Influence of Compartmentalisation in Blood Upon Oral Carriage of HCV in Brazilian Patients From the City of Recife - Brazil

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Abstract

Hepatitis C virus (HCV) is an increasingly common cause of liver disease worldwide. HCV may be present in saliva suggesting possible transmission via oral fluids. However the precise influence of peripheral blood carriage of HCV upon the frequency and load of HCV in oral fluids remains unknown.

The objectives of the study were to determine the frequency of oral carriage of HCV and determine the influence of plasma and peripheral blood mononuclear cells (PBMCs) HCV levels upon HCV levels in oral fluids.

The study group comprised 85 patients with treated (18) or untreated (67) HCV infection resident in northern Brazil. HCV 5'-NCR and NS5b regions were detected by quantitative Real Time PCR and nested block based PCR respectively in whole saliva, plasma, CD2+, CD14+, CD19+, and CD45+ PBMCs for both methods.

Approximately 32% of the total group had detectable HCV RNA in whole saliva. In contrast HCV was present in 82.5% of plasma and up to 82.1% of PBMC. There was no significant difference in the genotypes between oral and blood compartments and no evidence of genetic diversity within the oral compartment. There was no correlation between the salivary prevalence of HCV with load of plasma. HCV RNA viral load in saliva was almost always below the level of quantification.

Given the low viral load presented in patient with a high plasma titration in the 30% saliva positive patients, HCV transmission via the saliva is highly unlikely, but cannot be discarded. There is no evidence for HCV compartmentalization as sequences from different compartments were closely related, although

mutations were identified more frequently within genotype 1b in all compartments. This finding suggest a new trend towards hepatitis C evolution, as genotypes 1a and 1b do not usually differentiate in terms of treatment outcome, being both traditionally related to poor antiviral response.

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Declaration

The findings reported in this thesis result entirely from my own work. Colleagues who helped in various aspects of the work are listed in the Acknowledgments. The work has not previously been submitted, in part or in full, for a degree or diploma of this or any other University or examination board.

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Abbreviations

3’-UTR	3’ untranslated region
5’-UTR	5’ untranslated region
ALT	Alanine Aminotransferase
ALP	Alkaline phosphatase
AST	Aspartate Aminotransferase
BMI	Body mass index
BMV	Brome mosaic virus
C	Core
C1	Complement 1
CD14 cells	Monocytes isolation via dynabeads
CD19 cells	B lymphocytes isolation via dynabeads
CD2 cells	T lymphocytes isolation via dynabeads
CD45 cells	Pan leukocytes isolation via dynabeads
cDNA	Complementary DNA
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
E1	Envelope 1
E2	Envelope 2
ER	Endoplasmatic reticulum
EVR	Early virological response
FDA	Food and drug administration
GCF	Gingival crevicular fluid
GGT	Gamma-glutamyltransferase (gamma-glutamyl transpeptidase)

gpE1	Glycoprotein E1
gpE2	Glycoprotein E2
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCVpp	HCV pseudotype particles
HAI	Histologic activity Index
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSA	Human serum albumin
HVR1	Hypervariable region 1
HVR2	Hypervariable region 2
IL-10	Interleukin-10
IL-28B	interleukin-28B
ISDR	Interferon-sensitivity-determining region
LCMV	Lymphocytic Choriomeningitis Virus
LDL-C	Low-density-lipoprotein cholesterol
LDLr	Low-density lipoprotein receptor
LiPA	Line probe assay
LT	Liver transplantation
MALDI-TOF	Matrix-assisted laser description ionisation time-of-flight spectrometry
MC	Mixed cryoglobulinaemia
N/A	Not available
NANBH	Non-A non-B hepatitis
NHL	Non Hodgkin’s lymphoma

NHS	National Health Service
NICE	National Institute for Clinical Excellence
NS	Non structural
NS2	Non structural 2
NS3	Non structural 3
NS4A	Non structural 4A
NS4B	Non structural 4B
NS5A	Non structural 5A
NS5B	Non structural 5B
OLP	Oral lichen planus
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PBST	Phosphate-buffered saline, containing 0.05% Tween 20
PCR	Polymerase chain reaction
PEG-IFN	Polyethylene glycol (pegylated) interferon
PSEA	Primer-specific extension analysis
R	Receptor
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-PCR
SNP	single nucleotide polymorphism
SR-B1	Scavenger receptor class B type I
STAT-C	Specifically targeted antiviral therapies for hepatitis C
SS	Sjogrën Syndrome
SVR	Sustained virological response

TC	Lymphocyte T cytotoxic
TGF	Transforming growth factor
TH	Lymphocyte H cytotoxic
UK	United Kingdom
US	United States of America
UTR	Untranslated region
WHO	World Health Organisation
WK	Week
WMS	Whole mouth saliva

Chapter 1

Introductory Considerations

1.1. Background

Viral hepatitis research started in the 1950s and 1960s with the "infectious" and "serum" hepatitis, later proven to be caused by the hepatitis A virus (HAV) and hepatitis B virus (HBV), respectively (reviewed by Houghton, 2009).

The hepatotropic viruses (A, B, C, D and E) are a diverse group of micro-organisms that share the ability to cause hepatic inflammation and different degrees of necrosis. Each of these viruses can cause acute hepatitis, although only hepatitis B, D (which requires hepatitis B virus for its replication) and C give rise to chronic hepatitis (Aguilera *et al.*, 2006). The putative viruses hepatitis F and G (HGV, GBV-C) have also been described. The earlier could be part of the *Togaviridae* family, but no specific virus was identified as hepatitis F, while the later is a positive sense RNA virus distantly related to HCV (HGV and GBV-C being virtually identical). There is little evidence that hepatitis G can cause significant liver disease, despite persistent viraemia. While hepatitis A and E are transmitted via faecal-oral route, the others are blood-borne viruses (Kelly *et al.*, 2002).

For many years, the hepatitis laboratory of Chiron Biotechnology Company had attempted to identify the NANBH (see section 1.2) genome by cloning nucleic acids either from infected human or chimpanzee materials. This was followed by the identification of viral clones by showing their specific hybridization to radioactive cDNA probes derived from infected liver or blood samples.

After exhaustive attempts, one clone out of six clones screened (the others were shown to be derived from host genes encoding proteins apparently eliciting an autoimmune response) was shown to have been truly derived from the putative HCV genome, containing around 150 base-pairs (Houghton, 2009). The others were show to have been derived from host genes encoding proteins, apparently eliciting an autoimmune response.

1.2. Hepatitis C Virus

When serological tests to detect HAV and HBV were developed in the mid-1970s, it rated that many cases of parenterally transmitted hepatitis were not due to either virus, hence heralding the term Non-A, Non-B hepatitis (NANBH), later named hepatitis C virus (Houghton, 2009). Hepatitis C virus belongs to a separate genus of the Flaviviridae family of viruses (Simmonds, 2004), it was first described in detail in 1989, and is the principal cause of the previously termed group of NANBH (Choo *et al.*, 1989).

The elucidation of the genetic organization of HCV was possible after the complete sequence of the virus was identified (see Figure 1.1). From sequence comparison with other RNA viruses, it was apparent that HCV encodes a conserved serine-like protease domain, a helicase domain and a RNA polymerase domain (Choo *et al.*, 1991).

The main challenges for the identification of HCV resided in three main aspects: HCV is genetically heterogeneous, has low titres in blood and has a natural history comprising of a lengthy asymptomatic phase during the early

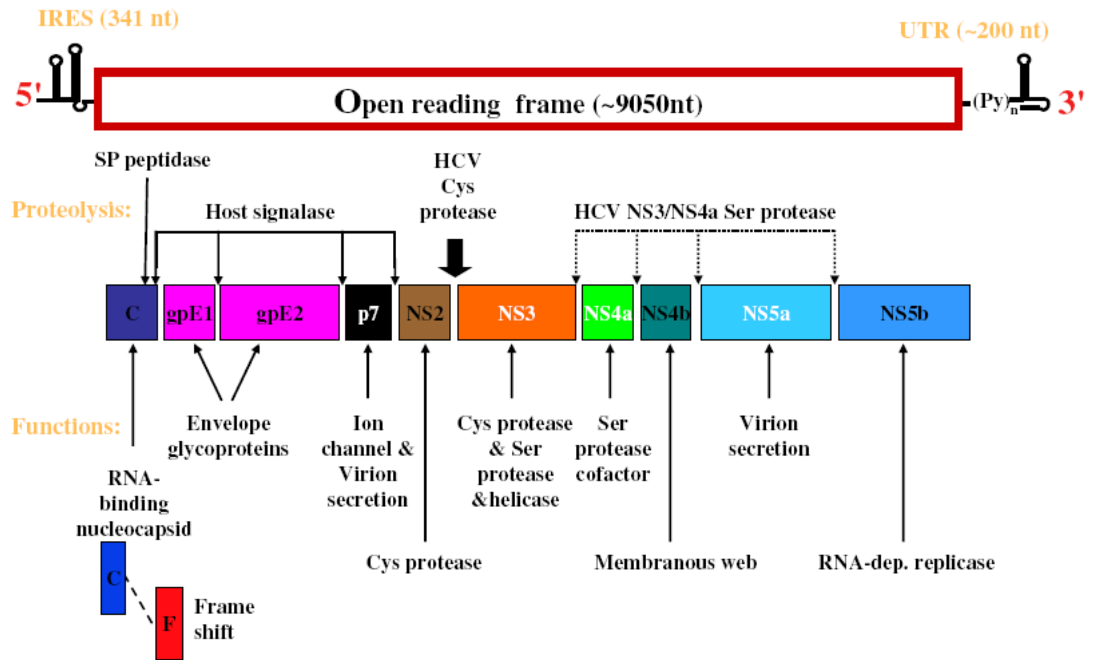
years of chronic infection. It was thus difficult to detect the genome and virus-encoded proteins by conventional methods and there was a lack of pathological landmarks to utilize in the development of chronic hepatitis (Feitelson, 2006).

1.3. Genome Structure and Organisation

The HCV viral particle is about 50nm in diameter and consists of an envelope derived from host cell membrane into which are inserted virally encoded glycoproteins E1 and E2. The membrane surrounds a nucleocapsid and a positive sense, single stranded RNA genome of about 9,600 nucleotides (Choo *et al.*, 1989).

The genome comprises highly conserved untranslated regions (UTRs) at both the 5' and 3' termini which flank a single Open Reading Frame (ORF) that encodes a polyprotein of about 3010-3033 amino acids (Chambers *et al.*, 1990). The genetic organization of the virus is such that the amino-terminal end of this polyprotein encodes the structural proteins of HCV while the remainder encodes a family of non structural (NS) proteins that are involved in virus maturation and replication (Feitelson, 2006). This polyprotein is processed co-translationally and post-translationally by cellular and viral proteases to produce a series of specific viral gene products that consist of the structural (core, E1 and E2) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). The structural proteins are at the N-terminal end of the polyprotein. The different components of the virus, which have different ratios of preservation between the various genotypes, (Simmonds, 2004) are summarised in Table 1.1 (Lauer *et al.*, 2001).

Figure 1.1. Open reading frame, proteolysis and protein functions of HCV (Houghton, 2009)



The C terminus of each structural protein is composed of a hydrophobic amino acid sequence, which acts as a signal peptide to target the proteins located downstream to the endoplasmic reticulum (ER). Cleavage at the C/E1, E1/E2, E2/p7 and p7/NS2 sites is mediated by ER-resident host signal peptidase(s). Although cleavage at the C/E1 and E1/E2 sites proceeds to completion rapidly after translation, cleavage at E2/p7 and p7/NS2 is delayed resulting in an E2p7NS2 species (Dubuisson *et al.*, 1994). Furthermore, cleavage at the E2/p7 site has been shown to be incomplete, resulting in two E2-specific species, E2 and E2p7 (Lin *et al.*, 1994, Mizushima *et al.*, 1994). The significance of these two forms of E2 has not been established, although it is conceivable that both play functional roles in the virus life cycle (Isherwood *et al.*, 2005).

Although recent reports indicated that p7 forms channels conducting ions across membranes and is essential for HCV infection, its exact role in the viral life cycle remained elusive. Steinmann *et al.* (2007) illustrated that HCV relies on p7 function for efficient assembly and release of infectious progeny virions from liver cells. Conversely, entry of HCV particles into new host cells is independent of p7. This new evidence supports the recent proposal to include p7 into the family of viroporins that comprises proteins from diverse viruses, for instance, HIV-1 and influenza A virus. Members of this group of functionally related proteins form membrane pores that promote virus release and in some cases also virus entry. Moreover, conserved p7 residues crucial for the efficient functioning of this protein have been identified. These amino acids possibly stabilize the structure of p7 or directly participate in channelling of ions. Interestingly, p7 variants from divergent patient isolates differ with

regard to their ability to promote virus production, suggesting that p7 also modulates viral fitness, i.e., its ability to cause disease (Steinmann *et al.*, 2007).

All cells permissive to HCVpp (pseudotype particles that display functional E1 E2 glycoprotein complexes) co-express low-density lipoprotein receptor (LDLr), CD81 and scavenger receptor class B type I (SR-B1), and are of hepatic origin. However, other cell types also express these receptors yet are not, or poorly, permissive to HCVpp infection. This suggests that additional liver-specific factors are necessary for HCV cellular entry (Bartosch *et al.*, 2003, Szabo *et al.*, 2003).

Low density lipoprotein receptor (LDLr) is involved at an early stage in infection of normal human hepatocytes by serum-derived HCV virions playing a critical role at this early step of infection (Molina *et al.*, 2007).

As noted above, the 5'-UTR and the 3'-UTR are the most conserved regions of the HCV genome. Within E2, there are two hypervariable regions (HVR1 and HVR2), each of which have considerable sequence variability. This variability is thought to be the result of selective pressure by virus-specific antibodies. E2 also contains the binding site for CD81 – one putative receptor or co-receptor for HCV. As denoted in Table 1.1, the non-structural proteins principally function as proteases (NS2, NS3 and NS4A), helicases (NS3) or RNA-dependent RNA polymerase (NS5B). A region within NS5A has been linked to the response to interferon alpha, and is thus termed interferon-sensitivity-determining region (ISDR) (Lauer *et al.*, 2001). Compared to the DNA-

dependent DNA polymerases found in eukaryotic cells, the HCV RNA-dependent RNA polymerase is a relatively small, simple enzyme that lacks accessory functions such as proof-reading. As a result, replication of HCV, in common with most RNA viruses, is relatively error-prone, and is associated with rapid genetic change. Nevertheless, Simmonds and Smith (1997) found that throughout the genome, most nucleotide changes were silent i.e. did not affect the sequence of the encoded amino acid sequence. The relative infrequency of non-silent changes is found in most coding sequences of all organisms, and reflects constraints upon the extent to which proteins may vary in sequence and yet remain functional (Simmonds *et al.*, 1997).

The life cycle of HCV has only recently been elucidated. Five years ago, a Japanese research group achieved robust hepatitis C virus infection *in vitro* for the first time. This was achieved by the transfection of Huh-7 cells with *in vitro*–transcribed JFH-1 (Japanese fulminant hepatitis 1) RNA, leading to the production of viral particles that are infectious for naive Huh-7 cells and can be serially passaged, thereby allowing study of the entire life cycle of HCV in tissue culture. Enveloped virus particles bind to specific surface receptors of host cells and are then internalised (Zhong *et al.* 2005). Putative receptors for Hepatitis C virus are CD81 tetraspanin, SR-B1, mannose-binding lectins DC-SIGN and L-SIGN, LDLr, heparan sulphate proteoglycans and the asialoglycoprotein receptor (Cocquerel *et al.*, 2006).

