotohno, K. & Takaku, H. (2005) Suppression of HCV RNA replication by baculovirus-mediated shRNA expression. *Nucleic Acids Symposium Series (2004),* (49) (49), 339-340.

Tajima (1989) Statistical Method for Testing the Neutral Mutation Hypothesis by DNA Polymorphism *Genetics* 123 (3): 585

Takaki, A., Koike, K. & Shiratori, Y. (2004) Mechanisms of the immune surveillance against HCV infection. *Nippon Rinsho.Japanese Journal of Clinical Medicine,* 62 Suppl 7 (Pt 1), 143-146.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. *Molecular Biology and Evolution,* 24 (8), 1596-1599.

Taylor, D. R., Shi, S. T., Romano, P. R., Barber, G. N. & Lai, M. M. (1999) Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. *Science (New York, N.Y.),* 285 (5424), 107-110.

Telfer, P., Sabin, C., Devereux, H., Scott, F., Dusheiko, G. & Lee, C. (1994) The progression of HCV-associated liver disease in a cohort of haemophilic patients. *British Journal of Haematology,* 87 (3), 555-561.

Thimme, R., Oldach, D., Chang, K. M., Steiger, C., Ray, S. C. & Chisari, F. V. (2001) Determinants of viral clearance and persistence during acute hepatitis C virus infection. *The Journal of Experimental Medicine,* 194 (10), 1395-1406.

Thomas, D. L., Thio, C. L., Martin, M. P., Qi, Y., Ge, D., O'Huigin, C., Kidd, J., Kidd, K., Khakoo, S. I., Alexander, G., Goedert, J. J., Kirk, G. D., Donfield, S. M., Rosen, H. R., Tobler, L. H., Busch, M. P., McHutchison, J. G., Goldstein, D. B. & Carrington, M. (2009) Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature,* 461 (7265), 798-801.

Thomas, D. L., Astemborski, J., Rai, R. M., Anania, F. A., Schaeffer, M., Galai, N., Nolt, K., Nelson, K. E., Strathdee, S. A., Johnson, L., Laeyendecker, O., Boitnott, J., Wilson, L. E. & Vlahov, D. (2000) The natural history of hepatitis C virus infection: host, viral, and environmental factors. *JAMA : The Journal of the American Medical Association,* 284 (4), 450-456.

Thomson, E. C., Nastouli, E., Main, J., Karayiannis, P., Eliahoo, J., Muir, D. & McClure, M. O. (2009) Delayed anti-HCV antibody response in HIV-positive men acutely infected with HCV. *AIDS (London, England),* 23 (1), 89-93.

Thomson, M., Nascimbeni, M., Havert, M. B., Major, M., Gonzales, S., Alter, H., Feinstone, S. M., Murthy, K. K., Rehermann, B. & Liang, T. J. (2003) The clearance of hepatitis C virus infection in chimpanzees may not necessarily correlate with the appearance of acquired immunity. *Journal of Virology,* 77 (2), 862-870.

Thursz, M., Yallop, R., Goldin, R., Trepo, C. & Thomas, H. C. (1999) Influence of MHC class II genotype on outcome of infection with hepatitis C virus. The HENCORE group. Hepatitis C European Network for Cooperative Research. *Lancet,* 354 (9196), 2119-2124.

Timm, J., Lauer, G. M., Kavanagh, D. G., Sheridan, I., Kim, A. Y., Lucas, M., Pillay, T., Ouchi, K., Reyor, L. L., Schulze zur Wiesch, J., Gandhi, R. T., Chung, R. T., Bhardwaj, N., Klenerman, P., Walker, B. D. & Allen, T. M. (2004) CD8 epitope escape and reversion in acute HCV infection. *The Journal of Experimental Medicine,* 200 (12), 1593-1604.

Tindall, K. R. & Kunkel, T. A. (1988) Fidelity of DNA synthesis by the Thermus aquaticus DNA polymerase. *Biochemistry,* 27 (16), 6008-6013.

Tomei, L., Failla, C., Santolini, E., De Francesco, R. & La Monica, N. (1993) NS3 is a serine protease required for processing of hepatitis C virus polyprotein. *Journal of Virology,* 67 (7), 4017-4026.

Torriani, F. J., Rodriguez-Torres, M., Rockstroh, J. K., Lissen, E., Gonzalez-Garcia, J., Lazzarin, A., Carosi, G., Sasadeusz, J., Katlama, C., Montaner, J., Sette, H.,Jr, Passe, S., De Pamphilis, J., Duff, F., Schrenk, U. M., Dieterich, D. T. & APRICOT Study Group. (2004) Peginterferon Alfa-2a plus ribavirin for chronic hepatitis C virus infection in HIV-infected patients. *The New England Journal of Medicine,* 351 (5), 438-450.

Toussi, S. S., Joseph, A., Zheng, J. H., Dutta, M., Santambrogio, L. & Goldstein, H. (2009) Short communication: Methamphetamine treatment increases in vitro and in vivo HIV replication. *AIDS Research and Human Retroviruses,* 25 (11), 1117-1121.

Trkola, A., Purtscher, M., Muster, T., Ballaun, C., Buchacher, A., Sullivan, N., Srinivasan, K., Sodroski, J., Moore, J. P. & Katinger, H. (1996) Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. *Journal of Virology,* 70 (2), 1100-1108.

Tuplin, A., Evans, D. J. & Simmonds, P. (2004) Detailed mapping of RNA secondary structures in core and NS5B-encoding region sequences of hepatitis C virus by RNase cleavage and novel bioinformatic prediction methods. *The Journal of General Virology,* 85 (Pt 10), 3037-3047.

Tural, C., Fuster, D., Tor, J., Ojanguren, I., Sirera, G., Ballesteros, A., Lasanta, J. A., Planas, R., Rey-Joly, C. & Clotet, B. (2003) Time on antiretroviral therapy is a protective factor for liver fibrosis in HIV and hepatitis C virus (HCV) co-infected patients. *Journal of Viral Hepatitis,* 10 (2), 118-125.

Ueda, E., Enomoto, N., Sakamoto, N., Hamano, K., Sato, C., Izumi, N. & Watanabe, M. (2004) Changes of HCV quasispecies during combination therapy with interferon and ribavirin. *Hepatology Research : The Official Journal of the Japan Society of Hepatology,* 29 (2), 89-96.

Urbani, S., Amadei, B., Fisicaro, P., Tola, D., Orlandini, A., Sacchelli, L., Mori, C., Missale, G. & Ferrari, C. (2006) Outcome of acute hepatitis C is related to virus-specific CD4 function and maturation of antiviral memory CD8 responses. *Hepatology (Baltimore, Md.),* 44 (1), 126-139.

Urbani, S., Amadei, B., Tola, D., Pedrazzi, G., Sacchelli, L., Cavallo, M. C., Orlandini, A., Missale, G. & Ferrari, C. (2008) Restoration of HCV-specific T cell functions by PD-1/PD-L1 blockade in HCV infection: effect of viremia levels and antiviral treatment. *Journal of Hepatology,* 48 (4), 548-558.

van de Laar, T., Pybus, O., Bruisten, S., Brown, D., Nelson, M., Bhagani, S., Vogel, M., Baumgarten, A., Chaix, M. L., Fisher, M., Gotz, H., Matthews, G. V., Neifer, S., White, P., Rawlinson, W., Pol, S., Rockstroh, J., Coutinho, R., Dore, G. J., Dusheiko, G. M. & Danta, M. (2009) Evidence of a large, international network of HCV transmission in HIV-positive men who have sex with men. *Gastroenterology,* 136 (5), 1609-1617.

van de Laar, T. J., van der Bij, A. K., Prins, M., Bruisten, S. M., Brinkman, K., Ruys, T. A., van der Meer, J. T., de Vries, H. J., Mulder, J. W., van Agtmael, M., Jurriaans, S., Wolthers, K. C. & Coutinho, R. A. (2007) Increase in HCV incidence among men who have sex with men in Amsterdam most likely caused by sexual transmission. *The Journal of Infectious Diseases,* 196 (2), 230-238.

van den Berg, C. H., Ruys, T. A., Nanlohy, N. M., Geerlings, S. E., van der Meer, J. T., Mulder, J. W., Lange, J. A. & van Baarle, D. (2009) Comprehensive longitudinal analysis of hepatitis C virus (HCV)-specific T cell responses during acute HCV infection in the presence of existing HIV-1 infection. *Journal of Viral Hepatitis,* 16 (4), 239-248.

Vandelli, C., Renzo, F., Romano, L., Tisminetzky, S., De Palma, M., Stroffolini, T., Ventura, E. & Zanetti, A. (2004) Lack of evidence of sexual transmission of hepatitis C among monogamous couples: results of a 10-year prospective follow-up study. *The American Journal of Gastroenterology,* 99 (5), 855-859.

Vento, S., Di Perri, G., Cruciani, M., Garofano, T., Concia, E. & Bassetti, D. (1993) Rapid decline of CD4+ cells after IFN alpha treatment in HIV-1 infection. *Lancet,* 341 (8850), 958-959.