The recent development of functional models to analyze the early steps of the hepatitis C virus (HCV) life cycle has highlighted that HCV entry is a slow and complex multistep process involving the presence of several entry factors.

Initial host cell attachment may involve glycosaminoglycans and the low-density lipoprotein receptor, after which the particle appears to interact sequentially with three entry factors: the scavenger receptor class B type I, the tetraspanin CD81 and also the tight-junction protein claudin-1. Several serum components may also modulate HCV entry, while the recently discovered CD81 partner EWI-2wint can block the interaction of the viral particle with CD81, potentially preventing infection in the cell types in which it is expressed. After binding to the host cell, the HCV particle is internalized by clathrin-mediated endocytosis, with fusion likely occurring in early endosomes (Helle *et al.*, 2008).

The claudin-1 receptor (CLDN1) must play a role in a late step of the entry process, probably after virus binding and interaction with CD81. Thus far, no direct interaction between CLDN1 and the HCV particle has been reported, but such an interaction may require a conformational change in the envelope glycoproteins triggered by an initial interaction between E2 and another entry factor, e.g.CD81 or SRBI. The precise role of CLDN1 in HCV entry remains to be determined. However, since CLDN1 is strictly localized to the tight junctions in polarized hepatocytes, the authors of this study felt tempted to speculate that CLDN1 acts after lateral migration of a virus-receptor complex to the tight junctions (Helle *et al.*, 2008).

The fusion of the viral and cellular membranes leads to release of the single stranded RNA HCV genome into the cytoplasm of the cell. This RNA (thus) forms the template for the translation of viral proteins and the production of new viral RNA (Lindenbach *et al.*, 2005, Zeisel *et al.*, 2006). HCV replication

occurs in a specific membrane alteration – the membranous web induced by the NS4B protein (Egger *et al.*, 2002, Hugle *et al.*, 2001). The virons are thought to form by budding into the endoplasmic reticulum of the cell and then exit from the cell via its secretory pathway. Some aspects of this life cycle remain unknown (Zeisel *et al.*, 2006, Lindenbach *et al.*, 2005).

A Swedish research group indicated that single amino acid substitutions in the N terminus of NS4B can have a great impact on replication, both positively and negatively (Lindstrom *et al.*, 2006). In another paper from the same group, they have shown that translocation of the N terminus may be influenced by the presence of NS5A and may be associated with the ability of NS4B to rearrange the intracellular membranes. Together, these facts suggest an important role for the N-terminus of NS4B in the viral life cycle (Lundin *et al.*, 2006).

Based on the fact that most HCV proteins are membrane-binding proteins it has been postulated that there are at least two replication steps of HCV, i.e., RNA replication and virion formation are endoplasmatic reticulum - membrane associated. Egger et al. demonstrated that HCV proteins have the propensity to induce distinct alterations of endoplasmatic reticulum membranes, which may represent, in productively infected cells, the scaffolds necessary for virus multiplication (Egger *et al.*, 2002).

Table 1.1. Hepatitis C Virus Proteins (from amino- to carboxy-terminus, the polyprotein that encodes C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B)

Protein	Function	Reference
C	It is a structural protein, important in encapsulation of viral RNA. This protein may also interact with cellular proteins to aid transactivation and suppression	(Feitelson, 2006).
E1 & E2	E1 and E2 are structured proteins that lie within the membrane of the virus. E1 interacts with E2 to form a stable complex. Important in binding to host cells and aiding viral entry	(Feitelson, 2006).
E2-P7	The degree of E2-P7 cleavage by host signal peptidases vary but are reported to be incomplete in genotype 1. The C terminus of P7 in E2-P7 showed to be cytoplasmatically orientated suggesting that P7 adopts a dual transmembrane topology	(Isherwood *et al.*, 2005).
P7	P7 is primarily involved in the late phase of the HCV replication cycle. Promote virus production, modulates fitness and in turn virus persistance and pathogenesis	(Steinmann *et al.*, 2007).
NS2	NS2 is a non-structural protein that binds to N3 to form an NS2-3 complex essential for autoproteolysis of the polyprotein	(Feitelson, 2006).
NS3	NS3 is a non-structured protein that binds with NS2 to form NS2-3. NS3 also has serine protease, helicase and NTPase activity	(Feitelson, 2006).
NS4A	NS4A is a non-structural protein that functions as a co-factor for NS3 serine protease activity	(Feitelson, 2006).
NS4B	NS4B is a non-structural protein that induces formation of membranous web	(Egger *et al.*, 2002, Hugle *et al.*, 2001)
NS5A	NS5A is a non-structural protein, may play a role in RNA replication influencing the topology of NS4B	(Lundin *et al.*, 2006).
NS5B	NS5B is a non-structured protein which functions as a RNA dependent RNA polymerase	(Feitelson, 2006).

Cellular immune responses, particularly those mediated by CD8+ cytotoxic T cells, are important components of protective immunity against many viral infections, including hepatitis C. In HCV infection, the role of CD8+ cells in protecting against viral persistence is unknown (Zein, 2000). It has been identified that the core and E2 proteins are targets for HCV-specific CD8+ cells in chronically infected persons. HCV-specific cytolytic activity is present in bulk-expanded liver-infiltrating lymphocytes in the absence of any *in vitro* antigenic stimulation. Such activity could not be detected in bulk-expanded PBMC, suggesting a tissue-specific localization of HCV-specific CD8+ cell (Koziel *et al.*, 1993).

The escape of HCV from CD8+ cells-induced immunity may be related to the virus heterogeneity. For example, in a chronically infected chimpanzee, CD8+ cells obtained from the liver were initially able to recognize an epitope in the NS3 region. Over a period of several years, a new strain of the virus emerged with a mutation in the CD8+ cell epitope that was no longer recognized by the CD8+ cells isolated earlier. An extraordinarily high rate of nucleotide change that frequently resulted in codon changes has also been found in hypervariable region 1 of the E2 protein of the HCV genome (Zein, 2000). The presence of these rapidly changing regions may permit a mechanism by which HCV evades host immune surveillance and establishes and maintains persistent infection (Simmonds, 2004).

HCV does not integrate into the genome of the hepatocytes. Several viral proteins, however, have been suggested to be associated with hepatocarcinogenesis, especially the core and NS5 (Szabo *et al.*, 2003).

1.4. Genotypes

Hepatitis C virus exhibits considerable genetic heterogeneity. The virus can be phylogenetically differentiated into 6 main types or genotypes within which are a number of variable, but closely related, genetically distinct subtypes or subgenotypes (using letters in order of discovery, e.g. 1a, 1b, etc) (Simmonds *et al.*, 1993). Genotypes beyond genotype 6 have been described, these principally being isolates from the developing world. Novel variants of HCV from Vietnam, Thailand and Indonesia have been classified as types 7, 8, 9, 10 and 11, many of which were subdivided into between two to four subtypes (Simmonds *et al.*, 1996). However, it is now recognised that many of these newer genotypes bear close similarities to sequences of existing genotypes and thus can be ascribed to one of the genotypes 1 to 6 (Simmonds *et al.*, 2005). Indeed recently it has been recommended that the viruses be assigned to one of six clades. Clades 1, 2, 4 and 5 refer to genotypes 1, 2, 4 and 5 respectively, while genotypes 3 and 10 form clade 3, and genotypes 6, 7, 8, 9 and 11 form clade 6 (Simmonds *et al.* 2005).

Early dates for the divergence of HCV genotypes may help explain several features of their geographical distribution. In certain geographical regions, a single HCV type occurs but this is represented by numerous subtypes, a pattern suggestive of a long period of endemic infection. In contrast, in other parts of the world, more than one virus type is present, but each type is represented by only a few different subtypes. This pattern is consistent with relatively recent and limited introductions of HCV from endemic areas.

Examples include Northern Europe and North America where types 1a, 1b, 2a, 2b and 3a are common, and Japan where only types 1b, 2a and 2b occur. The history of these introductions can be partially reconstructed by comparing the frequency of HCV genotypes in different age groups. For example, types 1a and 3a are found in younger individuals who often have drug injection as a risk factor, and their recent dissemination is supported by the reduced diversity within these subtypes (Smith *et al.*, 1997).

Genotypes of HCV differ from each other by 31 to 33% at the nucleotide level, while the subtypes within a genotype differ by 20-25%. Despite this sequence diversity, all the genotypes show 60-70% homology and share an identical complement of co-linear genes of similar or identified size in the large open reading frame (Simmonds *et al.*, 2005).

1.4.1. Genotype Distribution

The clade (or genotype) of HCV is of relevance in both the investigation of epidemiology of HCV disease and its therapy. For example, clade 1 is widely distributed in Northern Europe and the USA and is generally associated with injecting drug use. Clade 2 is found predominantly in the older HCV-infected individuals in Mediterranean countries and the Far East. Clade 3 predominantly occurs in the Indian subcontinent, Europe and the US. Clade 4 is widely distributed in the Middle East and associated with unsafe therapeutic injections. Clade 5 is distinct to South Africa, while clade 6 (which contains the former genotypes 6 to 11) has been associated with injecting drug use in Hong Kong, Vietnam and Australia (Simmonds *et al.* 2005). Clade 1 is the

most commonly found in Brazil followed by clade 3, as listed in Table 5.1 (Chapter 5).

Knowledge of the geographic distribution of the different HCV clades is epidemiologically important as it allows infection to be tracked back to its source (e.g. following a nosocomial accident). In addition it may allow prediction of future health care burden as some genotypes may influence disease and/or response to therapy. Steatosis, the accumulation of fat in hepatocytes may reduce with therapy in patients who have genotype 3a, whereas all other genotypes are not associated with this phenomenon. Genotype 1b may be associated with an increased risk of cirrhosis and the need for liver transplantation, and recurrence of hepatic disease following transplantation (Hnatyszyn, 2005). The HCV genotype is considered to be a strong independent prediction of response to therapy. Genotype 4 infection responds poorly to interferon-based therapy whereas genotype 5 infection responds well. Furthermore, infection with genotype 6 is intermediate in response (Nguyen *et al.*, 2005). Genotype 1 is more resistant to interferon and ribavirin than genotypes 2 and 3 (Simmonds *et al.*, 2005). The dosage regime of interferon and ribavirin may be dependent upon the genotype, such that patients with genotypes 2 and 3 may require reduced dosages of interferon and ribavirin than other genotype infections. The response to therapy may be influenced not only by the genotype, but by mutations leading to quasispecies generation. Resistance to interferon has been suggested to be due to mutations within NS5A (Hnatyszyn, 2005).

Demographic and migration analysis histories of the prevalent HCV subtypes 1a and 1b were inferred from viral gene sequences samples in 5 countries. The parsimony migration analysis suggested that the global subtype 1a and 1b epidemics are geographically structured, with asymmetrical movement of HCV strains among the sampled countries. The coalescent analysis indicated that subtype 1a infections in the United States, Brazil, and Indonesia began to increase exponentially during the 1940s and 1950s, whereas in Vietnam the increase began after the 1970s. In contrast, subtype 1b infections in these 4 countries and in Japan began to increase exponentially between 1880 and 1920, with a possible recent decrease in infection rates in Indonesia and Japan. In the United States, Brazil, and Vietnam, the epidemic growth rates for subtype 1a strains were higher than those for subtype 1b strains, whereas the growth rates were similar in Indonesia (Nakano *et al.*, 2004).

1.4.2. Methods for Genotyping

Restriction fragment length polymorphism (RFLP) analysis - that by electrophoresis demonstrates banding patterns of digested nucleotide sequences - is considered to be an accurate and efficient means of determining the genotype of HCV isolates (Furione *et al.*, 1999). However, it does require enzymes that can recognise specific sites. Allele-specific oligonucleotide hybridisation methods require expensive probes to identify mutated regions, and may not detect single nucleotide polymorphisms (SNP). Matrix-assisted laser description ionisation time-of-flight spectrometry (MALDI-TOF) can sequence nucleotides allowing accurate discrimination between oligonucleotides 10 to 30 bases in size (Ilina *et al.*, 2005).

Genotyping assays are usually based upon an analysis of the sequence of an amplified section of the genome, typically the 5' UTR (the site with the highest degree of conservation (Cha *et al.*, 1991). Although this region is highly conserved, a number of polymorphisms are predictable and therefore allow genotypes to be determined. Nevertheless, some genotype 6 variants have 5' UTR identical to those of genotype 1a or 1b and thus this site cannot always be utilised for genotyping (Hnatyszyn, 2005).

Relative to genotyping in the NS5B region – considered the gold standard for genotyping determination – mis-typing seems to be rare, whereas mis-subtyping is frequent (approaching 10% of cases). This however has no clinical consequences, as clinical decisions are not currently based on the HCV subtypes (Pawlotsky, 2002). After nested PCR, this part of NS5B region about 300 base pairs long (see methods) is sequenced via Sanger method, which means DNA sequencing with chain-terminating inhibitors (Sanger *et al.*, 1977). Another sequencing method is based on E1 region (Forns *et al.*, 1999).

Typically, RNA viruses show high mutation frequencies because of an error-prone viral RNA polymerase that lacks proof-reading 3′-5′ exonuclease activity (Freeman *et al.*, 2001). For HCV, the rate of nucleotide misincorporation has been calculated as approximately 10^{-3} base substitutions per genome site per year (Ogata *et al.*, 1991). The resulting genetic diversity defines the evolution of a quasispecies: a complex population of closely related HCV variants circulating simultaneously in each individual patient (Martell *et al.*, 1992). The quasispecies encompass a predominant 'master' genome, which is believed to

have a superior replicative capacity, and a multitude of distinct 'minor' genomes. Generally, HCV quasispecies vary by less than 5% in nucleotide sequence. The generation and evolution of the quasispecies is likely to have important implications for escape from immune surveillance, generation of drug resistance and vaccine failure. Considering the potential role of the HVR1 in cell entry and its high rate of nucleotide diversity, it is conceivable that different components of the quasispecies within a single patient may display different cellular tropism. This may explain the observation that circulating quasispecies do not always reflect those of the intrahepatic virus (Freeman *et al.*, 2001).

Fourteen years ago, Ronaghi *et al.* (1996) showed that natural nucleotides can be used to obtain efficient incorporation during a sequencing-by-synthesis protocol. The detection was based on the pyrophosphate (PPi) released during the DNA polymerase reaction, the quantitative conversion of pyrophosphate to ATP by sulfurylase, and the subsequent production of visible light by firefly luciferase. However, this PPi-based sequencing method is not without drawbacks: The template must be washed thoroughly between nucleotide additions to remove unincorporated nucleotides. Also, templates not bound to a solid support are difficult to sequence, and the addition of new enzymes to each cycle of deoxynucleotide (dATP, dTTP, dGTP, and dCTP) is required (Ronaghi *et al.*, 1996).

This technique allows rapid detection of single nucleotide polymorphisms and the reading of small lengths of nucleotides from PCR amplicon. This is based on the quantitative detection of inorganic pyrophosphate released following

nucleotide incorporation into a growing DNA chain. DNA sequencing-based method for quantitating SNPs from a very heterogenic RNA pool such as HCV is a promising scientific effort. Hepatitis C virus is known to not only have heterogenic genome sequences among different genotypes and sub types, but also to acquire mutations with drug treatment (Qiu *et al.*, 2003).

One hundred and forty nucleotide-long regions of the HCV genome can be correctly sequenced in three Pyrosequencing reactions (Elahi *et al.*, 2003). For typing of novel subtypes, the length needs to be extended beyond this region (Elahi *et al.*, 2003, Stuyver *et al.*, 1996). Pyrosequencing is a sequencing method based upon real-time monitoring of DNA synthesis. In this method the DNA is sequenced as it is synthesised, and unlike some of the other methods, does not require the use of probes to identify already known sequences. Pyrosequencing does not require labelled nucleotides or labelled primers and is an automated system; hence the system of large numbers of samples can be examined in real time (Ahmadian *et al.*, 2006). It is attractive for the study of HCV genotyping. A recent study utilising multiplex pyrosequencing allowed accurate identification of the genotypes of HCV isolates from US and Iranian patients when compared with conventional sequencing. In this study the sequence of a 237 nucleotide long fragment of 5'-UTR was examined (Elahi *et al.*, 2003). In contrast, it has been suggested that pyrosequencing may not be as accurate as DNA sequencing quantitation for the detection of single nucleotide polymorphisms (SNPs) of HCV (Qiu *et al.*, 2003). Nowadays pyrosequecing is a novel strategy which can be used to identify multiple infections and quasispecies diversity.

Denaturing gradient gel electrophoresis (DGGE) is another molecular biology technique that permits intrahost screening and can be used for detecting HCV quasispecies diversity (Harris *et al.*, 2001).

Line probe assay (LiPA) technology is based on the reverse hybridization principle: biotinylated PCR fragments are hybridized to a selection of highly specific immobilized probes. In a second step, the biotin group in the hybridization complex is revealed by incubation with a streptavidin- alkaline phosphatase complex and the appropriate chromogen compounds (Stuyver *et al.*, 1993). The second-generation version of this test and its ability to discriminate among seven HCV types (types 1 to 6 and 10) and their most relevant subtypes was described 3 years later (Stuyver *et al.*, 1996). A new generation LiPA has been recently developed (Versant HCV Genotype 2.0 assay). It is based on the simultaneous detection of 5'-UTR and Core regions for genotypes 1 and 6 to provide correct HCV genotypes characterized by phylogenetic analysis. Using the new LiPA assay, genotypes 6c to 6l and 1a/b samples were more accurately genotyped than with the previous test only targeting the 5-UTR (sensitivity of 96% versus 71%, respectively) (Noppornpanth *et al.*, 2006). In a set of HCV strains mainly encountered in Western countries, Versant HCV Genotype 2.0 assay provided results that in conclusion classified it as a useful tool for clinical practice when only the discrimination between major HCV genotypes is necessary (Bouchardeau *et al.*, 2007).

Antonishyn *et al.* have developed a fluorescence-based genotyping assay called primer-specific extension analysis (PSEA) for the most prevalent HCV

genotypes and have demonstrated the capacity of PSEA-HCV for detecting mixed genotype HCV infections. The authors claim PSEA-HCV can detect as many as five genotypes in one specimen (Antonishyn *et al.*, 2005).

1.5. Multitypic HCV infection and quasispecies

Infection with more than one type of HCV (multitypic HCV infection) can occur, particularly in individuals at risk of multiple or repeated HCV exposures (e.g. via injecting drug use or repeated blood transfusion or blood product donations). There is, however, considerable variation in the frequency of such multitypic infection: in recipients of blood products, 0 to 25% of patients may have multitypic infection (Viazov *et al.*, 2000, Toyoda *et al.*, 1999, Preston *et al.*, 1995, Isobe *et al.*, 1995). This wide range may reflect the true frequency of such infection (e.g. as a consequence of variable numbers of blood product administrations) or reflect the varying different assay methods.

A new method to detect multitypic infection (and thus identify minority species that would otherwise not be detected by PCR-based methods) entails real-time nucleotide sequencing to characterise PCR clones after amplicons of the dominant HCV genotype in a sample of body tissue/fluid have been digested by genotype-specific endonuclease action. This method eliminates the dominant HCV genotype and permits characterisation of the PCR clones originating from the residual genomes (i.e. the minority genotypes). The identity of the minority genotypes can be achieved rapidly by pyrosequencing. This method permits detection of minority genotypes even when representing only 1% of the total HCV population. The disadvantage of this method is that it

is only qualitative, providing no data on the viral load of the minority genotype. In addition there remains no endonucleases to specifically cleave amplicons from genotypes 2 and 4, and the region of the HCV sequence used in this technique cannot discriminate between genotypes 1 and 6 (Buckton *et al.*, 2006).

Harris and Teo provided evidence that HCV genetic complexity and diversity in blood donors and IDUs did not differ significantly from those in haemophilia patients. This observation led them to hypothesize that multiple HCV infection is uncommon because persistent infection by one HCV strain prevents the establishment of infection by subsequently introduced strains (Harris *et al.*, 2001).

Nevertheless, in another study, working with samples, again from two patients, with mixed genotype infection determined by RFLP/LiPA, it was verified that the patients did indeed have mixed genotype infection instead of a new sequence/genotype in which the genotype-specific nucleotide variations for both genotypes were present. However, these data do show that mixed infection identified by RFLP/LiPA through the genotype-specific conserved variations indeed reflect mixed genotype infection, at least in both cases that were examined in that study (Qian *et al.*, 2000).

Ducoulombier *et al.* showed that during chronic HCV infection, B cells and/or monocytes cells (PBMCs) frequently harbor specific HCV variants. The data also suggest that these variants are subjected to lesser constraints. This phenomenon could be involved in the persistence of HCV and might be an

important determinant of the natural history and therapeutic outcome of the infection (Ducoulombier *et al.*, 2004).

1.6. Quasispecies

Circulating HCV is not a homogeneous population of identical viral particles, but rather a pool of genetically distinct, but clearly related variants referred to collectively as a quasispecies (Martell *et al.*, 1992). The quasispecies arise from the combination of the high error rate of viral RNA polymerase, the rapid replication rate of HCV (10^{12} virions per day in infected adults) and the large viral population size (Pawlotsky, 2006a). The quasispecies nature of HCV infection confers a survival advantage by permitting rapid selection of mutants best suited for the environmental conditions (e.g. immune response and concomitant anti-viral therapy). Of greatest clinical relevance is the opportunity that quasispecies have in influencing treatment. Resistance to IFN-α or ribavirin have each been found to reflect changes within the quasispecies (Pawlotsky, 2006b).

There are constraints upon the composition of quasispecies. Nucleotide mutation can occur at any position on the HCV genome, but different regions are subject to different selection constraints upon the functional rate of the encoded protein and its specificity to the immune response. The NS5A, NS3, NS3 helicase and RdRp regions are well conserved, as any change in the nucleotide (and hence amino acid) sequence will alter viral function. In contrast the 27 amino acid section at the N-terminus of the E2 envelope glycoprotein (the hypervariable region - HVR1) is subject to rapid change such

that the HVR1 quasispecies changes rapidly, e.g. with IFN-α therapy (Pawlotsky, 2006b).

The consequences of HCV quasispecies distribution are unclear. However, it is known that quasispecies from the liver, general circulation and peripheral blood mononuclear cells can be slightly different (Roque Afonso *et al.*, 1999). After studying PBMCs and a wide range of autopsy tissues from HCV-infected immunocompetent subjects, Laskus *et al.* have found that a number of samples contained 5'-UTR quasispecies differing from those found in the liver or circulating in the serum. The presence of these tissue-unique sequences is compatible with independent viral replication at extrahepatic sites, although the lineage of the infected cells was unclear. These results also suggest that PBMCs, and probably various tissues, can selectively adsorb viral subpopulations differing in the E2 region. That can be easily understood as E2 region is typically highly divergent, resulting in the presence of multiple viral quasispecies (Laskus *et al.*, 2000). This compartmentalisation of quasispecies probably reflects differences in tissue tropism.

PBMCs have a limited life span and the most plausible explanation for the persistence of compartmentalized quasispecies, as demonstrated in 2004, is replication and propagation of HCV RNA within these cells (Roque-Afonso *et al.*, 2005). HCV also seems to replicate in the PBMC of patients with occult HCV infection. As it has been shown that HCV can propagate in lymphoid cell cultures and that the virus derived is infectious, it should be determined whether these patients (although they do not have detectable circulating virus) are potentially infectious (Castillo *et al.*, 2005, Lins *et al.*, 2005)

1.7. Compartmentalization

Genetic diversity and tissue compartmentalization of the Hepatitis C virus are described in the literature more then 10 years ago in PBMC, liver and serum, (Navas *et al.*, 1998) and it continues to be a great mystery and promising field to explore towards a better understanding of the pathogenesis of the disease. Liver is the main site of viral replication; however, HCV also may be found in extrahepatic locations such as peripheral blood mononuclear cells (PBMCs) (Maylin *et al.* 2008).

Until recently, the “gold standard” tool for the diagnosis of occult HCV infection was the detection of HCV RNA in the liver since viral RNA in PBMCs, is detectable in only 70% of patients with this occult infection. However with simultaneous testing for HCV RNA in PBMCs and in ultracentrifuged serum samples, an occult HCV infection can be identified in up to 87% of the cases. Therefore, in light of these results, this is a substantial improvement in the accurate diagnosis of occult HCV infection without the need for a liver biopsy (Bartolome *et al.*, 2007).

B cells (CD19 positive) and monocytes (CD14 positive) are the two main cell types involved in HCV compartmentalization according to Ducoulombier et al. (Ducoulombier *et al.*, 2004). In a study with chronically infected patients, HCV RNA was present in the CD19 cells of all 15 patients, but only 5 (35.7%) of 14 and 5 (41.6%) of 12 showed HCV RNA in CD3 and CD14 cells, respectively (Zehender *et al.*, 1997). An increased number of CD14-positive cells are

associated with high inflammatory activity and in advanced fibrosis in hepatitis C (Leicester *et al.*, 2006).

The distribution pattern of the HCV genome in lymphocyte subpopulations study by Torres et al. was as follows: 87% presence by PCR in the B cell line (CD19), either accompanied by presence at other sub population locations (45%) or alone (42%); considerable presence in CD14 monocytes (45%); and scarce and isolated presence in the lymphocytes CD8 (12%) and CD4 (12%) (Torres *et al.*, 2000).

PBMCs are frequently infected by hepatitis C virus (HCV) variants that are not found in plasma. (Di Liberto *et al.*, 2006) The consequences of this compartmentalization on the natural and therapeutic outcome of hepatitis C are currently unknown.

One study of immunocompetent subjects with chronic hepatitis C demonstrated that HCV quasispecies compartmentalization among plasma and BMC subsets, based on analysis of the HVR1 region, is a common phenomenon (Ducoulombier *et al.*, 2004).

Panasiuk *et al* (2003) showed that in minimal hepatitis, G1, there is a statistically significant increase of B lymphocytes CD19+ in peripheral blood. At the initiation of inflammatory activity, CD19+ count is primarily strongly decreased (G2). Patients with advanced inflammatory changes with periportal hepatocyte necrosis showed B lymphocytes such as those in the control group. There is no statistical relationship between the stage of fibrosis and B

cells in peripheral blood (Panasiuk *et al.*, 2003). Another study showed no association of cleared or chronic HCV infection with altered levels of CD4+ or CD8+ T cells. Subjects with high level, chronic HCV viremia, but not those with cleared HCV infection, had reduced CD19+ B-cell levels (Zhang *et al.*, 2005). Jirillo *et al* (1998) showed that soluble CD14 and interferon γ serum levels were significantly higher in chronic hepatitis C patients than those detected in normal donors. On the other hand, CD4+/CD8+ antibacterial activity was depressed (Jirillo *et al.*, 1998).

Detection of the HCV-RNA positive strand by RT-PCR and fluorescent in situ hybridisation confirmed the existence of HCV infection in the PBMC of the 18 patients with occult HCV infection included in a study. Analysis of the HCV-RNA negative strand showed that HCV was replicating in the PBMC of the majority (61%) of patients with occult HCV infection, with concordant results between the two different techniques (Castillo *et al.*, 2005). Significant compartmentalization of HCV quasispecies was observed in the PBMC of four of nine subjects (three with Mixed Cryoglobulinemic Syndrome) and seven of nine cryoprecipitates, which are common findings of the disease (Zehender *et al.*, 2005).

Roque-Afonso *et al* (2005) have shown that a significant proportion of HCV-infected subjects harbour, in their PBMC, highly divergent variants which are not detectable in plasma and which are likely acquired through coinfections or superinfections. In 9 out of 109 analyzable patients, viral sequences detected in PBMC differed enough to be assigned to a genotype different from the predominant strain in plasma. This genotypic compartmentalization was

confirmed by analysis of different regions. It has persisted for years in three patients, including post-liver transplantation in two (Roque-Afonso *et al.*, 2005).

Two out of 11 hemodialysis study participants who lost HCV RNA in serum at the 30-month follow-up visit retained the virus in their PBMC population. The authors have suggested that HCV outbreak investigation and control in the hemodialysis setting could consider testing of PBMCs for HCV RNA in addition to serum HCV RNA and HCV antibody testing (Thongsawat *et al.*, 2008).

Approximately 30% of women were found to harbor HCV negative strand in their genital tract, in two different studies of HCV/HIV co-infected patients (Minosse *et al.*, 2006, Nowicki *et al.*, 2005) and positive strand (no-replicating) in another study with chronically HCV-infected women (Belec *et al.*, 2003a). It's important to highlight that the methods of the study performed by Minosse have been criticized due to a possible artefact of resampling the same viral templates or possible contamination (Bull *et al.*, 2007).

HCV RNA was detectable in up to 36% of cases in HCV seropositive homeless men. Men with HCV RNA in semen had higher RNA viral load in plasma than the ones that did not shed the virus in the semen. On the other hand, some men with very high HCV load in plasma did not have detectable HCV RNA in their semen, suggesting that passive transfer of viral particles from blood into semen could not be the only considerable factor to justify the RNA presence in the semen (Pekler *et al.* 2003). The prevalence of HCV RNA

detection in semen of HCV-HIV co-infected men was higher than in HCV infected individuals alone according to Briat *et al* (Briat *et al.*, 2005).

Three of 5 HCV/HIV-coinfected women showed evidence of HCV compartmentalization in PBMCs. Additionally, signature sequence analysis identified PBMC-specific amino acids in all HCV/HIV-coinfected persons (Blackard *et al.*, 2007).

A polymorphism in exon 4 (C77G) of CD45 that alters CD45 splicing has been associated with autoimmune and infectious diseases in humans and changes in CD45 isoform expression can alter immune function in human C77G variants and CD45 transgenic mice. The C77G allele may influence the outcome of HCV infection. CD45 isoforms also show altered immune function. These data provide convincing evidence that CD45 expression patterns can have profound effects on immune function and human diseases (Dawes *et al.* 2006).

A study in the setting of liver transplantation (LT) has demonstrated frequent HCV compartmentalization between plasma and PBMCs in LT and leukotropic variants with PBMC-related amino acid residues. These were not considered signatures by the authors due to signature variations observed in amino acid alignments by time point and patient, with the corresponding residues possibly present in plasma in this study or in a previous study (Ducoulombier *et al.*, 2004) as defined by VESPA computer software (Schramm *et al.*, 2008). Nevertheless, the functional relevance of the identified amino acids to cell

tropism, HCV-host interactions, and the pathogenesis of HCV infection is still not well established.

By infecting several peripheral blood cell types, such as B and T lymphocytes, monocytes, macrophages, and dendritic cells, HCV could interfere with antiviral cellular and humoral immune responses (Blackard *et al.* 2006; Roque-Afonso *et al.* 2005).

It has been proposed that HCV infection of immune cells could contribute to viral persistence by altering the ability of these cells to mount an immune response (Rodrigue-Gervais *et al.* 2007).

HCV infection of lymphocytes could contribute to the pathogenesis of extrahepatic disease. If PBMCs serve as a reservoir for HCV, they might contribute to re-infection of the graft after liver transplant. Furthermore, reports that HCV RNA persists in association with PBMCs after successful antiviral therapy raise the possibility that PBMC-associated HCV could contribute to virologic relapse. Thus, it is important to clarify the mechanisms by which HCV RNA may associate with PBMCs and to determine whether these cell subsets support viral replication (Marukian *et al.*, 2008).