Verma, S., Wang, C. H., Govindarajan, S., Kanel, G., Squires, K. & Bonacini, M. (2006) Do type and duration of antiretroviral therapy attenuate liver fibrosis in HIV-hepatitis C virus-coinfected patients? *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America,* 42 (2), 262-270.

Vispo, E., Barreiro, P., Pineda, J. A., Mira, J. A., Maida, I., Martin-Carbonero, L., Rodriguez-Novoa, S., Santos, I., Lopez-Cortes, L. F., Merino, D., Rivero, A. & Soriano, V. (2008) Low response to pegylated interferon plus ribavirin in HIV-infected patients with chronic hepatitis C treated with abacavir. *Antiviral Therapy,* 13 (3), 429-437.

Vogel, M., Bieniek, B., Jessen, H., Schewe, C. K., Hoffmann, C., Baumgarten, A., Kroidl, A., Bogner, J. R., Spengler, U. & Rockstroh, J. K. (2005) Treatment of acute hepatitis C infection in HIV-infected patients: a retrospective analysis of eleven cases. *Journal of Viral Hepatitis,* 12 (2), 207-211.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H. G., Mizokami, M., Bartenschlager, R. & Liang, T. J. (2005) Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nature Medicine,* 11 (7), 791-796.

Walker, B. D. (2007) Elite control of HIV Infection: implications for vaccines and treatment. *Topics in HIV Medicine : A Publication of the International AIDS Society, USA,* 15 (4), 134-136.

Wardell, A. D., Errington, W., Ciaramella, G., Merson, J. & McGarvey, M. J. (1999) Characterization and mutational analysis of the helicase and NTPase activities of hepatitis C virus full-length NS3 protein. *The Journal of General Virology,* 80 (Pt 3) (Pt 3), 701-709.

Weiner, A. J., Brauer, M. J., Rosenblatt, J., Richman, K. H., Tung, J., Crawford, K., Bonino, F., Saracco, G., Choo, Q. L. & Houghton, M. (1991) Variable and hypervariable domains are found in the regions of HCV corresponding to the flavivirus envelope and NS1 proteins and the pestivirus envelope glycoproteins. *Virology,* 180 (2), 842-848.

Wiese, M., Grungreiff, K., Guthoff, W., Lafrenz, M., Oesen, U., Porst, H. & East German Hepatitis C Study Group. (2005) Outcome in a hepatitis C (genotype 1b) single source outbreak in Germany--a 25-year multicenter study. *Journal of Hepatology,* 43 (4), 590-598.

Wilson, J. D., Ogg, G. S., Allen, R. L., Davis, C., Shaunak, S., Downie, J., Dyer, W., Workman, C., Sullivan, S., McMichael, A. J. & Rowland-Jones, S. L. (2000) Direct visualization of HIV-1-specific cytotoxic T lymphocytes during primary infection. *AIDS (London, England),* 14 (3), 225-233.

Wolk, B., Sansonno, D., Krausslich, H. G., Dammacco, F., Rice, C. M., Blum, H. E. & Moradpour, D. (2000) Subcellular localization, stability, and trans-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines. *Journal of Virology,* 74 (5), 2293-2304.

Woollard, D. J., Grakoui, A., Shoukry, N. H., Murthy, K. K., Campbell, K. J. & Walker, C. M. (2003) Characterization of HCV-specific Patr class II restricted CD4+ T cell responses in an acutely infected chimpanzee. *Hepatology (Baltimore, Md.),* 38 (5), 1297-1306.

Worobey, M., Gemmel, M., Teuwen, D. E., Haselkorn, T., Kunstman, K., Bunce, M., Muyembe, J. J., Kabongo, J. M., Kalengayi, R. M., Van Marck, E., Gilbert, M. T. & Wolinsky, S. M. (2008) Direct evidence of extensive diversity of HIV-1 in Kinshasa by 1960. *Nature,* 455 (7213), 661-664.

Yasumi, Y., Takikawa, Y., Endo, R. & Suzuki, K. (2007) Interleukin-17 as a new marker of severity of acute hepatic injury. *Hepatology Research : The Official Journal of the Japan Society of Hepatology,* 37 (4), 248-254.

Yu, Q., Zhang, D., Walston, M., Zhang, J., Liu, Y. & Watson, R. R. (2002) Chronic methamphetamine exposure alters immune function in normal and retrovirus-infected mice. *International Immunopharmacology,* 2 (7), 951-962.

Zajac, A. J., Blattman, J. N., Murali-Krishna, K., Sourdive, D. J., Suresh, M., Altman, J. D. & Ahmed, R. (1998) Viral immune evasion due to persistence of activated T cells without effector function. *The Journal of Experimental Medicine,* 188 (12), 2205-2213.

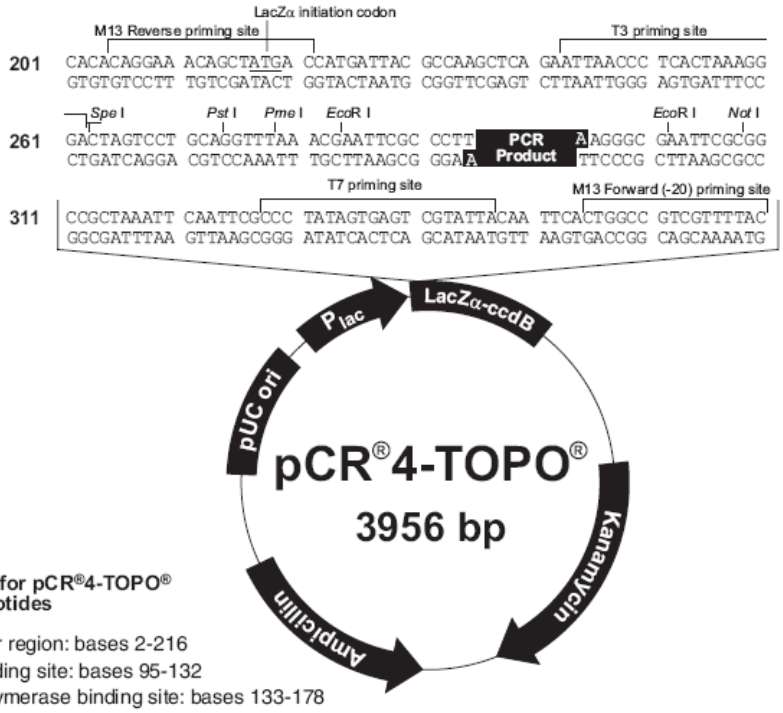
Zhang, J., Randall, G., Higginbottom, A., Monk, P., Rice, C. M. & McKeating, J. A. (2004) CD81 is required for hepatitis C virus glycoprotein-mediated viral infection. *Journal of Virology,* 78 (3), 1448-1455.

Zhang, J. Y., Zhang, Z., Lin, F., Zou, Z. S., Xu, R. N., Jin, L., Fu, J. L., Shi, F., Shi, M., Wang, H. F. & Wang, F. S. (2010) Interleukin-17-producing CD4(+) T cells increase with severity of liver damage in patients with chronic hepatitis B. *Hepatology (Baltimore, Md.),* 51 (1), 81-91.

Zibert, A., Kraas, W., Ross, R. S., Meisel, H., Lechner, S., Jung, G. & Roggendorf, M. (1999) Immunodominant B-cell domains of hepatitis C virus envelope proteins E1 and E2 identified during early and late time points of infection. *Journal of Hepatology,* 30 (2), 177-184.

Zimmerli SC, Harari A, Cellerai C, Vallelian F, Bart PA & Pantaleo G. HIV-1-specific IFN-gamma/IL-2-secreting CD8 T cells support CD4-independent proliferation of HIV-1-specific CD8 T cells. (2005) *Proc Natl Acad Sci*, 102(20), 7239-44.

Appendix 1: Vector map TOPO-4



Comments for pCR®4-TOPO®
3956 nucleotides

lac promoter region: bases 2-216
  CAP binding site: bases 95-132
  RNA polymerase binding site: bases 133-178
  Lac repressor binding site: bases 179-199
Start of transcription: base 179
M13 Reverse priming site: bases 205-221
LacZα-*ccdB* gene fusion: bases 217-810
  LacZα portion of fusion: bases 217-497
  *ccd*B portion of fusion: bases 508-810
T3 priming site: bases 243-262
TOPO® Cloning site: bases 294-295
T7 priming site: bases 328-347
M13 Forward (-20) priming site: bases 355-370
Kanamycin promoter: bases 1021-1070
Kanamycin resistance gene: bases 1159-1953
Ampicillin (*bla*) resistance gene: bases 2203-3063 (c)
Ampicillin (*bla*) promoter: bases 3064-3160 (c)
pUC origin: bases 3161-3834
(c) = complementary strand