1.8. HCV Epidemiology

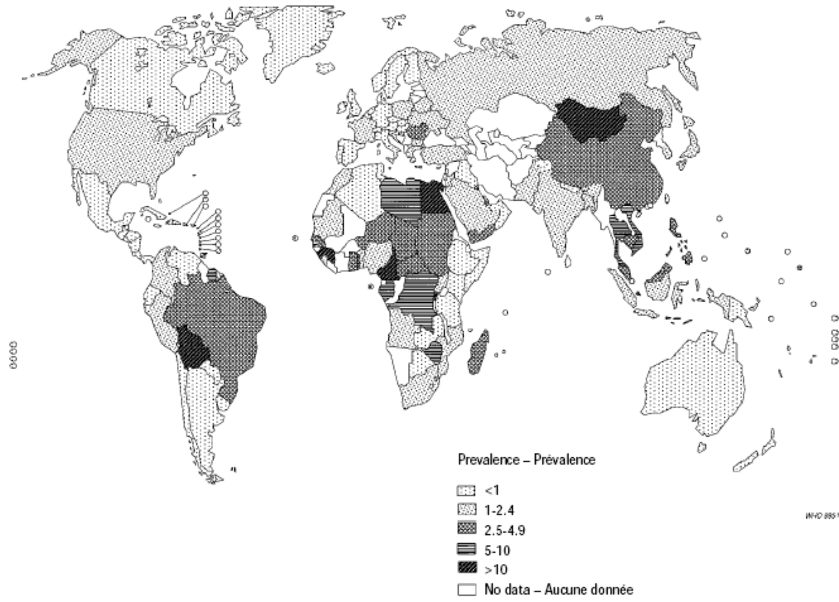
Hepatitis C infection is a major health care problem worldwide. It is estimated that the worldwide prevalence of HCV infection is 2%, thus affecting from 123 million (Shepard *et al.*, 2005) to 170 million individuals worldwide. Of these,

only 22 million (13%) are found in the Americas and Europe. The majority of remaining infected individuals (87%) are from Western Pacific countries (62.2 million), Southeast Asia (32.3 million), Africa (31.9 million), and Eastern Mediterranean countries (21.3 million) (Nguyen *et al.*, 2005).

In the developed world HCV is the leading cause of liver transplantation and the prevalence of HCV infection varies from 0.5% (e.g. UK) to 2.3% (Japan). There is a wide range of prevalence estimates among developing countries, and generally less data available to validate assumptions about the burden of disease than in the developed world. This range in prevalence is reflected in reviewing the estimates from developing countries that are among the world's most populous nations. China, whose citizen's account for one-fifth of the world's population, has a reported seroprevalence of 3.2%. In India, which holds an additional one-fifth of the world's population, one community-based survey reported an overall rate of 0.9%. Indonesia's rate is 2.1%, but is based on serosurveys of voluntary blood donors. More thorough data exist on the seroprevalence in Pakistan, where most reported rates range between 2.4% and 6.5% (Shepard *et al.*, 2005).

The World Health Organization has published a report with studies by 131 countries/areas as of June 1999. It is not recent, although it is the best general world HCV map available to date. Even though prevalence data is shown, it does not necessarily represent the true prevalence in each country, due to differences in the population groups studied, methods of data collection, interpretation between countries and limitation of data availability in several countries (Figure 1.2) (Who, 2000).

Figure 1.2. Overview of the prevalence of HCV around the globe in percentage (Who, 2000)



Several co-factors are associated with accelerated HCV-related hepatic disease (and resultant complications). In particular, male gender, older age at HCV acquisition, obesity, co-infection with HIV or HBV and alcohol consumption (Feitelson, 2006, Shepard *et al.*, 2005).

Chronic infection with HCV is the major infectious cause of chronic liver disease in Western countries. A difficulty in understanding the epidemiology of HCV is the lack of symptoms associated with both initial infection and for prolonged periods of chronic infection (Simmonds, 2001a, Simmonds, 2001b).

Neal *et al.* (2007) have shown that HCV-infected persons have a death rate three times higher than that of the general population. Mortality in HCV-infected patients is substantially higher than that of control populations particularly in those younger people where the lifestyles associated with the acquisition of HCV carry increased risk. As patients age, however, there is clear evidence of excess mortality from liver-related death and this is likely to significantly increase as the cohort (9 HCV-infected individuals) ages and liver damage progresses (Neal *et al.*, 2007).

Previous cohort studies exploring the relationship between HCV infection and mortality have demonstrated an interesting dichotomy. In several studies conducted in selected population subgroups, namely, those who acquired HCV infection through blood transfusion or contaminated intravenous immunoglobulin, or in military recruits, no significant increase in mortality risk from HCV infection was demonstrated. In contrast, community-based studies have tended to show that individuals with HCV infection have a demonstrably

increased mortality rate compared with the general population over time (Pungpapong *et al.*, 2008).

1.8.1. Modes of Transmission

Hepatitis C virus is transmitted principally via parenteral routes such as injecting drug use, blood products, unsafe therapeutic injections and a number of other health care related procedures (Shepard *et al.*, 2005). The institution of blood-screening measures in developed countries has decreased the risk of transfusion-associated hepatitis to a negligible level (Lauer *et al.*, 2001) but injecting drug use remains the predominant mode of transmission of HCV (Ferreiro *et al.*, 2005, Shepard *et al.*, 2005). Indeed in long-term injecting drug users, the prevalence may be as high as 94%. In the developing world, unsafe therapeutic injections (e.g. non-availability of sterile syringes necessitating re-use) have been a major route of spread of the HCV and contaminated equipment used in healthcare-related procedures (Hermida *et al.* 2002; Shepard *et al.* 2005). In Egypt for example, transmission of HCV was attributed to contaminated glass syringes used in national schistosomiasis treatment campaigns between 1960 and 1987. Blood transfusion was a common mode of HCV transmission in the developing world (Ferreiro *et al.* 2005; Shepard *et al.* 2005), although better screening methods of blood donations have reduced this risk (Shepard *et al.*, 2005). Transmission via nosocomial sharps injury is rare. Unknown factors for HCV acquisition may play a significant role in HCV epidemiology. Those would more likely be represented by sexual transmission, which can occur (Ferreiro *et al.*, 2005), but is rare, as is transmission via oral fluids (e.g. bites) (see

later), perinatal exposure (from an infected mother to her baby during birth) (Ferreiro *et al.* 2005; Shepard *et al.* 2005) and percutaneous or mucous-membrane exposure (Lauer *et al.*, 2001).

It is interesting to take into account the study by Mastromoteo *et al.*, when there was a prevalence of 5% HCV plasma RNA and 3.6% of HCV saliva RNA infected individuals within family members or co-habitants of chronic hepatitis C patients raising the possibility of non-sexual transmission between families (Mastromatteo *et al.* 2001).

Saliva and vaginal secretions from HCV seropositive individuals with detectable plasma HCV-RNA showed the unique association of HCV-specific humoral immunity directed to viral surface glycoproteins, undetectable free viral RNA, and occasional non-replicating strand, cell-associated HCV. These observations suggested a resulting poor infectivity of saliva or cervicovaginal fluid in chronically HCV-infected individuals, and suggested a biological basis for the low risk of non-parenteral transmission of HCV infection. However, blood-contamination of body fluids may increase the mucosal shedding of cell free HCV, and inflammation of mucosa may increase the mucosal passage of mononuclear cell-associated HCV (Belec *et al.* 2003b).

A study conducted in Cairo found strong correlations in HCV seroprevalence between first degree relatives which can be explained by a combination of specific modes of intrafamilial viral transmission and genetic predisposition to HCV infection. The respective contribution of direct HCV transmission between relatives by close contacts or exposure to an unidentified common

source of virus to the intrafamilial clustering of viral strains remains to be determined. Genetic epidemiological studies based upon genome-wide linkage analyses are twice as fast to underly intrafamilial transmission. The outcome of further studies could have major implications for the development of new HCV control strategies, particularly in developing countries (Plancoulaine *et al.*, 2008).

Vertical transmission of HCV is of concern, particularly in women who are co-infected with HCV and HIV. Polis *et al.* have conducted a meta-analysis study showing that the odds of vertical transmission of hepatitis C virus (HCV) infection are approximately 90% higher for HIV-HCV–coinfected women than for HCV-monoinfected women (Polis *et al.*, 2007). Schackman *et al.* previously conducted a decision analysis and found that a recommendation for elective cesarean delivery among HIV-HCV–coinfected mothers in the United States with suppressed HIV RNA levels could avoid up to 90 perinatal HCV transmissions per year and incur a risk of 1 additional maternal death per 50 years (Schackman *et al.*, 2004). On the basis of the findings from both of these studies, Schackman et al. suggested that elective cesarean delivery to prevent transmission of HCV infection should now be offered to HIV-HCV co-infected mothers who would not otherwise be offered this delivery option (Schackman *et al.*, 2007).

Sexual transmission of HCV among men who have sex with men (MSM) appears to be increasing. This was more clearly observed within HIV-HCV co-infected patients in Amsterdam (van de Laar *et al.*, 2007) but significant sexual transmission of HCV to MSM whose HIV status was either negative or

unknown at the time of their first HCV test has also been reported, suggesting that the sexual transmission of HCV in MSM may not be confined to those with HIV infection (Richardson *et al.*, 2008).

Matching sentinel surveillance reports with HIV diagnoses revealed that in England and Wales in 2003 nearly 5% of HIV-diagnosed MSM tested HCV positive where the only risk was sex with another man. Reports of sexually-transmitted HCV infection were not confined to London. Enhanced surveillance could clarify the exact likelihood of sexual transmission of HCV (Dougan *et al.*, 2007).

1.9. Clinical features of HCV

1.9.1. Diagnosis of HCV infection

Chronic HCV infection is usually asymptomatic, although occasionally patients may present with nonspecific symptoms such as fatigue or malaise. Some of them may have arthralgia and myalgia. Patients with decompensated disease may display peripheral manifestations of cirrhosis, such as palmar erythema, Dupuytren’s contracture, gynaecomastia, parotid enlargement (see section 1.12.1), temporal muscle wasting, ascites, hepatosplenomegaly, testicular atrophy and spider nevi (Modi et al., 2008). These signs may be a presenting feature of chronic liver disease (Vedamurthy et al., 2008) and are described as markers of portal hypertension if more than 6 are present (Sharma et al., 2009).

Clinical course of HCV infection is influenced by several factors. Older age at HCV acquisition (patients over 40 years old), male gender, coinfection with hepatitis B virus or HIV, steatohepatitis, immunosuppression, and predisposing human leukocyte antigen (HLA) haplotypes have all been associated with progression of fibrosis and possible development of cirrhosis. Alcohol consumption remains the main risk factor for faster progression to cirrhosis in HCV infection. Furthermore, many intravenous drug users, the main population still at risk of HCV infection in developed countries, consume alcohol regularly (Vento et al., 2006).

1.9.2. Staging of HCV infection (grading and staging liver biopsies)

Laboratory tests are the most frequently used means of monitoring and grading severity of chronic hepatitis. Histologic examination is stil the gold standard for staging chronic liver diseases like HCV infection. Notwithstanding, limitations related to sampling and interpretation have to be carefully considered. Knodell scoring system (Knodell *et al.*, 1981) was published three decades ago (Table 1.2) and is still the current method of choice for this purpose. The only modification in the scoring system relates to fibrosis (Desmet *et al.*, 1994), which is currently viewed as a dynamic process that can progress or regress over time. Even the excess fibrous tissue of cirrhotic livers has been found to sometimes regress over time (Germani *et al.*, 2011). Chronic hepatitis staging relates to its time course and has significant prognostic and therapeutic ramifications. Its histological evaluation is based on the extent of fibrosis and development of cirrhosis, which is the final and irreversible stage of chronic hepatitis (Desmet *et al.*, 1994).

Table 1.2. Histology activity index (HAI) numerical scoring of liver biopsy specimen used for HCV infection staging (Knodell et al., 1981)

I. Periportal +/− bridging necrosis	Score	II. Intralobular degeneration and focal necrosis[b]	Score	III. Portal inflammation	Score	IV. Fibrosis	Score
A. None	0	A. None	0	A. No portal inflammation	0	A. No fibrosis	0
B. Mild piecemeal necrosis	1	B. Mild (acidophilic bodies, ballooning degeneration and/or scattered foci of hepatocellular necrosis in $<\frac{1}{3}$ of lobules or nodules)	1	B. Mild (sprinkling of inflammatory cells in $<\frac{1}{3}$ of portal tracts)	1	B. Fibrous portal expansion	1
C. Moderate piecemeal necrosis (involves *less* than 50% of the circumference of most portal tracts)	3	C. Moderate (involvement of $\frac{1}{3}$–$\frac{2}{3}$ of lobules or nodules)	3	C. Moderate (increased inflammatory cells in $\frac{1}{3}$–$\frac{2}{3}$ of portal tracts)	3	C. Bridging fibrosis (portal-portal or portal-central linkage)	3
D. Marked piecemeal necrosis (involves *more* than 50% of the circumference of most portal tracts)	4	D. Marked (involvement of $>\frac{2}{3}$ of lobules or nodules)	4	D. Marked (dense packing of inflammatory cells in $>\frac{2}{3}$ of portal tracts)	4	D. Cirrhosis[c]	4
E. Moderate piecemeal necrosis *plus* bridging necrosis[d]	5						
F. Marked piecemeal necrosis *plus* bridging necrosis[d]	6						
G. Multilobular necrosis[e]	10						

[a] HAI score is the combined scores for necrosis, inflammation, and fibrosis.
[b] Degeneration—acidophilic bodies, ballooning; focal necrosis—scattered foci of hepatocellular necrosis.
[c] Loss of normal hepatic lobular architecture with fibrous septae separating and surrounding nodules.
[d] Bridging is defined as ≥2 bridges in the liver biopsy specimen; no distinction is made between portal-portal and portal-central linkage.
[e] Two or more contiguous lobules with panlobular necrosis.

1.9.3. Future directions in the staging of HCV infection

Polymorphisms in the region of the interleukin-28B (IL-28B) gene were associated with genotype 1 hepatitis C virus (HCV) clearance in patients treated with pegylated interferon-alfa and ribavirin. Spontaneous clearance of HCV in untreated patients was also associated with IL-28B polymorphisms. IL-28B genotype and HCV clearance may impact decisions regarding initiation of current therapy and the design and interpretation of clinical studies (Afdhal et al., 2011).

1.9.4. Hepatic manifestations of HCV infection

HCV causes chronic hepatitis in up to 80% of cases and may give rise to hepatic cirrhosis and hepatocellular carcinoma in a significant proportion of cases (Lodi *et al.*, 1998).

The incubation time of HCV, defined as the average time from acquisition to onset of symptoms, is about 6 to 8 weeks. Antibodies to HCV can be detected at the end of or just after, this period. In contrast, circulating HCV RNA becomes detectable 1 to 2 weeks after exposure to HCV. Only 60-70% of infected individuals have clinically detectable acute symptoms (and only 20-30% have jaundice) and 10-20% of patients only have non-specific features such as anorexia, malaise or abdominal pain. Clinical features of true acute hepatitis are very uncommon, but when these do occur, affected individuals will have clinical illness that lasts about 3-12 weeks, exhibiting elevated alanine transaminase levels, yet low levels of HCV RNA (Zoulim *et al.*, 2003).

The vast majority of HCV-infected individuals are thus symptomless carriers of the virus (Zoulim *et al.*, 2003). They have only mild fatigue (Freeman *et al.*, 2001), manifesting the clinical consequences of chronic hepatic disease, and/or the extra-hepatic manifestations of HCV infection. It is suggested that there are 3 patterns of chronic HCV disease: chronic hepatitis with normal ALT levels, mild chronic hepatitis, and moderate to severe chronic hepatitis. The first group have persistently normal ALT levels, asymptomatic disease, histopathological evidence of mild chronic hepatitis and do not have progressive disease. The second group have elevated ALT levels, histopathological evidence of mild necroinflammation of the liver and progress slowly, if at all, to cirrhosis. In contrast, however, the third group have marked necroinflammation of the liver and are likely to progress to cirrhosis (Zoulim *et al.*, 2003).

Hepatic cirrhosis arises in as many as 35% of patients with HCV infection and is most likely to occur in those individuals with moderate to severe chronic hepatitis. Disease is often asymptomatic, however, the clinical features when they do present are not different to hepatic cirrhosis of any other cause. Hepatocellular carcinoma (HCC) arises in about 3% of all HCV-infected individuals, and is the consequence of the HCV-related cirrhosis (Zoulim *et al.*, 2003).

Ohki *et al* (2008) have shown that there is an association between the body mass index (BMI) and the risk of hepatocarcinogenesis in chronic hepatitis C patients in a wide range of BMIs. Although the mechanism of this phenomenon

remains to be investigated, obesity is considered an independent risk factor for HCC development in chronic hepatitis C patients (Ohki *et al.*, 2008).

HCV (with the possible exception of HIV) is distinct and somewhat unusual for an RNA virus in being able to establish persistent infection in the majority of exposed individuals. Furthermore, long-term, chronic infections underlie its disease manifestations (Simmonds, 2004).

1.9.5. Extra-hepatic manifestation of HCV infection

HCV infected patients often have extrahepatic manifestations, which significantly contribute to HCV-related morbidity (Carrozzo *et al.*, 2002). It is well established that HCV can give rise to essential mixed cryoglobulinaemia (MC), this association being most common in patients in Italy (Pozzato *et al.*, 1994). The cryoglobulinaemia may lessen with interferon therapy indicating that there is a causal relationship with HCV infection. MC is characterised by a B-cell lymphoproliferative disorder. The definition of MC is provided by laboratory findings: presence of serum immunoglobulins that precipitate at low temperatures (less than 37 degrees Celsius). Type II MC is characterised by polyclonal IgG and monoclonal IgM with rheumatoid factor (RF) activity (Zignego *et al.*, 2007). Chronic infection with HCV is strongly associated with MC type II, which can evolve into overt lymphoma in some patients (De Sanjose *et al.*, 2008). HCV may also be causally associated with certain types of non-Hodgkin's lymphoma. Other possible extra-hepatic features of HCV infection are indicated in Table 1.2 (Carrozzo, 2008).

Table 1.3. Classification of extrahepatic manifestations of HCV infection (Carrozzo, 2008)

A: Association defined on the basis of high prevalence and pathogenesis	*B: Associations defined on the basis of higher prevalences than in controls*	*C: Associations to be confirmed/characterised*	*D: Anedoctal observations*
Mixed cryglubulinemia	B-cell non-Hodgkin lymphoma	Autoimmune thyroiditis	Psoriasis
	Monoclonal gammopathies	Thyroid cancer	Peripheral/central neuropathies
	Porphyria cutanea tarda	Sialadenitis (Sjogren-like)	Chronic polyarthritis
	Lichen planus	Alveolitis-lung fibrosis	Rheumatoid arthritis
		Diabetes mellitus	Polyartheritis nodosa
		Non-cryoglobulinaemic nephropathies	Bechet's syndrome
		Aortic atherosclerosis	Poly/dermatomyositis
			Fibromyalgia
			Chronic urticaria
			Chronic pruritus
			Kaposi's pseudo-sarcoma
			Vitiligo
			Cardiomyopathies
			Mooren corneal ulcer
			Erectile dysfunctions
			Necrolytic acral erythema
			Erythema multiforme
			Eythema nodosum
			Malakoplakia
			Still's disease
			Unilateral nevoid telangectasia
			Disseminated superficial porokeratosis
			Acquired epidermolysis bullosa
			Pulmonary fibrosis
			CRST (calcinosis cutis, Raynaud's phenomenon, sclerodactyly, and telangiectasia) syndrome
			Antiphospholipide syndrome
			Oral carcinoma

1.10. Treatment of HCV infection

Although HCV infection is a chronic viral infection, an advantage of this disorder over many other viral infections is that replication is generally confined to the cytoplasm of hepatocytes (Chung, 2006). As a consequence delivery of a specific antiviral agent to its site of action should be possible, and hence a sustained virological response may be achievable.

There are currently two different treatment regimens for chronic hepatitis C: monotherapy (using a single drug, interferon) and combination therapy (using two drugs, interferon and ribavirin) recommended by The Food and Drug Administration (FDA) from the US and the National Institute for Clinical Excellence (NICE) – National Health Service (NHS) from the United Kingdom (UK). Interferon alfa, a cytokine with immunomodulatory and antiviral activity which is injected into the bloodstream, works by bolstering the immune response to HCV (Bren, 2001). Ribavirin, a synthetic guanosine nucleoside analogue with *in vitro* antiviral activity (Freeman *et al.*, 2001) taken orally, may work by preventing the virus from reproducing (viral replication) (Bren, 2001).

The goal of antiviral therapy of HCV infection is to induce a sustained virological response (SVR) in which there is clearance of serum HCV RNA by the end of therapy that is further sustained for the subsequent 6 months (Hughes *et al.*, 2006). This is generally thought to represent “cure” of the infection, as the vast majority of subjects that reach this therapeutic goal go on to have a clinical, biochemical, and sometimes histological remission of their chronic hepatitis (Gallegos-Orozco *et al.*, 2008). A variety of factors influence

the ability to achieve SVR, these being both viral- or host-associated. In a large cohort of chronic hepatitis C patients with an SVR, followed up for up to 18 years after treatment cessation, HCV RNA remained undetectable in serum and PBMCs in all the patients, and was detected in 2 of 114 (1.7%) liver tissues tested. SVR was associated with sustained biochemical response (94%), stability or regression of fibrosis (88%), and with rare liver-related clinical events (3 hepatocelular carcinomas). These results suggest that SVR durable regardless of the type of treatment and should be considered to represent eradication of HCV infection (Maylin *et al.*, 2008).

A favourable response is likely when the genotype is 2 or 3, the viral RNA levels are low, there is lowest quasispecies diversity (Pawlotsky, 2006b) and the disease is acute rather than chronic. Genotypes 2 and 3 typically respond to treatment in 24 weeks or less (Heathcote and Main, 2005; Hughes and Shafran, 2006). Dalgard *et al.* (2008) have shown in a recent trial that SVR rate after 14 weeks of treatment is high, and although longer treatment may give slightly better SVR rate there are considerable economical savings, good response to retreatment, and fewer side effects making it rational to treat patients with genotype 2 or 3 and RVR for only 14 weeks (Dalgard *et al.* 2008).

Genotype 1 is more resistant, requiring treatment for 48 weeks and maybe more (Heathcote *et al.*, 2005). Variable treatment duration ensures an SVR rate similar to that of the standard treatment duration, with significant potential reductions in cost and side effects. Approximately a quarter of HCV genotype 1 patients may be cured by therapy in only 24 weeks and an approximately

comparable rate of patients may require extended treatment for 72 weeks. HCV RNA should be monitored qualitatively at week 4 to identify patients with the highest likelihood of response and at weeks 8 and 12 to determine if extended treatment duration may be required (Mangia *et al.* 2008).

The host factors that favour an SVR are female gender, young age, little hepatic fibrosis, low body weight and body mass, and lack of co-morbidity (e.g. alcohol abuse, renal disease, HIV infection) (Feld *et al.*, 2005). A favourable SVR results in a durable HCV response and an improvement in hepatic histology (e.g. lessening of inflammation and fibrosis). Three patterns of response to antiviral therapy have been observed: SVR (as detailed above), an end of treatment response with subsequent relapse, and non-response. In a typical SVR there is a two-phase virological response in which there is an initial drop of HCV RNA levels of 90-99% in the first two days, followed by a slower, but sustained decrease in HCV RNA levels (the latter reflecting clearance of virus from the hepatocytes) (Feld *et al.*, 2005).

Only patients with low HCV complexity, i.e., a small quasispecies sequence repertoire, appear to be able to have sustained HCV clearance after therapy. In contrast, patients with a large quasispecies sequence repertoire are very unlikely to achieve SVR, probably because there is a higher chance that one or several pre-treatment variants will escape the action of interferon alfa effectors and will proliferate (Pawlotsky, 2006a).

The attachment of polyethylene glycol to interferon alfa (peginterferon alfa) extends the half-life and duration of therapeutic activity of interferon alfa. In

contrast to interferon alfa, peginterferon alfa is given only once a week, and the individual dose is calculated according to the patient's weight. Treatment with peginterferon alfa results in a higher rate of response than does conventional monotherapy with interferon alfa. Large clinical trials are under way, at least over the course of the last decade, to evaluate the combination of peginterferon and ribavirin (Lauer *et al.*, 2001, Rai *et al.*, 2011). The results will determine the role of these agents in the treatment of HCV infection.

Two forms of pegylated (PEG-IFN) interferon alpha are available (PEG-IFN alpha 2a and PEG-IFN alpha 2b), each of which have a longer duration of action than conventional IFN alpha. Overall sustained virological response (SVR, i.e. the absence of detectable HCV RNA in blood 6 months after end of treatment) can be achieved in 25-40% of patients receiving PEG-IFN monotherapy, the typical duration of therapy being 24 or 48 weeks (Hughes *et al.*, 2006). Interferon alpha exerts its anti-HCV action by inducing IFN-stimulated genes of the host cell that then interfere in the translation of viral proteins (Feld *et al.*, 2005).

A wide of adverse side effects can arise with IFN therapy including fatigue, flu-like illness, gastrointestinal disturbances, haematological changes, neuropsychiatric disturbances (especially depression), thyroid dysfunction, cutaneous disease (e.g. alopecia and pruritis) (Pawlotsky, 2006a) and lichenoid reaction (Guillermo *et al.*, 2009, Sookoian *et al.*, 1999).

Gurguta *et al.* (2006) observed lingual hyperpigmentation, an unreported condition so far, in 5 of 171 patients participating in two clinical trials with 180

*µ*g PEG-Interferon *α*2a once weekly and various doses of Ribavirin (400–1,200 mg per day) given for 24–72 wks. All five were dark-skinned patients (out of 22). No hyperpigmentation occured in the 149 Caucasian patients(Gurguta *et al.*, 2006). Although two years later Fernández *et al.* (2008) reported a tongue hyperpigmentation resulting from peginterferon alfa-2a and ribavirin treatment in a Caucasian patient with chronic hepatitis C (Fernandez *et al.*, 2008).

Ribavirin will inhibit HCV replication via early chain termination and weak inhibition of RNA polymerase. The principal clinical effect of ribavirin is to prevent relapses in patients who respond to IFN-alpha (Pawlotsky, 2006b). Ribavirin monotherapy may not have a significant effect upon the SVR, but may improve the histopathological and biochemical responses in HCV infection (Brok *et al.*, 2006). When given in combination with IFN-alpha, ribavirin does however improve short-term and long-term outcome, although its antiviral action seems to be of greatest impact in the later phases of therapy, reducing the likelihood of rebound infection. As with IFN-alpha, a wide variety of adverse side effects can arise with ribavirin and include anaemia, dermatitis, anorexia, dyspepsia, insomnia, dyspnoea and malaise (Hughes *et al.*, 2006).

An unexpected case of severe pulmonary tuberculosis after 7 month combined pegylated interferon-ribavirin for Chronic HCV was observed in a patient with a severe underlying immunosuppression caused by the cited combination therapy (Sabbatani *et al.*, 2006).

The long-term effects of IFN alpha and ribavirin are unclear, however, certainly many patients do clear HCV from blood and have a fall in markers of HCV infection and hepatitis. In addition there may be an improvement in the histopathological features of hepatitis. Despite these, perhaps optimistic findings, the therapy of HCV infection is expensive and thus unlikely to be available to many HCV-infected individuals in the developing world. In addition, co-infection with hepatitis viruses (e.g. HBV, HDV and others) (Gaeta *et al.*, 2006) and HIV infection may hinder anti-HCV therapy being effective (Hughes *et al.*, 2006).

Weight-based ribavirin dosing must be taken into consideration to maximize treatment outcomes (Hrachovec *et al.*, 2003). In addition, certain other factors impact upon virological outcomes, such as high viral load, sex, race, weight, age at the time of infection, degree of hepatic fibrosis, and adherence (Heathcote *et al.*, 2005, Kim *et al.*, 2005).

Amantadine (1-aminoadamantanamine) is a water soluble tricyclic amine that has been used clinically to prevent influenza A viral infection, and has known antiviral effects against other families of RNA viruses such as togavirus, myxovirus, coronavirus and flavivirus, of which hepatitis C is a member, as explained before (De Clercq, 2001). Amantadine may be useful as a safe and well-tolerated adjunct to combination interferon/ribavirin therapy in the treatment of some patients who have failed interferon or interferon/ribavirin for chronic hepatitis C infection. The major advantage of amantadine is its favourable safety and tolerability profile and low cost. Although it is a weak antiviral agent with indirect effects on viral replication, multiple observational

studies and randomized trials suggest that combination regimens with amantadine may significantly increase rates of sustained viral clearance in non responders, responder/relapsers, and breakthrough responders to interferon and interferon/ribavirin. In the absence of available therapies which target the HCV viral replication cycle, amantadine may represent a suitable option in the properly selected patient (Lim *et al.*, 2005).

In a recent randomized clinical trial the sustained virological response (SVR) rate was 8% higher in the triple therapy group - pegylated interferon/ribavirin, plus amantadine - (24%) compared with the double therapy group - pegylated interferon/ribavirin - (16%), although the difference was not statistically significant (Maynard *et al.*, 2005). Further clinical trials with a larger population group may give us some hope about the benefits that amantadine could achieve in terms of effective doses for combined therapy in the future.

Akuta et al. identified amino acid substitutions in the core region and serum LDL-C as precursors of early virological response (EVR) and sustained virological response (SVR) to Peginterferon-Ribavirin therapy in Japanese patients infected with HCV genotype 1b (Akuta *et al.*, 2007).

Liver transplantation is the only available treatment option for patients with decompensated HCV-related cirrhosis and is also indicated for some patients with early stages of hepatocellular carcinoma (Lauer *et al.*, 2001).

A large scale Egyptian study provides strong evidence in favour of a higher HCV clearance rate in females compared to males, but it’s important to bear in

mind that 98,5% of the study population was genotype 4. There would be higher chances of spontaneous resolution of infection among females compared to males (Bakr *et al.*, 2006).

Histological evaluation of a liver-biopsy specimen remains the gold standard for determining the activity of HCV-related liver disease, while histological staging remains the only reliable predictor of prognosis and the likelihood of disease progression. A biopsy may also help to rule out other concurrent causes of liver disease. Therefore, biopsy is generally recommended for the initial assessment of persons with chronic HCV infection. However, a liver biopsy is not considered mandatory before the initiation of treatment, and some recommend a biopsy only if treatment does not result in sustained remission (Lauer *et al.*, 2001, Germani *et al.*, 2011).

HCV infection can be treated, but the treatment is still costly and requires long-term medical support and follow-up; current therapies are impractical for the majority of HCV carriers worldwide. The development of a protective vaccine was considered, at best, a distant prospect (Simmonds, 2004). This panorama may have started to change when Klade et al. showed that the HCV peptide vaccine IC41 can induce HCV-specific Th1/Tc1 responses in a subset of difficult to treat HCV nonresponder patients despite persisting viremia. However, changes in HCV RNA occurred only in single patients. Because strongest T-cell responses were associated with HCV RNA decline, further studies with optimized vaccine regimens and combination therapies have been initiated (Klade *et al.*, 2008).