Appendix 2: Python programme for cropping sequences

```
#!/usr/local/bin/python
################################################################################
####
# Sequence parsing module
#
# Crops sequence files in the following way:
# 1) removes left and right primer portions
# 2) copies "good" sequences
# 3) output is three text files:
#       a) cropped_sequences.fa (fasta file containing all cropped good sequences)
#       b) list_good_sequences.log
#       c) list_bad_sequences.log
#       d) bad_sequences.fa (fasta file with all bad seqs)
# must be run from the directory containing the sequence files.
#
# Sequence files must be recognizable by a unique extension, e.g. ".seq"
#
# 2009 edition by e.thomson@imperial.ac.uk based on programme by p.cherepanov@imperial.ac.uk
2007
################################################################################
####

print "sequence parsing module; will search through a list of sequence files, looking for forward and
reverse primer seuences and count/crop them"
print "requires sequence recognizable by a unique extention, for example:"
print "sequence1.seq, sequence2.seq, run125.seq, blabla.seq, etc"

import os, re, string
from string import maketrans, translate, join

seq_file_extention = raw_input('sequence file extension? For example, type seq for
sequence.seq:\n')
seq_file_extention = '.*' + seq_file_extention + '$'

cropped_sequences = open('cropped_sequences.fa', 'w')
bad_sequences = open('bad_sequences.fa', 'w')
#primer_product_sequences = open('primer_product_sequences.fa', 'w')
list_good_sequences = open('list_good_sequences.log', 'w')
list_bad_sequences = open('list_bad_sequences.log', 'w')
#list_primer_product_sequences = open('list_primer_product_sequences', 'w')
list_good_sequences.write('list of good files:')
list_good_sequences.write('\n')
list_good_sequences.write('-'*60)
list_good_sequences.write('\n')
list_bad_sequences.write('list of bad files:')
list_bad_sequences.write('\n')
list_bad_sequences.write('*'*60)
list_bad_sequences.write('\n')
number_of_good_sequences = 0
number_of_bad_sequences = 0
```

```
number_of_primer_product_sequences = 0

seq_file_count = 0    #sequence counter

trans = maketrans("acgttumrwsykvhdbnACGTTUMRWSYKVHDBN",
"TGCAAAKYWSRMBDHVNTGCAAAKYWSRMBDHVN")  #reverse complement rules

def reversecomp(sequence):
        sequence_list = list(sequence)
        sequence_list.reverse()
        reversed_sequence = join(sequence_list, '')
        reverse_complement_sequence = translate(reversed_sequence, trans)
        return reverse_complement_sequence

forward1 =
re.compile('(ATGATGAACTGG)|(NTGATGAACTGG)|(ANGATGAACTGG)|(ATNATGAACTGG)|(ATGNTG
AACTGG)|(ATGANGAACTGG)|(ATGATNAACTGG)|(ATGATGNACTGG)|(ATGATGANCTGG)|(ATGATGAA
NTGG)|(ATGATGAACNGG)|(ATGATGAACTNG)|(ATGATGAACTGN)', re.IGNORECASE)
forward2 =
re.compile('(GTCCTGGCGGGC)|(NTCCTGGCGGGC)|(GNCCTGGCGGGC)|(GTNCTGGCGGGC)|(GTCNTG
GCGGGC)|(GTCCNGGCGGGC)|(GTCCTNGCGGGC)|(GTCCTGNCGGGC)|(GTCCTGGNGGGC)|(GTCCTGG
CNGGC)|(GTCCTGGCGNGC)|(GTCCTGGCGGNC)|(GTCCTGGCGGGN)', re.IGNORECASE)

reverse1 =
re.compile('(ACATCAA[CTN]AGCA)|(ANATCAA[CT]AGCA)|(ACNTCAA[CT]AGCA)|(ACANCAA[CT]AGCA)
|(ACATNAA[CT]AGCA)|(ACATCNA[CT]AGCA)|(ACATCAN[CT]AGCA)|(ACATCAA[CT]NGCA)|(ACATCAA[
CT]ANCA)|(ACATCAA[CT]AGNA)|(ACATCAA[CT]AGCN)', re.IGNORECASE)
reverse2 =
re.compile('(GGCATATAAATA)|(GNCATATAAATA)|(GGNATATAAATA)|(GGCNTATAAATA)|(GGCANAT
AAATA)|(GGCANATAAATA)|(GGCATNTAAATA)|(GGCATANAAATA)|(GGCATATNAATA)|(GGCATATAN
ATA)|(GGCATATAANTA)|(GGCATATAAANA)|(GGCATATAAATN)', re.IGNORECASE)


for fname in os.listdir(os.getcwd()):
        if re.compile(seq_file_extention).match(fname):
                seq_file_count = seq_file_count + 1
                current_seq_file = open(fname, 'r')
                seq_dirty = current_seq_file.read()
                oseq = seq_dirty.replace('\n', '')    #removing new line characters, if any; oseq = the
original sequence
                LTR_end = -99
                LINKER_pos = -99
                seq = oseq
                rseq = reversecomp(seq)

                if (forward1.search(seq) or forward2.search(seq) or forward1.search(rseq) or
forward2.search(rseq)) and (reverse1.search(seq) or reverse2.search(seq) or reverse1.search(rseq)
or reverse2.search(rseq)):
                        if forward1.search(rseq) or forward2.search(rseq):
                                seq = rseq
                        if forward1.search(seq):
```

```
                        LTR_end = seq.find(forward1.search(seq).group()) + 96
                        print LTR_end
                elif forward2.search(seq):
                        LTR_end = seq.find(forward2.search(seq).group())
                if reverse1.search(seq):
                        LINKER_pos = seq.find(reverse1.search(seq).group()) - 3
                elif reverse2.search(seq):
                        LINKER_pos = seq.find(reverse2.search(seq).group())

                if LINKER_pos > 0 and LTR_end > 0 and (LINKER_pos - LTR_end > 40): #last
cond removes primer product observed in some clones
                        right = LINKER_pos
                        left = LTR_end
                        number_of_good_sequences = number_of_good_sequences + 1
                        #print '>', fname, 'cropped', left+12, ':', right
                        cropped = seq[left+12:right]
                        fa_record= '>' + fname + '\n' + cropped +'\n'
                        cropped_sequences.write(fa_record)
                        list_good_sequences.write(fname)
                        list_good_sequences.write('\n')
                        print fname, 'good'
                else:
                        number_of_bad_sequences = number_of_bad_sequences + 1
                        list_bad_sequences.write(fname)
                        bad_fa_record = '>' + fname + '\n' + oseq + '\n'
                        bad_sequences.write(bad_fa_record)
                        list_bad_sequences.write('\n')
                        print fname, 'baad'
        else:
                number_of_bad_sequences = number_of_bad_sequences + 1
                list_bad_sequences.write(fname)
                bad_fa_record = '>' + fname + '\n' + oseq + '\n'
                bad_sequences.write(bad_fa_record)
                list_bad_sequences.write('\n')
                print fname, ' bad'

print 'total files parsed: ', seq_file_count
print 'number of potentially usable sequences:', number_of_good_sequences
print 'number of bad sequences:', number_of_bad_sequences

cropped_sequences.close()
bad_sequences.close()
list_bad_sequences.close()
list_good_sequences.close()
raw_input('Done! Press enter to exit...')
```

Appendix 3a: CD8+ restricted epitopes (adapted from the Los Alamos Immunology databse http://hcv.lanl.gov/content/immuno/tables/ctl_summary.html)

Epitope	Region	HLA restriction	Location
MSTNPKPQK	Core	A11	1-9
STNPKPQK	Core	A11	2-9
STNPKPQKK	Core	A11	2-10
GVYLLPRRGPRLGVR	Core		33-47
YLLPRRGPRL	Core	A2	35-44
YLLPTTGPRL	Core	A2	35-44
LLPRRGPRL	Core	A2	36-44
GPRLGVRAT	Core		41-49
GPRLGVRAT	Core	B7	41-49
GPRLGFRAT	Core	B7	41-49
RLGVRATRK	Core	A3	43-51
KTSERSQPR	Core	A3	51-59
PLYGNEGCGWAGWLL	Core		84-98
NEGLGWAGW	Core	B44	88-96
NEGCGWAGW	Core	B44	88-96
NEGCGWMGW	Core	B44	88-96
ADLMGYIPLV	Core	A2	111-119
DPRRRSRNL	Core	B7	111-119
GFADLMGYIPLVGAP	Core		129-143
ADLMGYIPLV	Core	A2	131-140
DLMGYIPLV	Core	A2	132-140
LPGCSFSIF	Core	B7	169-177
LLALLSCLTV	Core	A*0201	178-187
LLALLSCLTV	Core	A2	178-187
CPNSSIVY	E1	B35	207-214
ILHTPGCV	E1	A2	220-227
NASRCWVAM	E1	B35	234-242
QLRRHIDLLV	E1	A2	257-266
QLRRIDLLV	E1	A2	257-266
VFLVSQLFTF	E1		284-293
QLFTFSPRR	E1	A3	289-297
SIYPGHITGH	E1		307-316
WDMMMNWSPTAALVV	E1		320-334
MMMNWSPTT	E1		322-330
LVMAQLLRI	E1	A2	332-340
SMVGNWAKV	E1	A2	363-371
SMVGNWAKVL	E1	A2	363-372
SLLAPGAKQNV	E2	A2	401-411
CRPLTDFDQGW	E2	B53	459-469
DFAQGWGPISYANGS	E2		464-478