Recent studies in the LCMV model of infection have also highlighted the key regulatory role of the IL-10/IL-10 receptor (R) pathway because IL-10-R blockade with specific antibodies was able to restore protective antiviral responses, preventing chronic evolution of infection. Also in human HCV infection, HCV-specific regulatory CD8 cells that are able to suppress T-cell responses by production of IL-10 or TGF-Beta have been isolated from the liver and the peripheral blood of patients with chronic hepatitis C. Because the impact of IL-10/IL-10-R blockade on LCMV, infection was more dramatic when the pathway was blocked at early stages of infection, studies are needed to understand whether this strategy can be envisaged for chronic infections in humans, where T cells have been exposed to viral antigens for years (Ferrari, 2008).

The specifically targeted antiviral therapies for hepatitis C (STAT-C) drugs target host proteins or influence the intrinsic host antiviral response through interferon-responsive pathways. In addition, new interferon compounds have been developed in order to further optimize pharmacokinetics, dosing frequencies, bioavailability, and potency against HCV. While the development of some compounds has discontinued due to safety or efficacy concerns, such as taribavirin, a prodrug of ribavirin with decreased haemolytic anemia, many promising drugs remain in various stages of development (Amorosa, 2010).

The constant development of new therapeutic approaches reveal promissing alternatives such as the traditional path of interferon related systems or modified forms of ribavirin, internal ribosome entry site (HCV IRES) inhibitors, NS3/4A serine protease (telaprevir and boceprevir) and NS5A inhibitors, novel

immunomodulators, specifically targeted anti-viral therapy for hepatitis C compounds, caspase inhibitors, anti-fibrotic agents, antibody treatment and vaccines (De Clercq, 2001, Feld *et al.*, 2005, Pawlotsky, 2006a, Munir *et al.*, 2010). Another novel class of agents in development is the entry inhibitors. SR-B1 is the hepatocyte membrane protein that is the primary substrate for HCV attachment followed by internalization. It is important to target this host receptor as well as identify and analyse other compounds that directly target the viral envelope (Bartosch *et al.*, 2003). Disadvantageously, no recent studies have explored further development of any entry inhibitor compounds.

Anti-NS4B molecules such as the clemizole hydrochloride, an old drug that has previously been clinically approved as an antihistamine, have also a great potential to be helpful in HCV therapy, either alone or in combination with other drugs. Clemizole hydrochloride could be synergistic with protease inhibitors and with interferon, ribavirin, nucleoside and non-nucleoside NS5B polymerase inhibitors. If clemizole hydrochloride or any other anti-NS4B compound further demonstrates efficacy in human clinical trials, evaluating their combination with other clinical candidates will considerably broaden the potential treatment options for HCV-infected patients (Rai *et al.*, 2011).

1.11. Oral aspects of HCV infection

1.11.1. HCV in oral fluids

The epidemiology of HCV disease does not suggest that saliva is a common vehicle for the transmission of HCV (Shepard *et al.*, 2005). In addition the prevalence of HCV infection among dental health care workers exposed to saliva is similar to that of local populations (Leao *et al.*, 2006). While these latter observations were made where the local population prevalence of HCV was likely to be low and infection control measures likely to be effective, the results of a recent study with a large cohort that included HCV-infected individuals did not reveal that orogenital contact (and thus contact with saliva) was a route of transmission of HCV (Marincovich *et al.*, 2003). Nevertheless HCV RNA and antibodies to HCV are present in saliva, HCV binds to, and may possibly replicate within oral epithelium and may give rise to salivary gland disease (Carrozzo *et al.*, 2003).

It has been more then two decades since eminent papers has been published regarding the diagnostic uses of saliva. (Ferguson, 1987; Malamud, 1992; Mandel, 1990; Streckfus and Bigler, 2002) It is not one of the popular bodily fluids. It “lacks the drama of the blood, the sincerity of sweat and the emotional appeal of tears” (Mandel, 1990). However, a growing number of health care workers are finding that saliva provides an easily available, non-invasive diagnostic medium for a rapidly widening range of diseases and clinical situations (Ferguson, 1987, Mandel, 1990).

Salivary transmission of HCV was suggested by the report of a male developing HCV infection one month after receiving a bite injury. However, the HCV status of the aggressor was not known, and thus it cannot be concluded that the bite injury lead to HCV acquisition (Dusheiko *et al.*, 1990). Certainly saliva may be infective, (as discussed below), and transmission of NANBH via innoculation of NANBH-infected saliva into a chimpanzee has been demonstrated (Abe *et al.*, 1991).

The salivary antibodies to HCV have immunoreactivity different to that of the serum antibodies. The antibodies of saliva have greatest activity to complement component C1, whereas those of serum show greater activity to antigen NS3. These differing immunoreactivities might reflect the existence of local viral replication, the carriage of viral mutants or the present of viral inhibitors in oral fluids (Maticic *et al.*, 2003).

RT-PCR is an accurate assay for the detection of HCV-RNA in saliva (Lins *et al.*, 2005). Furthermore, HCV-RNA detection in the saliva of HCV infected patients may be independent of the viral load (Fabris *et al.*, 1999, Lins *et al.*, 2005) and any oral disease. Saliva might, therefore, play a significant role in the non-parenteral transmission of HCV (Lins *et al.*, 2005) and explain intrafamilial transmission of HCV.

A study in Japan showed that 14 out of 18 patients (78%) whose saliva specimens were negative had HCV RNA in their gingival crevicular fluid (GCF). Most patients (20 of 26; 77%) also had higher HCV RNA levels in their GCF than in their saliva. Although the numbers of specimens were limited,

they have quantitatively determined HCV RNA in oral fluids from dental patients, including some patients with oral diseases, and demonstrated frequent detection of HCV in the saliva and GCF (Suzuki *et al.*, 2005).

There have been many studies of the detection of HCV RNA in oral fluids, particularly whole saliva. The frequency of detection of HCV-RNA in whole saliva has ranged from 0-100% (Goncalves *et al.*, 2005, Hermida *et al.*, 2002) with a mean of about 50% (Ferreiro *et al.* 2002; Leao *et al.* 2006) as detailed in chapter 3. The notable variation in the results of these studies probably reflects differences in the population groups, country of residence of patients, methods of collection of saliva and the assays employed to detect HCV RNA.

The presence of HCV in saliva usually correlates with high load of circulating virus. The HCV genotype in saliva seems to be identical to that in the blood of the same individual (Roy *et al.*, 1998). It has been suggested that HCV occurs mainly in the cellular element of saliva (Belec *et al.* 2003b; Chen *et al.* 1995), however, some workers have found HCV RNA to be present in the cell-free fraction (Roy *et al.* 1998; Hermida *et al.* 2002; Roy *et al.* 1996).

Hepatitis C virus particles have been detected in oral mucosal epithelial cells (Arrieta *et al.* 2000; Carrozzo *et al.* 2002). Both the non-replicating (positive) (Belec *et al.* 2003b) and replicating (negative) strand of HCV have been detected in oral epithelial cells (Carrozzo *et al.*, 2002), thus there is no conclusive evidence that HCV replicates in the oral epithelium. It would seem logical that HCV in saliva is not the consequence of local replication, but is passed to the mouth via gingival crevicular fluid (GCF). However, although

HCV RNA has been detected in up to 59% of GCF samples (Maticic *et al.* 2001), this cannot be the only local source of HCV as it has been detected in saliva of edentate individuals (Roy *et al.*, 1998). HCV is present in circulating monocytes – known to pass into GCF – thus it would be expected that the oral carriage of HCV would be increased in gingivitis and periodontitis, however, a study of patients in Brazil did not find any association between the present of HCV in saliva and their dental status (Lins *et al.*, 2005).

In contrast to HCV RNA, antibodies to HCV are frequently detected in saliva, indeed at least 90% of viraemic HCV seropositive individuals have detectable HCV antibodies in saliva (Lucidarme *et al.*, 2003). Several studies have established that the sensitivity of detection of HCV antibodies in saliva can approach 98% and recent epidemiological studies have indicated that the detection of antibodies to HCV in saliva may be more effective, and less invasive, than the detection of antibodies in serum. In particular studies of haemodialysis recipients (Yaari *et al.*, 2006), prisoners (Champion *et al.*, 2004) and children (Chatzipantazi *et al.*, 2004) have utilised saliva rather than serum to estimate the prevalence of HCV infection.

HCV may persist in the mouth despite clearance from blood (Diz *et al.*, 2005). This data suggests that there is the need for further studies in order to clarify the significance of the HCV virus in the saliva (Hermida *et al.*, 2002, Diz *et al.*, 2005, Goncalves *et al.*, 2005) as HCV RNA detection in saliva may be useful for monitoring anti-viral treatment in the future (Diz *et al.*, 2005).

1.12. Oral manifestations of HCV infection

The oral manifestations of HCV disease principally centre upon HCV sialadenitis. Associations between HCV and oral lichen planus have been extensively investigated and there remains no clear evidence of such an association. Likewise links between HCV infection and Behcet's disease have been proposed, but as of yet there is little evidence that conclusively supports such an association (Carrozzo *et al.*, 2002).

1.12.1. Sialadenitis

Sialadenitis presenting as xerostomia and/or salivary gland enlargement – usually the parotid glands (Vedamurthy *et al.*, 2008) – can affect up to 80% of examined individuals with HCV infection (Carrozzo *et al.*, 2002). Given the strong association recently discussed between HCV infection and essential MC and the high prevalence of Sjogrën Syndrome (SS) in essential MC, an association between HCV infection and the SS has been postulated (Gumber *et al.*, 1995). While lacrimal gland dysfunction can also occur, HCV does not cause SS (as only 0-19% of patients with SS are HCV infected). The histopathology of HCV sialadenitis does not include the same pattern of lymphocytic infiltrates as SS. However, a transgenic mouse model carrying HCV envelope genes E1 and E2 did develop sialadenitis with lymphocytic infiltrate that resembled that of SS (Haddad *et al.*, 1992). The exact pathogenic mechanism of HCV sialadenitis remains unknown.

In association with salivary gland tissue inflammation, the decreased production of salivary total IgA at a level nearly twofold less than the normal could be caused by possible dysfunction of salivary glands related to HCV infection itself. In three patients with clinical symptoms resembling primary SS, the salivary productions of total IgA and IgG were both decreased, but the local inflammation was probably enhanced, resulting in a pattern of highly increased salivary levels of HSA and immunoglobulins, a well known feature occurring during xerostomia (Belec *et al.* 2003b).

Even though sialadenitis is frequently observed in chronic hepatitis C, it seems to be not directly related to HCV *per se*. The presence of a common epitope between antigenic protein in the salivary gland and the HCV-structured protein could be a target for immunological attack (Ohoka *et al.*, 2003).

1.12.2. Oral lichen planus

Oral lichen planus (OLP) has been observed in some patients with HCV infection. The exact, if any, aetiological link between HCV-associated OLP and this viral infection remains controversial. Circulating HCV RNA has been detected in 0-34.7% of examined patients with OLP, while HCV antibodies have been detected in 0-55% of examined patients (Carrozzo *et al.*, 2002).

It has been recently suggested that the hepatitis C virus exerts an indirect effect, mediated possibly by the modulation of cytokines and lymphokines in the pathogenesis of oral erosive lichen planus (Femiano *et al.*, 2005).

The frequency of HCV RNA and antibodies is increased in patients with OLP in comparison with appropriate controls in some, but not all, studies. In particular, a marked relationship between HCV and OLP has been observed in studies with patients from Mediterranean coutries, Japan and USA, but not in the UK or Brazil (Cunha *et al.*, 2005, Jaber *et al.*, 2003, Lodi *et al.*, 2010). In some instances, the association of HCV with OLP is likely to simply reflect the high prevalence of HCV disease in local populations, however, a study of patients with OLP in Egypt (the country with the highest prevalence of HCV disease) did not find any significant association between HCV and OLP (Ibrahim *et al.*, 1999).

Associations between OLP and chronic liver disease (e.g. HBV infection) have previously been reported (Carrozzo *et al.*, 1996), and cutaneous lichen planus can arise following HCV vaccination (Rebora *et al.*, 1999). However, the associations between non-HCV-related chronic liver disease have predominantly been observed in patients in France, Italy and Spain, but not Scandinavia or the UK (el Kabir *et al.*, 1993).

Some instances of OLP associated with chronic liver disease reflect drug therapy (e.g. penicillamine) and similarly OLP can arise in HCV infection secondary to interferon therapy(Guijarro *et al.*, 2001).

It remains unclear if HCV truly gives rise to OLP. Anti-epithelial antibodies have been detected in patients with HCV-related OLP (Lodi *et al.*, 1997), however, these may not be of aetiological relevance as interferon alpha therapy can induce such antibodies (Fleischmann *et al.*, 1996). Both positive

and negative strands of HCV have been detected in 82-93% and 21-36% of examined HCV-associated lesions (Nagao *et al.*, 2000, Carrozzo *et al.*, 2002), but HCV RNA has also been detected in normal oral epithelia of HCV infected individuals (Arrieta *et al.* 2000). There may be some compartmentalisation of HCV specific CD8+ T cells in OLP (Pilli *et al.*, 2002).

While an association between HCV and OLP might exist, the geographic variation in the prevalence of HCV-related OLP is striking. A recent study demonstrated that there was an increased frequency of HLA-DR6 in Italian individuals with HCV-related OLP when compared with Italians with HIV, but not OLP, and UK individuals with OLP only. It has thus been suggested that the development of OLP in HCV disease reflects host immunogenetic factors, rather than any specific viral factor (Carrozzo *et al.*, 2005).

A recent review with meta-analysis on the prevalence of HCV in lichen planus patients and on the prevalence of lichen planus in chronic HCV infected patients has shown that HCV positive patients have significantly higher chances than controls of being HCV seropositive. In addition, significantly higher chances of having lichen planus were found among HCV patients. Variability of HCV/lichen planus association was only partially dependant on geographic distribution (Lodi *et al.*, 2010).

1.12.3. Non-Hodgkin's lymphoma

A study with high statistical power (more than 10000 patients including the control group) has confirmed the association between HCV infection and non-

Hodgkin's lymphoma and specific B-non Hodgkin's lymphoma subsets (diffuse large B-cell lymphoma, marginal zone lymphoma and lymphoplasmacytic lymphoma) (de Sanjose *et al.* 2008). Thus, perhaps as expected, NHL within parotid glands has been observed, but in a small number of patients with HCV disease (De Vita *et al.* 1995; Luppi *et al.* 1996; Ascoli *et al.* 1998).

1.12.4. Oral Squamous cell carcinoma

Oral squamous cell carcinoma (OSCC) has been observed in small numbers of patients with HCV infection (Nagao *et al.* 2000; Nagao *et al.* 1996; Nagao *et al.* 1995), however, as HCV infection is not common in oral epithelial dysplasia (Jaber *et al.*, 2003), it seems unlikely that HCV has any aetiological relevance to OSCC.

1.12.5. Behcet's disease

Associations between Behcet's disease and HCV infection have been suggested, but there is no clear evidence of such a link (Farajzadeh *et al.*, 2005).

1.13. Scope of studies and aims

There remains uncertainty as to wether HCV is present in saliva and hence a possible source of transmission of the virus. In addition, there is a need for a greater understanding of the possible influences upon oral carriage of HCV. This present study was thus undertaken to determine the frequency of oral carriage of HCV and the influence of oral factors (e.g. oral health status), systemic factors (e.g. presence of HCV in plasma and peripheral blood mononuclear cells) and viral factors (genotype and quasispecies) upon oral carriage.

Aims are:

1) To determine the prevalence and viral load of HCV in whole saliva of patients with HCV disease attending clinics in Recife in Northeast Brazil.
2) To determine the influence of HCV in plasma and peripheral blood mononuclear cells (PBMCs) upon the salivary presence of this virus.
3) To determine the prevalence and viral load of HCV in peripheral blood mononuclear cells (PMBCs) of patients with HCV disease attending clinics in Recife in Northeast Brazil.
4) To determine the influences of oral health on root of transmission and treatment.
5) To determine the influence of HCV in plasma upon the PMBC presence of this virus.
6) To determine if the genotype distribution of HCV of patients resident in the Recife area of Northern Brazil is consistent with other studies from South America.
7) To determine if there were any genotype discordances between plasma, peripheral blood mononuclear cells and whole saliva taken from the same individuals.
8) To establish if there are different quasispecies of HCV within plasma, saliva and peripheral blood mononuclear cells by means of analysing the compartments nucleotide changes and aminoacid variabilities.

Chapter 2

Materials and methods

2.1. Patients

The group comprised 86 individuals (52 males, 34 females; mean age 49.2 years; range 20 to 79 years) who were antibody positive for HCV attending the Hospital das Clínicas HC/UFPE, Recife, Brazil and/or the “Nefroclínica” (Haemodialysis clinic), Recife, Brazil; 23 of the patients required regular haemodialysis. The patients’ gender, age and HCV therapy are described in Table 2.1. Sixty six had not received anti-HCV therapy.

The untreated group comprised 40 males and 27 females, with a mean age of 49.1 years and a range of 20 to 79 years.

The treated group comprised 12 males and 7 females with a mean age of 49.3 years, and a range of 27 to 69 years. Therapy for the treated patients comprised interferon (5 patients), ribavirin (2 patients), interferon plus ribavirin (11 patients) and interferon plus amantadine (1 patient). Five patients had had retreatment of their HCV disease with interferon (1 patient) or interferon plus ribavirin (4 patients). All the samples were collected between July and August, 2006.

2.1.1. Inclusion criteria

Patients enrolled were either attending the Hospital das Clínicas HC/UFPE or Nefroclínica with HCV antibody positive status, and signed an informed consent agreeing to take part of the study between July and August, 2006. The patients were not necessarily tested for HCV RNA before commencing the study.

2.1.2. Oral health status

Each patient had a visual, non-invasive oral examination to detect their oral health status.

2.2. Sample Collection and Preparation

2.2.1. Collection and Preparation of whole blood

Peripheral blood was collected from the cubital fossa of each patient into EDTA-treated vacutainers and stored, for not more than 24 hours at 4ºC. Plasma was separated from blood by centrifugation at 3500 RPM for 15 minutes with 1 mL of plasma removed and stored at -20 ºC. The remaining blood was resuspended for isolation of individual cell types (see section 2.2.3).

Table 2.1. Details of the gender, age and HCV therapy of 86 patients with known HCV infection

Patient ID	Gender	Age	Treatment	Type (Duration)	Comment (re-treatment)
1	M	50	Yes	Peginterferon + Ribavirin	
2	M	34	No	N/A	
3	M	47	No	N/A	
4	F	37	Yes	Interferon + Ribavirin	
5	M	73	No	N/A	
6	M	33	No	N/A	
7	F	65	No	N/A	
8	F	56	Yes	Interferon + Ribavirin	Peginterferon +Ribavirin
9	F	22	No	N/A	
10	F	53	Yes	Interferon	
11	F	39	No	N/A	
12	F	43	No	N/A	
13	M	45	No	N/A	
14	F	69	Yes	Ribavirin	
15	M	55	No	N/A	

16	M	67	No	N/A	
17	M	50	No	N/A	
18	M	49	Yes	Interferon + Ribavirin	
19	M	73	No	N/A	
20	M	65	Yes	Interferon	
21	F	58	No	N/A	
22	F	71	No	N/A	
23	M	53	No	N/A	
24	M	30	Yes	Interferon + Ribavirin	
25	F	62	No	N/A	
26	M	52	No	N/A	
27	F	37	No	N/A	
28	M	36	No	N/A	
29	F	64	No	N/A	
30	M	57	No	N/A	
31	F	25	No	N/A	
32	M	27	No	N/A	

33	M	50	Yes	Interferon	Interferon + Ribavirin
34	F	27	No	N/A	
35	M	53	Yes	Interferon + Ribavirin	
36	F	51	No	N/A	
37	F	48	No	N/A	
38	F	60	No	N/A	
39	F	62	No	N/A	
40	M	55	No	N/A	
41	M	20	No	N/A	
42	M	47	No	N/A	
43	M	44	Yes	Interferon + Ribavirin	
44	F	30	No	N/A	
45	F	65	Yes	Ribavirin	
46	M	53	No	N/A	
47	F	49	No	N/A	
48	M	67	No	N/A	
49	M	43	No	N/A	

50	F	28	No	N/A	
51	F	64	No	N/A	
52	F	38	No	N/A	
53	M	45	No	N/A	
54	M	54	Yes	Interferon + Ribavirin	Interferon
55	F	61	No	N/A	
56	F	53	No	N/A	
57	M	45	No	N/A	
58	M	47	Yes	Interferon + Ribavirin	
59	M	44	Yes	Interferon	
60	F	48	No	N/A	
61	M	48	No	N/A	
62	F	39	No	N/A	
63	F	56	Yes	Interferon + Ribavirin	
64	F	51	No	N/A	
65	F	46	No	N/A	
66	M	57	No	N/A	

67	M	43	Yes	Interferon + Ribavirin	Peginterferon+ Ribavirin
68	M	51	No	N/A	
N1	M	44	Yes	Interferon (10 months)	
N2	M	40	No	N/A	
N3	M	52	No	N/A	
N4	M	45	No	N/A	
N5	F	34	No	N/A	
N6	M	73	No	N/A	
N7	M	74	No	N/A	
N8	M	47	No	N/A	
N9	M	26	No	N/A	
N10	M	49	No	N/A	
N11	F	27	Yes	Interferon	Interferon + Ribavirin for 1 year
N12	M	36	No	N/A	
N13	M	63	No	N/A	
N14	M	27	No	N/A	
N15	M	70	No	N/A	

N16	M	73	No	N/A	
N17	M	29	No	N/A	
N18	M	79	No	N/A	

* N denotes patients from the renal unit at Nefroclínica private dialysis centre.

2.2.2. Preparation of saliva samples

Unstimulated whole mouth saliva (WMS) was collected in 50 mL sterile centrifuge tubes (Sarstedt Ltd.). Samples were frozen immediately and shipped to the UK for laboratory analysis. After the arrival in the UK, samples were defrosted for analysis and the remaining stored in 2 mL microcentrifuge tubes and stored again in -80 degrees freezers.

2.2.3. Dynal Magnetic Bead cell isolation

Immunomagnetic cell isolation using Dynabeads allows isolation of predefined subsets directly from heterogeneous cell suspensions, such as whole blood. Dynabeads (Dynal A.S., Oslo, Norway) are small magnetically charged uniformly shaped beads coated with a specific monoclonal antibody. The Dynabeads were supplied as a suspension containing $4x10^8$ beads/mL in phosphate buffer saline (PBS), pH 7.4, containing 0.1% bovine serum albumin (BSA) and 0.02% sodium azide (NaN_3). As NaN_3 is a cytotoxic agent the beads required to be washed before use with a washing buffer (PBS/0.1 % BSA). Fifty microliters (50 µL) of Dynabeads was transferred into a washing tube that was then placed on a magnetic particle concentrator (Dynal MPC) for 60 seconds and the fluid removed with a pipette. After removing the tube from the magnetic device, 1 mL PBS/0.1 Bovine Serum albumin (BSA) was added and the beads resuspended. This procedure was repeated another two times until the Dynabeads are resuspended back into their original volume (50 µL) with washing buffer.

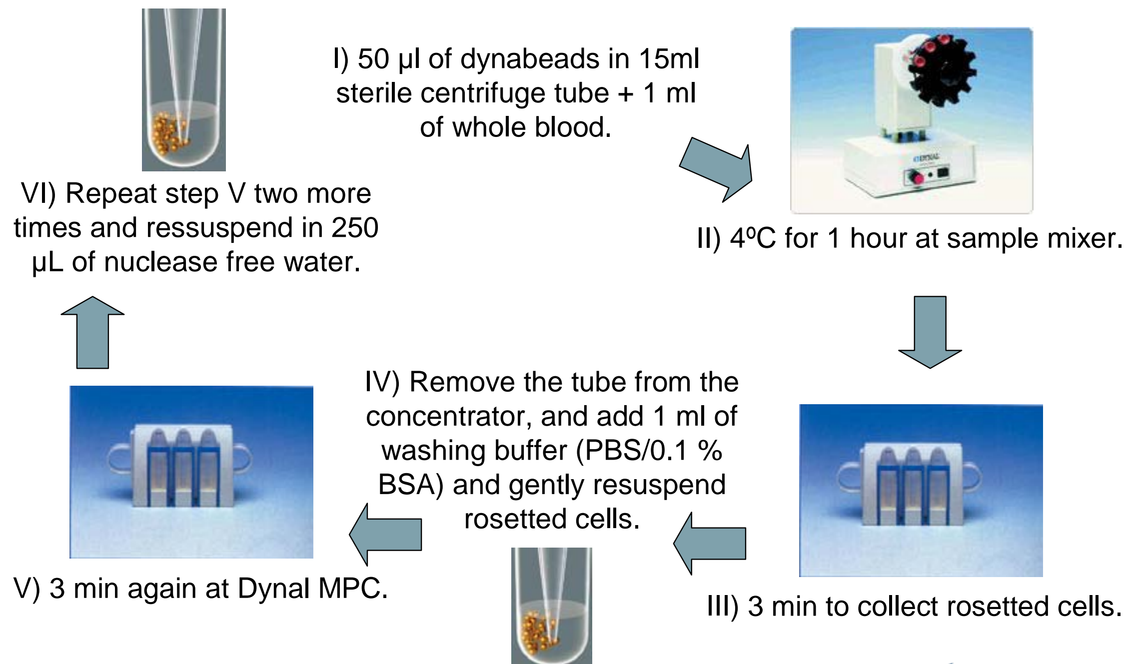
To separate different leukocyte fractions from the blood, Dynabeads coated with antibodies against CD19 (pan B leukocytes), CD14 (macrophages / monocytes), CD2 (pan T leukocytes) and CD45 (pan leukocytes) were used. To 1 mL of whole blood, 50 µL of dynabeads were added giving an approximate final concentration of 2 x 10^7 beads/mL. The whole blood and Dynabead mixture was then incubated at 4ºC for 1 hour with continuous rotation of the tubes using a Dynal sample mixer (Dynal A.S., Oslo, Norway). The tube was then positioned in the magnetic particle concentrator (Dynal MPC) for 3 minutes to collect the rosetted cells. The supernatant was pipetted carefully while the rosetted cells remained attached to the wall of the tube by the concentrator. The tube was removed from the concentrator, after which 1 mL of washing buffer was added and the rosetted cells gently resuspended. The tube was repositioned into the concentrator and the supernatant removed. The rosetted cells were washed three times with 1 mL washing buffer, resuspended in 250 µL of nuclease-free water and finally stored at -80 ºC until required. Figure 2.1 illustrates the process.

2.3. Molecular detection of HCV

2.3.1. Laboratory accommodation

To minimize contamination during polymerase chain reaction (PCR), nucleic acid extraction, PCR reagent preparation, thermocycling, and post-PCR procedures were conducted in dedicated rooms. Appropriate negative control specimens were also included in each PCR. The workflow was strictly unidirectional in order to prevent contamination with PCR products.

Figure 2.1. cell separation procedure using dynabeads



2.3.2. RNA Extraction

2.3.2.1. Manual RNA extraction - Qiagen QIAamp MinElute virus spin kit

Manual RNA extraction was employed for all samples, but CD45 from patients 1 to 4 due to lack of blood for cell separation. RNA was extracted from two hundred microlitres (200 µL) of plasma, whole saliva, as samples of CD19, CD14, CD2 or CD45 using the modified QIAamp kit (Qiagen, UK) following the manufacturer's instruction. The extraction was carried out using QIAamp MinElute columns in a standard microcentrifuge. The Qiagen protease was ressuspended in buffer AVE® (RNase free water containing 0.04% sodium azide) instead of the protease ressuspension buffer, which could coagulate cells. This modification was carried out to avoid clogging the columns as with the exception of plasma, there were cells in all of the analyzed body fluids.

Twenty five microliters (25 µL) of Qiagen protease, 200 µL of sample (plasma, whole saliva, CD19, CD14, CD2 or CD45) and then 200 µL of buffer AL (containing 28 µg / mL of carrier RNA) were pipetted into a 1.5 mL microcentrifuge tube. The microtubes were incubated at 56°C for 15 minutes in a heating block. Lysis was performed in the presence of Qiagen protease and lysis buffer (buffer AL®) in order to ensure the inactivation of RNAses. Samples were lysed under these elevated temperatures which were highly denaturing conditions.

The tubes were then briefly placed in a microcentrifuge to remove drops from the inside of the lid and 250 µL of ethanol (99.7-100%) added to the tubes.

Following thorough pulse-vortexing for 15 seconds the lysate was incubated with ethanol for 5 minutes at room temperature. Binding conditions were adjusted by adding ethanol to allow optimal binding of the viral RNA and DNA to the membrane. Lysates were then transferred onto a QIAamp MinElute column and viral nucleic acids were adsorbed onto the silica-gel membrane as the lysate was drawn through by centrifugation.

Four centrifugation steps were then performed, starting with the addition of 500 µL of buffer AW2 to the columns followed by centrifugation for 1 minute at 8000 RPM. After changing the collection tubes, 500 µL of ethanol was added to the columns followed by centrifuge for 1 minute at 8000 RPM. The collection tubes were changed and a dry spin at the centrifuge for 3 minutes at 13000 RPM performed to dry the membrane completely. Salt and pH conditions ensured that protein and other contaminants, which can inhibit PCR and other downstream enzymatic reactions, were not retained on the QIAamp MinElute membrane. The collection tube was replaced for a 1.5 mL microcentrifuge tube with a lid for storage purposes. The final elution volume added is 60 µL of buffer AVE (RNase free water, containing 0.04% sodium azide), after which the column is left at room temperature for 1 minute before being placed in the centrifuge at 13000 RPM for 1 minute. Figure 2.2 proposes a scheme representation of the process.

2.3.2.2. Automated extraction - Qiagen Universal QiAamp96 Virus Kit

Qiagen Universal QIAamp96 Virus nucleic acid purification procedure was used to perform RNA extraction for the quantitative Hepatitis C virus (HCV)

assay for whole saliva and plasma samples. Three hundred microliters (300 µL) of the samples were extracted by the Qiagen Universal QIAamp96 Virus Nucleic Acid Purification Robot. The HCV standard curve employed was based upon a 10 fold dilutions of an in-house 10000000 IU/mL standard shown in Table 2.2. One of 96 HCV window phase plasmas from a previous study (Tuke *et al.*, 2008) was used as the positive control. This plasma had been characterised as containing 3.58×10^7 IU / mL. One millilitre of this control was diluted with 2.58 mL of normal human plasma (NHP) to obtain a concentration of 10^7 IU / mL.