YPPKPCGI	E2	B51	489-496
GTTDKSGAPT	E2		517-526
TTDKSGAPTYSWGE	E2		518-531
GENDTDVFVL	E2	B60	530-539
VFVLNNTRPPLGNWF	E2		536-550
NTRPPLGNWF	E2	B57	541-550
CVIGGAGNNT	E2	B50	569-578
HYSYRLWHY	E2		610-618
DYPYRLWHY	E2	Cw7	610-618
YRLWHYPCTI	E2	Cw7	613-622
RLWHYPCTV	E2		614-622
TINYTIFK	E2	A11	621-628
RMYVGGVEHR	E2	A3	630-639
LEDRDRSEL	E2	B60	654-662
WAIKWEYVVLLFLLL	E2		712-726
EYVLLLFLL	E2	A*2402	717-725
FFCFAWYLKGRWVPG	p7		771-785
KWVPGAVYTFYGMWPLLLLL	p7		781-800
FYGMWPLL	p7	Cw7	790-797
FYGMWPLLL	p7	A29	790-798
LYGVWPLLL	p7	Cw*0801	790-798
MALTLSPY	NS2	A29	827-834
LSPYYKRYIS	NS2	A25	831-840
LSPYYKRYISW	NS2	A25	831-841
LSPYYKRYISWCLWWLQYFL	NS2		831-850
YISWCLWWL	NS2	A23	838-846
LTRVEAQLHVWVPPL	NS2		850-864
DAVILLMCAVHPTLVFDITK	NS2		871-890
HPALVFDITK	NS2		881-890
PYFVRAQGLI	NS2	Cw*0303	910-919
PYFVRAQGLI	NS2	Cw*0304	910-919
LGALTGTYVYNHLTPLRDWA	NS2		941-960
TYVYDHLTPL	NS2	B*4002	947-956
TYVYDHLTPL	NS2	B*4006	947-956
TPLRDWAHNGLRDLA	NS2		954-968
RDWAHNSL	NS2		957-964
RDWAHNGL	NS2	B37	957-964
INGLPVSARRGQEILL	NS2		997-1012
AYSQQTRGL	NS3	A24	1031-1039
VEGEVQIVSTAAQTF	NS3		1055-1069
QTFLATCINGVCWTV	NS3		1067-1081
ATCINGVCWTVYHGAGTRTI	NS3		1071-1090
CINGVCWTV	NS3		1073-1081
KLVALGINAV	NS3		1073-1081
CINGVCWTV	NS3	A2	1073-1081

CVNGVCWTV	NS3	A2	1073-1081
CINGVWCTV	NS3	A2	1073-1081
AGTRTIASPKGPVIQM	NS3		1085-1100
TRTIASPKGPVIQMY	NS3		1087-1101
DLVGWPAPQGSRSLT	NS3		1107-1121
RRRGDSRGSLLSPRP	NS3		1143-1157
LLCPAGHAV	NS3	A2	1169-1177
CPAGHAVGIFRAAVCTRGVA	NS3		1171-1190
HAVGLFRAA	NS3	A68	1175-1183
AYAAQGYKVL	NS3	Cw*0303	1243-1252
AYAAQGYKVL	NS3	Cw*0304	1243-1252
TLGFGAYMSK	NS3	A11	1261-1270
LGFGAYMSK	NS3	A3	1262-1270
GAYMSKAHGV	NS3	A11	1265-1274
TITTGAPVTYSTYGK	NS3	A2	1284-1298
TGSPITYSTY	NS3	A2	1287-1296
TGAPVTYSTY	NS3	A2	1287-1296
TYSTYGKFL	NS3	A*2402	1292-1300
CHAQDATTVL	NS3	B38	1318-1327
HPNIEEVAL	NS3		1359-1367
HPNIEEVAL	NS3	B*3501	1359-1367
HPNIEEVAL	NS3	B35	1359-1367
GEIPFYGKAI	NS3		1371-1380
FYGKAIPIEAI	NS3		1375-1385
LEVIKGGRHLIFCHSK	NS3		1382-1397
LIFCHSKKK	NS3	A3	1391-1399
LIFCHSKKKCDELAA	NS3		1391-1405
HSKKKCDEL	NS3		1395-1403
ATDALMTGY	NS3		1395-1403
HSKRKCDEL	NS3		1395-1403
HSKKKCDEL	NS3	B8	1395-1403
ELAAKLVAL	NS3	B8	1402-1410
LAAKLVALGINAVAY	NS3		1403-1417

LAAKLVALGINAVAY	NS3		1403-1417
KLVALGINAV	NS3	A2	1406-1415
GDVVVVSTDALMTGY	NS3		1430-1444
DVVVVATDALMTGYT	NS3		1431-1445
ATDALMTCY	NS3	A1	1436-1444
ATDALMTGY	NS3	A1	1436-1444
ALMTGYTGDFDSVI	NS3		1439-1452
GFTGDFDSV	NS3	A*0206	1443-1451
MFDSSVLCECYDAGC	NS3		1511-1525
VFTGLTHIDAHFLSQTKQSG	NS3		1561-1580
CLIRLKPTLHGPTPLLYR	NS3		1610-1627
DPRRRSRNL	NS3		1610-1627
LIRLKPTL	NS3	B8	1611-1618
EVTLTHPITKYIMTCMSA	NS3	B8	1634-1651
TLTHPITK	NS3	A11	1636-1643
TLTHPVTK	NS3	A11	1636-1643
STWVLVGGVLAALAA	NS4a		1658-1672
VLAALAAYCL	NS4a	A2	1666-1675
IPDREVLY	NS4a	B35	1695-1702
EVIAPAVQTNW	NS4b		1744-1754
VIAPAVQTNW	NS4b	A24	1745-1754
ETFWAKHMW	NS4b	A25	1758-1766
AFWAKHMWNF	NS4b		1759-1768
FWAKHMWNF	NS4b	A*2402	1760-1768
GIQYLAGLSTLPGNPAIASL	NS4b		1771-1790
SLMAFTAAV	NS4b	A2	1789-1797
LTTSQTLLF	NS4b	B57	1801-1809

LLFNILGGWV	NS4b	A2	1807-1816
ILAGYGAGV	NS4b	A2	1851-1859
GVAGALVAFK	NS4b	A3	1858-1867
VAGALVAFK	NS4b	A3	1859-1867
AARVTAIL	NS4b		1941-1948
SECCTPCSGSW	NS4b	B37	1966-1972
SECTTPCSGSW	NS4b	B37	1966-1976
VLSDFKTWL	NS5a	A2	1987-1995
VLDSFKTWL	NS5a	A2	1987-1995
DFKTWLQSKL	NS5a		1990-1999
LLREEVSFRV	NS5a	A2	2140-2149
HEYPVGSQL	NS5a	B60	2152-2160
PCEPEPDVAVL	NS5a	B35	2161-2171
EPEPDVAVL	NS5a	B35	2163-2171
ARGSPPSVASSSASQLSAPS	NS5a		2191-2210
SVASSSASQLSA	NS5a		2197-2208
QLSAPSLKATCTANH	NS5a		2205-2219
NHDSPDAEL	NS5a	B38	2218-2226
SPDAELIEANL	NS5a	A2	2221-2231
ELIEANLLW	NS5a	A25	2225-2233
ILDSFDPLV	NS5a	A2	2252-2260
REISVPAEIL	NS5a	B60	2266-2275
KFPPALPIW	NS5a	A*2402	2280-2288
VYSTTSRSASL	NS5b		2457-2467
TSRSACQRQKKVTFDRLQVL	NS5b		2461-2480
SLTPPHSAK	NS5b	A3	2510-2518
QPEKGGRKPA	NS5b	B55	2568-2577

RLIVFPDLGV	NS5b	A2	2578-2587
RVCEKMALY	NS5b	A3	2588-2596
MALYDVVSKLPLAVM	NS5b		2593-2607
ALYDVVTKL	NS5b	A2	2594-2602
KSKKTPMGF	NS5b	B57	2629-2637
KSKKTPMGFSYDTRC	NS5b		2629-2643
GLQDCTMLV	NS5b	A2	2727-2735
HDGAGKRVY	NS5b	A3	2794-2802
HDGAGKRVYYL	NS5b	B38	2794-2804
TARKTPVNSW	NS5b	A25	2819-2828
TARHTPVNSW	NS5b	A25	2819-2828
RMILMTHFF	NS5b	A*2402	2842-2850
CYSIEPLDL	NS5b	A*2402	2871-2879
SLHSYSPGEINRVAA	NS5b		2893-2907
SPGEINRVAA	NS5b	B55	2898-2907
LGVPPLRAWR	NS5b	B57	2912-2921

Appendix 3b: CD4+ restricted epitopes (adapted from the Los Alamos Immunology databse http://hcv.lanl.gov/content/immuno/tables/ctl_summary.html)

Epitope	Protein	HLA restriction	Location
MSTNPKPQIKTKRNTNRR	Core	DR	1-18
MSTNPKPQRKTKRNTNRRQD	Core		1-20
DVKFPGGGQIVGGVYLLPRR	Core		21-40
DVKFPGGGQIVGGVYLLPRR	Core	DRB1*1101, DQB1*0301	21-40
VGGVYLLPRRGPRLG	Core		31-45
VGGVYLLPRR GPRLG	Core	DR11	31-45
VGGVYLLPRRGPRLGVRATR	Core		31-50
RRRSRNLGKVIDTLT	Core		113-127
TLTCGFADLMGYIPL	Core		125-139
GAPLGGAARA LAHGV	Core	DR11, DR12	141-155
GAPLGGAARALAHGVRVLED	Core		141-160
APLGGAARALAHGVR	Core		142-156
SFSIFLLALLSCLTI	Core		173-187
LLSCLTIPASA	Core		181-191
HVTNDCPNSSIVYEA	E1		202-216
CPNSSIVYEAHDAIL	E1		207-221
SRCWVALTPTLAARN	E1		236-250
RCWVAMTPTVATRDG	E1		237-251
MTPTVATRDGKLPAT	E1		242-256
KLPATQLRRHIDLLV	E1		252-266
TIRRHVDLLVGAAAF	E1		257-271
GSATLCSALYVGDLC	E1		267-281
GSVFLIGQLFTFSPR	E1		282-296
TFSPRRHQTVQDCNC	E1		292-306

SIYPGHITGHRMAWD	E1		307-321
HITGHRMAWDMMMNW	E1		312-326
HVSGHRMAWDMMMNW	E1		312-326
MMMNWSPTAALVMAQ	E1		322-336
SPTAALVVSQLLRIP	E1		327-341
QAILDMIAGAHWGVL	E1		342-356
HWGVLAGLAYYSMVG	E1		352-366
FSMVGNWAKVLVVLL	E1		362-376
NWAKVLVVLLLFAGV	E1		367-381
GFATQRLTSLFALGPSQK	E2	DRB1*1101	393-410
CLWMMLLIAQAEQALL	E2		734-749
AMDREMAASCGGAVF	NS2		809-823
RDAIILLTCAVHPEL	NS2		870-884
GPLMVLQAGIIRAPY	NS2		897-911
PVSARRGREILLGPADGMVS	NS2		1001-1020
GQGWRLLAPITAYSQ	NS3		1020-1034
GPVIQMYTNVDQDLV	NS3		1095-1109
QGSRSLTPCTCGSSD	NS3		1115-1129
SSDLYLVTRHADVIP	NS3		1127-1141
YLVTRHADVIPVRRRGDSRG	NS3		1131-1150
SLLSPRPISYLKGSS	NS3		1151-1165
CPAGHAVGIFRAAVC	NS3		1171-1185
LETTMRSPVFTDNSSPPVVP	NS3		1201-1220
SPVFTDNSSP PAVPQ	NS3	DR11	1207-1221
VPQSFQVAHLHAPTG	NS3		1219-1233
FQVAHLHAPTGSGKS	NS3		1223-1237
KVPAAYAAQGYKVLV	NS3		1239-1253

PAAYAAQGYKVLVLNPSVAA	NS3		1241-1260
AAKGYKVLVL NPSVA	NS3	DR11	1245-1259
AQGYKVLVLNPSVAA	NS3		1246-1260
QGYKVLVLNPSVAAT	NS3		1247-1261
GYKVLVLNPSVAAT	NS3	DR	1248-1261
GYKVLVLNPSVAAT	NS3	DR4	1248-1261
GYKVLVLNPSVAAT	NS3	DRB1*1101,DRB1*1201,DRB1*0401,DRB1*1302,DRB1*1601	1248-1261
GYKVLVLNPSVAATLGFG	NS3		1248-1265
GYKVLVLNPSVAATLGFGAY	NS3	DQB1*0301	1248-1267
VLVLNPSVA	NS3	DR	1251-1259
VLVLNPSVA	NS3	DRB1*1101,DRB1*1201,DRB1*0401,DRB1*1302,DRB1*1601	1251-1259
ITYSTYGKFLADGGCSGGAY	NS3		1291-1310
YSTYGKFLADAGCSGGAY	NS3		1293-1310
TYGKFLADGGCSGGA	NS3		1295-1309
LADAGCSGGAYDIIICDE	NS3		1300-1317
LADAGCSGGAYDIIICDE	NS3	DR	1300-1317
GGAYDIIICDECHST	NS3		1307-1321
DIIICDECHSTDATS	NS3		1311-1325
TDATSILGIGTVLDQAETAG	NS3		1321-1340
ATSILGIGTVLDQAE	NS3		1323-1337
VIKGGRHLIFCHSKKKCD	NS3		1384-1401
VIKGGRHLIFCHSKKKCD	NS3	DR15	1384-1401
VIKGGRHLIFCHSKKKCD	NS3	DRB1*15	1384-1401
VIKGGRHLIFCHSKKKCD	NS3	DRB1*15	1384-1401
VIKGGRHLIFCHSKKKCD	NS3	HLA-DR15	1384-1401

VIKGGRHLIFCHSHHHCD	NS3	HLA-DRB1*15	1384-1401
GGRHLIFCHSKKKCD	NS3		1387-1401
GRHLIFCHSK	NS3	DRB1*1501/DRB5*0501	1388-1397
GRHLIFCHSKR	NS3	DRB1*1502/DRB1*1104	1388-1398
KCDELAAKLVALGIN	NS3		1399-1413
LAAKLVALGINAVAY	NS3		1403-1417
GINAVAYYRGLDVSV	NS3		1411-1425
GINAVAYYRGLDVSVIPTSG	NS3		1411-1430
VAYYRGLDVSV	NS3	DRB1*1501	1415-1425
VAYYRGLDVSVIPTS	NS3		1415-1429
IPTNGDVVVVSTDALMTG	NS3		1426-1443
ALMTGYTGDFDSVID	NS3		1439-1453
DFDSVIDCNTCVTQTVDF	NS3		1447-1464
DFDSVIDCNTCVTQTVDF	NS3		1447-1464
VIDCNTCVTQTVDFS	NS3		1451-1465
NTCVTQTVDFSLDPT	NS3		1455-1469
TQTVDFSLDPTFTIE	NS3		1459-1473
EQYVDFSLDPTFSIE	NS3		1459-1473
PTFTIETTTLPQDAVSRT	NS3		1468-1485
MFDSSVLCECYDAGC	NS3		1511-1525
YDAGCAWYELTPAETTVRLR	NS3		1521-1540
MPAETTVRLRAYMNTPG	NS3		1531-1547
MPAETTVRLRAYMNTPG	NS3	HLA-DR	1531-1547
TPAETTVRLRAYMNTPGLPV	NS3		1531-1550
TTVRLRAYMNTPGLP	NS3		1535-1549
LRAYMNTPGLPV	NS3	DRB1*1502/DRB1*1104	1539-1550
LRAYMNTPGLPVCQD	NS3		1539-1553

RAYMNTPGLPV	NS3	DRB1*0701	1540-1550
AYMNTPGLPV	NS3	DRB1*0101/DRB1*0701	1541-1550
ESVFTGLTHIDAHFL	NS3		1559-1573
TGLTHIDAHFLSQTK	NS3		1563-1577
ENLPYLVAYQATVCARAQAP	NS3		1581-1600
LPYLVAYQATVCARA	NS3		1583-1597
NLPYLVAYQATVCA	NS3	DRB1*0101/DRB1*0701	1585-1594
YLVAYQATVC	NS3	DRB1*0301/DRB1*1501	1585-1594
YLVAYQATVCA	NS3	DRB1*1501	1585-1595
VAYQATVCARAQAPP	NS3		1587-1601
TAYQATVCARAQAPP	NS3		1587-1601
AQAPPPSWDQMWKCL	NS3		1597-1611
DQMWLKCLIRLKPTLH	NS3		1605-1619
PTPLLYRLGAVQNEITLTHP	NS3		1621-1640
PVTKYIMACSADLES	NS3		1640-1655
PITKYIMTCMSADLEVVT	NS3		1640-1657
TSTWVLVGGVLAALA	NS3		1657-1671
NFISGIQYLAGLSTLPGNPA	NS4		1767-1786
TSTWVLVGGVLAA	NS4a	DRB1*0101/DRB1*0701	1657-1669
STWVLVGGVLAALAA	NS4a		1658-1672
TWVLVGGVLA	NS4a	DRB1*0101/DRB1*1301	1659-1668
AAYCLSTGCVVIVGRVVLSG	NS4a		1671-1690
VVLSGKPAIIPDREVLYREF	NS4a	DRB1*0301	1686-1705
GKPAIIPDREVLYRE	NS4a	DRB1*0301	1690-1704
IIPDREVLYR	NS4a	DRB1*0301/DRB1*1501	1694-1703
IAPAVQTNWQKLETFWAKHM	NS4b	DRB1*16 or DRB3*0202	1746-1765
NFISGIQYLAGLSTLPGNPA	NS4b	DRB1*1104	1767-1786

GIQYLAGLSTLPGNPAIASL	NS4b		1771-1790
QYLAGLSTLPG	NS4b	DRB1*1104	1773-1783
YLAGLSTLPG	NS4b	DRB1*1502/DRB1*1104	1774-1783
LAGLSTLPGNPAIASLMAFT	NS4b	DRB1*0402/DRB1*1101	1775-1794
GLSTLPGNPAIASLMAFT	NS4b	DR	1777-1794
LPGNPAIASLMAFTAAVTSP	NS4b		1781-1800
PAIASLMAFTA	NS4b	DRB1*1502/DRB1*1104	1785-1795
IASLMAFTAA	NS4b	DRB1*0102	1787-1796
LTTSQTLLFNILGGWVAAQL	NS4b		1801-1820
TQHTLLFNILGGWVA	NS4b		1803-1817
LFNILGGWVA	NS4b	DRB1*0101, DRB1*0102	1808-1817
FNILGGWVA	NS4b	DRB1*0101/DRB1*0701	1809-1817
FNILGGWVAAQL	NS4b	DRB1*0101/DRB1*1301	1809-1820
LGKVLVDILAGYGAG	NS4b		1844-1858
IMSGEVPSTEDLVNLLPA	NS4b	DR	1868-1885
GEVPSTEDLVNLLPAILSPG	NS4b		1871-1890
LVNLLPAILS	NS4b	DRB1*0101	1879-1888
NLLPAIL	NS4b	DRB1*0101, DRB1*0701	1881-1887
ALVVGVVCAAILRRHVGPGE	NS4b		1891-1910
GPGEGAVQWMNRLIAFASRG	NS4b	DRB1*1104, DQB1*0501	1907-1926
EGAVQWMNRLIAFAS	NS4b		1910-1924
GAVQWMNRLIAFASRGNHVS	NS4b		1911-1930
NRLIAFASRGNHVSPTHY	NS4b	DR	1917-1934
SQLSARSLKATCTANHDS	NS5a	DR	2204-2221
IEANLLWRQEMGGNI	NS5a		2227-2241
VSVPAEILRK SRRFA	NS5a	DR11	2268-2282
VSVPAEILRK SRRFA	NS5a	DR11	2270-2284

PAELRKSRFAQALPVWAR	NS5a		2271-2290
LRHHNLVYSTTSRSACQRQK	NS5b		2451-2470
KVTFDRLQVLDSHYQDVLKE	NS5b		2471-2490
SVWKDLLEDNVTPIDTTIMA	NS5b		2541-2560
KGGRKPARLIVFPDLGVRVC	NS5b		2571-2590
KPARLIVFPDLGVRVCEK	NS5b	DR	2575-2592
KPARLIVFPDLGVRVCEK	NS5b	HLA-DR	2575-2592
KLPLAVMGSSYGFQYSPGQR	NS5b		2601-2620
VEFLVQAWKSKKTPMGFSYD	NS5b		2621-2640
SDIRTEEAIYQCCDLDPQAR	NS5b		2651-2670
QCCDLDPQARVAIKSLTERL	NS5b		2661-2680
GYRRCRASGVLT	NS5b		2695-2706
CSSNVSVAHDGAGK	NS5b		2786-2799
SVAHDGAGKRVYYLTRDPTT	NS5b		2791-2810
RDPTTPLARAAWETA	NS5b		2806-2820
NIIMFAPTLWARMILMTHFF	NS5b		2831-2850
ARMILMTHFFSVLIARDQLE	NS5b		2841-2860
LMIHFFSILAQEQLE	NS5b		2845-2860
INRVAACLRKLGVPPLRAW	NS5b		2901-2920
CGKYLFNWAV RTKLK	NS5b	DR11	2941-2955
CGKYLFNWAVRTKLKLTPIA	NS5b		2941-2960

Appendix 4: Full flow cytometry results

Table 1: Total T cell responses; all peptides

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN	0.58	0.09,1.25	0.1	0,0.5	0	0,0.06	0.06
IL-17	0	0,0.09	0.15	0.09,0.26	0.04	0,0.43	0.02*
TNF	0.02	0,0.42	0.35	0.07,1.17	0.12	0,0.78	0.14
IL-2	0.02	0,0.29	0.03	0,0.1	0.05	0,0.17	0.85

Table 2: Total T cell responses by peptide pool

a) Peptide pool 1 (HCV core, envelope E1 and E2, p7, NS2)

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN	0.54	0.08,1.28	0.11	0,1.92	0.03	0,0.08	0.14
IL-17	0	0,0.61	0.11	0,0.43	0.59	0.01,1.12	0.10
TNF	0.07	0,4.94	0.57	0.01,3.93	0.3	0,2.01	0.40
IL-2	0.02	0,0.64	0	0,0.04	0	0,0.11	0.41

b) Peptide pool 2 (NS3 protease and helicase, NS4A and NS4B)

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN	0.44	0,7.42	0.23	0.01,1.42	0	0,0.13	1.00
IL-17	0	0,0.07	0.14	0,0.35	0	0,0.29	0.05*
TNF	0	0,1.7	0.36	0.09,2.62	0.18	0,1.4	0.07
IL-2	0	0,12.12	0.18	0.03,1.08	0.12	0.01,1.74	0.31

c) Peptide pool 3 (NS5A, NS5BI and II)

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN	0.75	0.03,3.95	0	0,0.46	0.01	0,0.67	0.05*
IL-17	0.08	0,1.18	0.2	0.01,0.76	0	0,0.45	0.60
TNF	0.09	0,6.12	0.12	0,3.17	0.05	0,1.37	0.80
IL-2	0.08	0,1.91	0.01	0,0.15	0.11	0,0.51	0.85

Table 3: Total CD4+ cells: all pools

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN	0.45	0.08,1.24	0.01	0, 0.27	0.02	0, 0.12	0.05*
IL-17	0	0,0.08	0.02	0,0.07	0	0,0.03	0.54
TNF	0.05	0,0.23	0.21	0,0.89	0	0,0.11	0.83
IL-2	0.04	0,0.19	0	0,0.01	0.02	0,0.04	0.82

Table 4: CD4 cell responses by peptide pool: a) Peptide pool 1, b) Peptide pool 2, and c) Peptide pool 3

a) Peptide pool 1 (HCV core, envelope E1 and E2, p7, NS2)

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN	0.25	0,0.95	0.02	0,0.37	0.02	0,0.09	0.34
IL-17	0	0,0.09	0.01	0,0.1	0	0,0.3	0.37
TNF	0.07	0,2.75	0.1	0,1.42	0.03	0,0.44	0.71
IL-2	0	0,0.19	0	0,0	0	0,0.03	0.33

b) Peptide pool 2 (NS3 protease and helicase, NS4A and NS4B)

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN	0.26	0,2.3	0.01	0,0.55	0	0,0.03	0.79
IL-17	0	0,0.03	0	0,0.04	0	0,0.02	0.35

TNF	0	0,0.68	0	0,0.18	0	0,0.48	0.66
IL-2	0	0,3.34	0.04	0,0.35	0.04	0,0.47	0.44

c) Peptide pool 3 (NS5A, NS5BI and II)

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN	0.58	0,2.73	0	0,0	0	0,0.05	0.04*
IL-17	0.04	0,0.63	0.02	0,0.09	0	0,0.05	0.51
TNF	0.1	0,3.4	0.02	0,0.94	0	0,0.6	0.65
IL-2	0.02	0,0.22	0	0,0.03	0.02	0,0.1	0.60

Table 5: Total CD8+ cells: all peptide pools

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN	0.11	0,0.67	0	0,0.13	0	0,0.06	0.75
IL-17	0	0,0.03	0.01	0,0.12	0	0,0.05	0.09
TNF	0	0,0.18	0.11	0,0.51	0.05	0,0.28	0.04*
IL-2	0.02	0,0.1	0	0,0.07	0	0,0.12	0.91

Table 6: CD8 responses by peptide pool: a) Peptide pool 1, b) Peptide pool 2, and c) Peptide pool 3

a) Peptide pool 1

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN	0.11	0,0.74	0.04	0,0.81	0	0,0.12	0.63
IL-17	0	0,0.54	0	0,0.22	0.02	0,0.94	0.20
TNF	0	0,2.19	0.18	0,2.46	0.1	0,1.58	0.26
IL-2	0.02	0,0.45	0	0,0	0	0,0.08	0.33

b) Peptide pool 2

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN	0	0,5.12	0.05	0,0.81	0	0,0.42	0.74
IL-17	0	0,0.04	0.05	0,0.15	0	0,0.25	0.14
TNF	0	0,1.02	0.16	0,2.21	0.05	0,0.68	0.11
IL-2	0	0,8.78	0.13	0,0.75	0.12	0,1.47	0.34

c) Peptide pool 3

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN	0.39	0,1.87	0	0,0.17	0	0,0.62	0.22
IL-17	0	0,0.6	0.04	0,0.73	0	0,0.16	0.60
TNF	0.09	0,2.72	0	0,1.25	0.04	0,0.67	0.98
IL-2	0.06	0,1.72	0	0,0.07	0.01	0,0.32	0.58

Table 7 Dual expression in CD4+ cells against all peptides

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN-ve TNF+ve	0.01	0,0.24	0	0,0.02	0	0,0.06	0.72
IFN+ve TNF+ve	0.15	0.01,0.39	0	0,0.04	0	0,0	0.01*
IFN+ve TNF-ve	0.5	0,1	0.01	0,0.14	0	0,0.02	0.03*
IFN-ve IL-17+ve	0	0,0	0	0,0.03	0	0,0	0.08
IFN+ve IL-17+ve	0.01	0.01,0.03	0	0,0	0	0,0	0.006**
IFN+ve IL-17-ve	0.7	0,1.48	0.01	0,0.16	0.01	0,0.04	0.09
IFN-ve IL-2+ve	0	0,0.05	0	0,0.01	0.02	0,0.03	0.40
IFN+ve IL-2+ve	0	0,0.02	0	0,0	0	0,0	0.40
IFN+ve IL-2-ve	0.14	0,0.36	0	0,0.01	0	0,0.01	0.02*

Table 8 Dual expression in CD4 positive cells by peptide pool: a) Peptide pool 1 b) Peptide pool 2 c) Peptide pool 3

a) Peptide pool 1

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN-ve TNF+ve	0.05	0,2.02	0	0,0.29	0.03	0,0.31	1.00
IFN+ve TNF+ve	0.15	0,1.33	0	0,0.25	0.01	0,0.08	0.28
IFN+ve TNF-ve	0.29	0,2.14	0.01	0,0.29	0.02	0,0.14	0.21
IFN-ve IL-17+ve	0	0,0.06	0	0,0.06	0	0,0.21	0.26
IFN+ve IL-17+ve	0.01	0,0.06	0	0,0.01	0.02	0,0.04	0.69
IFN+ve IL-17-ve	0.45	0,4.1	0.01	0,0.65	0.03	0,1.17	0.46
IFN-ve IL-2+ve	0	0,0.14	0	0,0	0	0,0.03	1.00
IFN+ve IL-2+ve	0	0,0.02	0	0,0.01	0	0,0	0.46
IFN+ve IL-2-ve	0.14	0,0.67	0	0,0.24	0	0,0.04	0.40

b) Peptide pool 2

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN-ve TNF+ve	0	0,0.51	0	0,0.02	0	0,0.15	0.87
IFN+ve TNF+ve	0.01	0,0.55	0	0,0.14	0	0,0	0.38
IFN+ve TNF-ve	0.31	0,4.61	0.01	0,0.68	0	0,0.03	0.63
IFN-ve IL-17+ve	0	0,0	0	0,0.06	0	0,0.02	0.05*
IFN+ve IL-17+ve	0.01	0,0.03	0	0,0.01	0	0,0.01	0.29
IFN+ve IL-17-ve	0.53	0,5.91	0.08	0,1.16	0	0,0.86	0.84
IFN-ve IL-2+ve	0	0,1.61	0.02	0,0.19	0.04	0,0.23	0.45
IFN+ve IL-2+ve	0.01	0,0.62	0	0,0.02	0	0,0.03	0.69
IFN+ve IL-2-ve	0.1	0,0.57	0	0,0.2	0	0,0.01	0.41

c) Peptide pool 3

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN-ve TNF+ve	0.01	0,2.29	0	0,0.19	0	0,0.06	0.62
IFN+ve TNF+ve	0.37	0,2.67	0	0,0.04	0	0,0.03	0.03*
IFN+ve TNF-ve	0.89	0,4.75	0.01	0,0.29	0	0,0.03	0.03*
IFN-ve IL-17+ve	0	0,0.55	0	0,0.09	0	0,0.05	1.00
IFN+ve IL-17+ve	0.03	0,0.12	0	0,0	0	0,0	0.0007**
IFN+ve IL-17-ve	1.53	0,9.34	0	0,0.08	0	0,0.16	0.05
IFN-ve IL-2+ve	0	0,0.12	0	0,0.01	0.02	0,0.09	0.50
IFN+ve IL-2+ve	0	0,0.03	0	0,0.01	0	0,0.01	0.68
IFN+ve IL-2-ve	0.37	0,0.88	0	0,0	0	0,0.01	0.03*

Table 9 Dual expression in CD8+ cells against all peptides

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN-ve TNF+ve	0	0,0.29	0	0,0.11	0.04	0,0.15	0.49
IFN+ve TNF+ve	0.14	0.01,0.38	0	0,0.05	0	0,0.01	0.04*
IFN+ve TNF-ve	0.31	0.03,0.83	0	0,0.16	0	0,0.01	0.01*
IFN-ve IL-17+ve	0	0,0.01	0.05	0,0.12	0	0,0.1	0.02*
IFN+ve IL-17+ve	0	0,0.01	0	0,0.01	0.01	0,0.02	0.33
IFN+ve IL-17-ve	0.67	0.06,1.34	0	0,0.26	0	0,0.11	0.02*
IFN-ve IL-2+ve	0	0,0.03	0	0,0.04	0	0,0.05	0.38
IFN+ve IL-2+ve	0	0,0.02	0	0,0.01	0	0,0.01	0.50

IFN+ve IL-2-ve	0.05	0,0.2	0	0,0.01	0	0,0.01	0.24

Table 10 Dual expression of by CD8 positive cells by peptide pool: a) Peptide pool, 1 b) Peptide pool 2, and c) Peptide pool 3

a) Peptide pool 1

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN-ve TNF+ve	0	0,2.37	0.06	0,0.54	0.09	0,0.93	0.45
IFN+ve TNF+ve	0.14	0.01,0.78	0.04	0,0.48	0.01	0,0.15	0.24
IFN+ve TNF-ve	0.31	0.01,0.73	0.01	0,0.45	0	0,0.06	0.19
IFN-ve IL-17+ve	0	0,0.5	0	0,0.19	0.04	0,0.98	0.31
IFN+ve IL-17+ve	0	0,0.02	0	0,0.01	0.01	0,0.12	0.25
IFN+ve IL-17-ve	0.67	0.1,1.05	0	0,0.87	0.01	0,0.89	0.12
IFN-ve IL-2+ve	0	0,0.03	0	0,0.01	0	0,0.06	0.79
IFN+ve IL-2+ve	0	0,0.05	0	0,0	0	0,0.01	0.43
IFN+ve IL-2-ve	0.07	0,0.46	0	0,0.47	0	0,0.07	0.41

b) Peptide pool 2

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN-ve TNF+ve	0	0,0.43	0	0,0.2	0.05	0,0.18	0.32
IFN+ve TNF+ve	0.02	0,1.03	0	0,0.19	0	0,0.05	0.57
IFN+ve TNF-ve	0.05	0,7.25	0.17	0,0.58	0	0,0.11	0.76
IFN-ve IL-17+ve	0	0,0	0.07	0,0.23	0	0,0.25	0.02*
IFN+ve IL-17+ve	0	0,0.03	0.01	0,0.03	0.01	0,0.21	0.28
IFN+ve IL-17-ve	0.04	0,9.09	0.12	0,0.88	0.02	0,0.23	0.80
IFN-ve IL-2+ve	0	0,4.34	0.12	0.02,0.34	0	0,0.67	0.34
IFN+ve IL-2+ve	0.01	0,2.73	0.01	0,0.07	0.02	0,0.31	0.81
IFN+ve IL-2-ve	0	0,2.62	0	0,0.01	0	0,0.01	0.56

c) Peptide pool 3

Cytokine	Cleared	95% CI	Fluctuating	95% CI	Plateau	95% CI	P value
IFN-ve TNF+ve	0.11	0,2.79	0	0,0.15	0	0,0.19	0.50
IFN+ve TNF+ve	0.28	0,1.26	0	0,0.09	0	0,0.04	0.09
IFN+ve TNF-ve	0.86	0.02,3.78	0	0,0.13	0	0,0.08	0.006**
IFN-ve IL-17+ve	0	0,0.54	0.06	0,0.23	0	0,0.4	0.47
IFN+ve IL-17+ve	0.01	0,0.12	0	0,0.01	0	0,0.12	0.51
IFN+ve IL-17-ve	1.54	0.06,6.96	0	0,0.69	0	0,0.62	0.02*
IFN-ve IL-2+ve	0	0,0.37	0	0,0.03	0	0,0.14	0.90
IFN+ve IL-2+ve	0	0,0.09	0.01	0,0.02	0	0,0.02	0.75
IFN+ve IL-2-ve	0.01	0,0.55	0	0,0.1	0	0,0.11	0.60

Table 11: Total T cell responses; all peptides.

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN	0.1	0.01,0.8	0.02	0,0.13	0.06
IL-17	0.01	0,0.17	0.09	0,0.13	0.02*
TNF	0.2	0.04,0.63	0.09	0,3.14	0.15
IL-2	0.05	0.01,0.11	0	0,0	0.85

Table 12: Total T cell responses by peptide: a) Peptide pool 1, b) Peptide pool 2, and c) Peptide pool 3.

a) Peptide pool 1 (HCV core, envelope E1 and E2, p7, NS2).

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN	0.09	0,1.23	0.05	0,0.5	0.25
IL-17	0.15	0,0.59	0.09	0,0.33	0.35
TNF	0.31	0,1.2	0.07	0,3.68	0.70
IL-2	0	0,0.04	0	0,0.13	0.54

b) Peptide pool 2 (NS3 protease and helicase, NS4A and NS4B).

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN	0.14	0,1.57	0.04	0,0.48	0.16
IL-17	0	0,0.15	0.11	0,0.19	0.84
TNF	0.2	0.02,0.96	0.05	0,7.56	0.72
IL-2	0.26	0.07,1.84	0	0,0.28	0.006**

c) Peptide pool 3 (NS5A, NS5BI and II).

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN	0.11	0,1.43	0	0,0.62	0.22
IL-17	0	0,0.31	0.09	0,0.18	0.90
TNF	0.08	0,0.66	0.21	0,10.92	0.35
IL-2	0.06	0.01,0.24	0	0,0.12	0.06

Table 13: Total CD4+ cells: all pools.

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN	0.01	0,0.1	0	0,0.04	0.26
IL-17	0	0,0.02	0	0,0.04	0.90
TNF	0.01	0,0.14	0	0,0.99	0.67
IL-2	0.01	0,0.03	0	0,0	0.0004**

Table 14: CD4 cell responses by peptide pool: a) Peptide pool 1, b) Peptide pool 2, and c) Peptide pool 3.

a) Peptide pool 1 (HCV core, envelope E1 and E2, p7, NS2).

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN	0.02	0,0.3	0	0,0.47	0.45
IL-17	0.01	0,0.15	0	0,0.09	0.44
TNF	0.02	0,0.39	0	0,1.43	0.37
IL-2	0	0,0	0	0,0	0.96

b) Peptide pool 2 (NS3 protease and helicase, NS4A and NS4B).

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN	0.01	0,0.6	0	0.16,	0.37
IL-17	0	0,0	0	0,0.07	0.49
TNF	0	0,0.19	0	0,2.94	0.86
IL-2	0.06	0.02,0.63	0	0,0.01	0.002**

c) Peptide pool 3 (NS5A, NS5BI and II).

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN	0	0,0.19	0	0,0.49	0.77
IL-17	0	0,0.1	0.01	0,0.1	0.80
TNF	0.01	0,0.32	0.1	0,4.38	0.38
IL-2	0.01	0,0.03	0	0,0	0.05*

Table 15: Total CD8+ cells: all pools.

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN	0.06	0,0.34	0	0,0.06	0.05*
IL-17	0	0,0	0	0,0.06	0.65
TNF	0.05	0,0.19	0	0,0.79	0.88
IL-2	0.01	0,0.07	0	0,0	0.01*

Table 16: CD8 cell responses by peptide pool: a) Peptide pool 1, b) Peptide pool 2, and c) Peptide pool 3.

a) Peptide pool 1 (HCV core, envelope E1 and E2, p7, NS2).

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN	0.05	0,0.6	0	0,0.1	0.32
IL-17	0	0,0.27	0	0,0.11	0.45
TNF	0.11	0,0.91	0	0,2.05	0.49
IL-2	0	0,0	0	0,0.13	0.99

b) Peptide pool 2 (NS3 protease and helicase, NS4A and NS4B).

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN	0.09	0,0.89	0.01	0,0.36	0.28
IL-17	0	0,0.03	0.03	0,0.14	0.72
TNF	0.11	0,0.68	0	0,4.6	0.88
IL-2	0.12	0.04,1.25	0	0,0.25	0.01*

c) Peptide pool 3 (NS5A, NS5BI and II).

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN	0.08	0,0.55	0	0,0.54	0.22
IL-17	0	0,0.1	0	0,0.09	0.79
TNF	0.01	0,0.21	0.15	0,6.18	0.31
IL-2	0.01	0,0.11	0	0,0	0.08

Table 17 Dual expression by CD4+ cells against all peptides

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN-ve TNF+ve	0	0,0.02	0	0,0.01	0.83
IFN+ve TNF+ve	0.01	0,0.04	0	0,0.04	0.83
IFN+ve TNF-ve	0.02	0,0.11	0.02	0,0.12	0.63
IFN-ve IL-17+ve	0	0,0.01	0	0,0.01	0.34
IFN+ve IL-17+ve	0	0,0.01	0	0,0.01	0.71
IFN+ve IL-17-ve	0.03	0,0.32	0.02	0,1.01	0.73
IFN-ve IL-2+ve	0.01	0,0.03	0	0,0	0.0001**
IFN+ve IL-2+ve	0	0,0.01	0	0,0	0.0003**
IFN+ve IL-2-ve	0	0,0.01	0	0,0.03	0.77

Table 18 Dual expression by CD4+ positive cells by peptide pool: a) Peptide pool 1, b) Peptide pool 2, and c) Peptide pool 3.

a) Peptide pool 1

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN-ve TNF+ve	0	0,0.01	0	0,1.29	0.69
IFN+ve TNF+ve	0	0,0.04	0	0,0.28	0.59
IFN+ve TNF-ve	0.02	0,0.12	0.03	0,1.31	0.86

IFN-ve IL-17+ve	0	0,0.01	0	0,0.05	0.21
IFN+ve IL-17+ve	0	0,0.01	0	0,0.03	0.32
IFN+ve IL-17-ve	0.02	0,1.01	0.02	0,3.65	0.74
IFN-ve IL-2+ve	0	0,0	0	0,0	0.13
IFN+ve IL-2+ve	0	0,0	0	0,0	0.09
IFN+ve IL-2-ve	0	0,0.03	0	0,0.32	0.84

b) Peptide pool 2

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN-ve TNF+ve	0	0,0.11	0	0,0	0.29
IFN+ve TNF+ve	0	0,0.14	0	0,0.38	0.86
IFN+ve TNF-ve	0.01	0,0.73	0	0,0.36	0.21
IFN-ve IL-17+ve	0	0,0	0	0,0.11	0.83
IFN+ve IL-17+ve	0	0,0.01	0	0,0.02	0.92
IFN+ve IL-17-ve	0.03	0,1.26	0.04	0,1.17	0.59
IFN-ve IL-2+ve	0.06	0.02,0.28	0	0,0.02	0.003**
IFN+ve IL-2+ve	0.01	0,0.02	0	0,0	0.01*
IFN+ve IL-2-ve	0	0,0.13	0	0,0.27	

c) Peptide pool 3

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN-ve TNF+ve	0	0,0.03	0	0,2.81	0.40
IFN+ve TNF+ve	0	0,0.05	0	0,0.42	1.00
IFN+ve TNF-ve	0.01	0,0.35	0.04	0,1.1	0.61
IFN-ve IL-17+ve	0	0,0.11	0	0,0.03	0.50
IFN+ve IL-17+ve	0	0,0	0	0,0.03	0.70
IFN+ve IL-17-ve	0.01	0,0.44	0.01	0,3.78	0.77
IFN-ve IL-2+ve	0	0,0.02	0	0,0	0.03*
IFN+ve IL-2+ve	0	0,0.01	0	0,0	0.04*
IFN+ve IL-2-ve	0	0,0.02	0	0,0.36	0.77

Table 19 Dual expression by CD8+ positive cells by peptide pool: a) Peptide pool 1, b) Peptide pool 2, and c) Peptide pool 3

Peptide pool 1

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN-ve TNF+ve	0	0,0.01	0	0,1.29	0.69
IFN+ve TNF+ve	0	0,0.04	0	0,0.28	0.59
IFN+ve TNF-ve	0.02	0,0.12	0.03	0,1.31	0.86
IFN-ve IL-17+ve	0	0,0.01	0	0,0.05	0.21
IFN+ve IL-17+ve	0	0,0.01	0	0,0.03	0.32
IFN+ve IL-17-ve	0.02	0,1.01	0.02	0,3.65	0.74
IFN-ve IL-2+ve	0	0,0	0	0,0	0.13
IFN+ve IL-2+ve	0	0,0	0	0,0	0.09
IFN+ve IL-2-ve	0	0,0.03	0	0,0.32	0.84

Peptide pool 2

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN-ve TNF+ve	0	0,0.11	0	0,0	0.29

IFN+ve TNF+ve	0	0,0.14	0	0,0.38	0.86
IFN+ve TNF-ve	0.01	0,0.73	0	0,0.36	0.21
IFN-ve IL-17+ve	0	0,0	0	0,0.11	0.83
IFN+ve IL-17+ve	0	0,0.01	0	0,0.02	0.92
IFN+ve IL-17-ve	0.03	0,1.26	0.04	0,1.17	0.59
IFN-ve IL-2+ve	0.06	0.02,0.28	0	0,0.02	0.003**
IFN+ve IL-2+ve	0.01	0,0.02	0	0,0	0.01*
IFN+ve IL-2-ve	0	0,0.13	0	0,0.27	0.95

Peptide pool 3

Cytokine	No IDU	95% CI	IDU	95% CI	P value
IFN-ve TNF+ve	0	0,0.03	0	0,2.81	0.40
IFN+ve TNF+ve	0	0,0.05	0	0,0.42	1.00
IFN+ve TNF-ve	0.01	0,0.35	0.04	0,1.1	0.61
IFN-ve IL-17+ve	0	0,0.11	0	0,0.03	0.50
IFN+ve IL-17+ve	0	0,0	0	0,0.03	0.70
IFN+ve IL-17-ve	0.01	0,0.44	0.01	0,3.78	0.77
IFN-ve IL-2+ve	0	0,0.02	0	0,0	0.03*
IFN+ve IL-2+ve	0	0,0.01	0	0,0	0.04*
IFN+ve IL-2-ve	0	0,0.02	0	0,0.36	0.77