Table 2.2. HCV standard 10 fold dilutions of the in-house control for real time RT-PCR of HCV RNA

In-house Control	Composition
10000000 IU/mL	Plasma "122" diluted 1mL + 2.58 mL NHP
1000000 IU/mL	50 µL 10000000 IU/mL control + 450 µL NHP
100000 IU/mL	50 µL 1000000 IU/mL control + 450 µL NHP
10000 IU/mL	50 µL 100000 IU/mL control + 450 µL NHP
1000 IU/mL	50 µL 10000 IU/mL control + 450 µL NHP
100 IU/mL	50 µL 1000 IU/mL control + 450 µL NHP
10 IU/mL	50 µL 100 IU/mL control + 450 µL NHP
Negative	450 µL normal human plasma (NHP)

The extraction was automated following the same principles as the QIAamp MinElute Virus Spin Kit with some modifications. An AW1 (guanidine and

ethanol) wash step was added before the AW2 wash, and instead of spinning in a centrifuge all fluid passed through the columns using a vacuum.

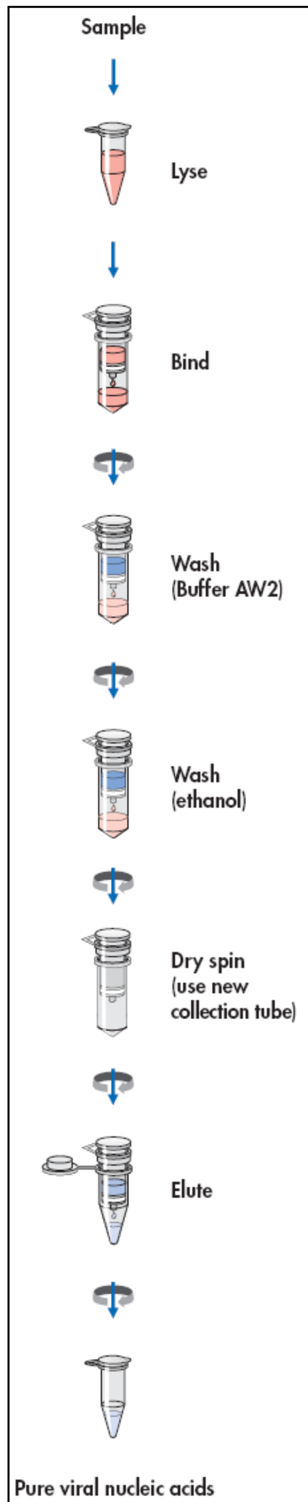


Figure 2.2. MinElute Virus Spin Procedure / manual extraction of HCV RNA

2.3.3. Microzone DNA OK Kit

DNA OK kit (Microzone, UK) was the housekeeping assay used for this study. It has confirmed the integrity of DNA, therefore highlighting that samples were adequate for molecular biology analysis.

In this kit five primer pairs were amplified from 5 different chromosomes to verify the integrity of the DNA of the extracts. The kit was used according to manufacturer's criteria testing all the samples. The gel picture (figure 2.3) shows results for intact DNA (G), degraded, fragmented or shortened DNA (B) and fully degraded DNA (U).

The protocol used comprised mixing 7.5 µL of Human DNAOK mix with 12.5 µL of MegaMix Gold. Five (5) µl DNA (5 to 50 ng) was then added and the reaction tubes were placed in a Thermal Cycler. The cycling profile comprised of an initial denaturation step of 95°C for 5 mins. Then this cycle was repeated 33 times:

Step 1: 95°C for 30 secs. Step 2: 62°C for 30 secs. Step 3: 72°C for 45 secs. After cycling, 10 µl was loaded onto a 2% agarose gel and electrophoresed alongside a 100 bp DNA Ladder. Expected fragment sizes were 100 bp, 200, 300, 400, 500 (internal control) and 600 bp. In this assay, the more negative bands present, the more DNA is degraded or not present, which was not the case in the present study.

Figure 2.3. Interpretation of DNAOK results (G, B and U explained above in text)

Modified from www.microzone.co.uk/images/HDNA.png.

2.3.4. cDNA Generation

RNA was reverse-transcribed using Murine Maloney Leukemia Virus reverse transcriptase (Invitrogen, UK), 10X buffer (Invitrogen, UK), 5 mM $MgCl_2$ (Invitrogen, UK), 10 mM deoxynucleotide triphosphates (dNTP's - Invitrogen, UK), RNasin (Promega, UK) and 20 units / μL random hexamers (Amersham Pharmacia, UK). Cycling conditions were as follows: 23°C for 10 min, 37°C for 45 min and 95°C for 5 min. The mix for the cDNA generation is shown in Table 2.3.

Table 2.3. Mix for the cDNA generation

	Amount
Nuclease free water	4.06 μL
10 x PCR Buffer	4 μL
$MgCl_2$ (50mM)	4 μL
dNTP's (10mM)	4 μL
RH (20 U/ μL)	0.4 μL
Rnasin (0.34 U / μL)	0.34 μL
RT (0.02 U / μL)	1 μL
RNA	22.2 μL
Final	40 μL

2.3.5. Amplification of HCV 5' Noncoding Region

This amplification, used for the identification of the virus, was performed in order to use final products to run the RFLP genotyping assay (refer to item 2.3.12). The HCV 5' noncoding region (5'-NCR) was amplified by nested PCR.

Primary amplification was carried out using 1 mM $MgCl_2$ (Invitrogen, UK), 10X buffer (Invitrogen, UK),10 mM each of the four dNTPs (Invitrogen, UK), 5 pmoles of sense primer 57 (P1) and of antisense primer 321 (P2), and 0.625 units of Taq polymerase (Invitrogen, UK). Reactions were heated to 94°C for 4 minutes followed by 35 cycles of 94°C for 20 seconds, 62°C for 40 seconds, 72°C for 35 seconds and final extension at 72°C for 3 minutes. Secondary amplification was from 2 µL of primary PCR product using 2 mM $MgCl_2$ (Invitrogen, UK), 10X buffer (Invitrogen, UK), 0.625 units of Taq polymerase (Invitrogen, UK), 20 pmoles of sense primer 126 (P3) and 20 pmoles of antisense primer 299 (P4) were used and cycling conditions were as follows: 25 cycles of 94°C for 20 seconds, 68°C for 40 seconds and 72°C for 30 seconds. Primer sequences (Lin *et al.*, 1992) as indicated in Table 2.4 and the PCR mixs are indicated in tables 2.5 and 2.6.

Table 2.4. NCR PCR primer sequences

57 (sense):	5’-AGCGTCTAGCCATGGCGT
321 (antisense):	5’ GCACGGTCTACGAGACCT
126 (sense):	5’-GTGGTCTGCGGAACCGG
299 (antisense):	5’-GGGCACTCGCAAGCACCC

Table 2.5. Mix for the first round HCV NCR PCR

	Amount
Nuclease free water	30.8 μL
10 x Buffer	4 μL
$MgCL_2$ (50mM)	1 μL
P1 (5 pmoles)	2 μL
P2 (5pmoles)	2 μL
Taq	0.2 μL
cDNA	10 μL
Final	50 μL

Table 2.6. Mix for the second round HCV NCR PCR

	Amount
Nuclease free water	37.8 μL
10 x Buffer	5 μL
$MgCL_2$ (50mM)	2 μL
dNTPs (10mM)	1 μL
P3 (20 pmoles)	1 μL
299 (20 pmoles)	1 μL
Taq	0.2 μL
Total	48 μL
HCV NCR-1	2 μL

2.3.6. Amplification of Non Structural 5 b Region (NS5b)

Non Structural 5 b Region PCR (NS5b) was employed in order to achieve direct sequence of plasma, whole saliva and PBMCs genome.

The NS5b was amplified by nested PCR. Primary amplification was carried out using 3.5 mM $MgCl_2$, 10X buffer, 10 mM each of the four dNTPs, 20 pmoles of sense primer P1203 and 20 pmoles of antisense primer P1204 (Mellor *et al.*, 1995), and 0.625 units of Taq polymerase (Invitrogen, UK). Reactions were heated to 94°C for 30 seconds followed by 35 cycles of 94°C for 30 seconds, 55°C for 40 seconds, 72°C for 50 seconds and final extension at 72°C for 30 seconds. Secondary amplification was from 2 μL of primary PCR product using megamix blue® (50 μL reaction – Microzone, UK) plus 20 pmoles of sense primer NS5bn2 (Reference Laboratory – Health Protection Agency, Colindale – UK) and 20 pmoles of antisense primer P1204 (Mellor *et al.*, 1995). The cycling conditions were as follows: 94°C for 30 seconds followed by 30 cycles of 94°C for 30 seconds, 54°C for 40 seconds, 72°C for 30 seconds and final extension at 72°C for 30 seconds. The primer sequences are indicated in Table 2.7 and the PCR mixs are indicated in Tables 2.8 and 2.9.

Table 2.7. NS5b PCR primer sequences

P1203 (sense):	5' ATGGGGTTCTCGTATGATACCCGCTG CTTTGACTC
P1204 (antisense):	5' GGAGGGGCGGAATACCTGGTCATAGCCTCCGTGAA
NS5bn2 (sense):	5' - TGATACCCGCTGCTTTGACTCNACNGTCAC

Table 2.8. Mix for the first round HCV NS5b PCR

	HCV NS5b-1
Nuclease free water	27.8 μL
10 x Buffer	3.5 μL
$MgCL_2$ (50mM)	2.5 μL
P1203 (20 pmoles)	0.5 μL
P1204 (20 pmoles)	0.5 μL
Taq	0.2 μL
cDNA	15 μL
Final	50 μL

Table 2.9. Mix for the second round HCV NS5b PCR with megamix blue®

	HCV NS5b-2
Megamix blue® mastermix	46 μL
P1204 (20 pmoles)	1 μL
NS5bn2 (20 pmoles)	1 μL
HCV NS5b-1	2 μL
Final	50 μL

2.3.7. Detection of the PCR product

Ten microliters (10 μL) of PCR product was eletrophoresed immediately after removed from the thermal cycler, as megamix blue® already contains the loading dye. After cloning and GFX cleanup, 8 μL of the PCR product was mixed with 2 μL of Blue/Orange 6X loading dye (Promega, UK) and

electrophoresed through a 2% agarose gel along with 1 µg of 1-kb ladder molecular weight marker (Invitrogen, UK) on either side of the test samples to assess the size of the amplified product. A positive and a negative control were also added. Electrophoresis was undertaken in a 1X tris-borate EDTA buffer (Invitrogen, UK). The gels were then stained with 10 mg/mL ethidium bromide solution (Invitrogen, UK) in TBE buffer (concentration of 5 µg / mL). DNA fragments were visualized using a short wave ultra-violet trans-illuminator and photographed using a photo printer.

2.3.8. Restriction Fragment Length Polymorphism (RFLP) and Analysis

The amplification product obtained in the 5'-NCR PCR was used for genotyping via RFLP. A 10 µL final volume restriction digestion assay was done for each enzyme, adding nuclease free water (NFW) and the appropriate buffer for each enzyme. The enzymes and the tubes in which they were mixed remained on ice, as 3 of these 4 enzymes work at 37°C (very close to room temperature). The tubes were incubated individually (for each enzyme) with 10U of the enzymes for 1.5 hour at 37°C for ScrfI, MvaI and HinfI and 60°C for BstUI and then at 80°C for 10 minutes. The following restriction enzymes were added in two different reactions: 1: ScrfI (New England Biolabs, UK) /MvaI (Roche, UK); 2: HinfI /BstUI (New England Biolabs, UK) for electrophoresis. The mix for the RFLP in indicated in Table 2.10.

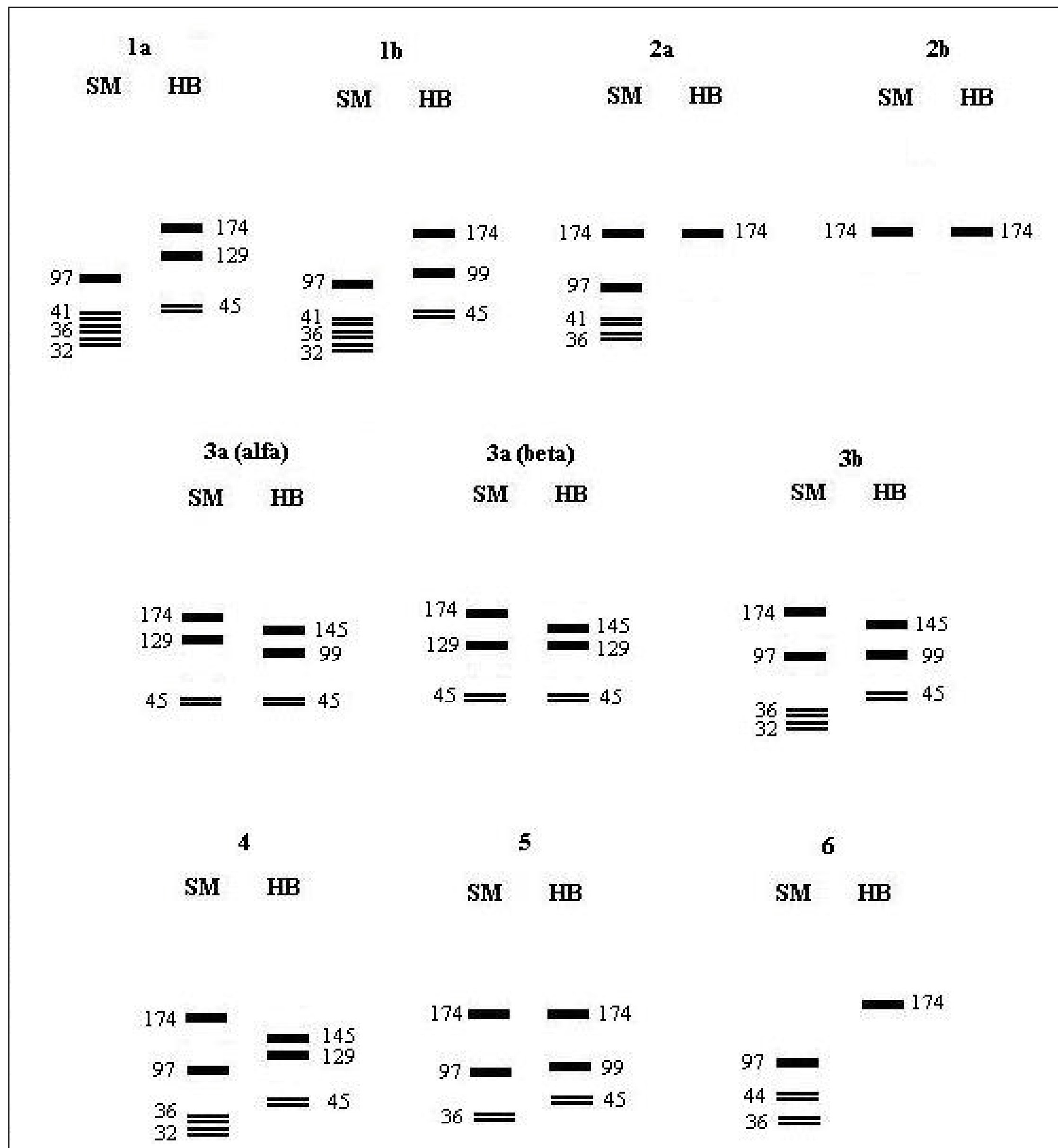
Table 2.10. Mix of the restriction fragment length polymorphism

	Amount
Nuclease free water	4 μL
10 X Buffer	1 μL
Restriction enzyme	0.5 μL
DNA	4.5 μL
Final	10 μL

2.3.9. Detection of the RFLP product

Four microliters of the each restriction digestion product was mixed following the previous pattern (ScrfI+MvaI and HinfI+BstUI) with 2 μL of Blue/Orange 6X loading dye (Promega, UK) and electrophoresed through a 3% agarose gel along with 1 μg of 100-bp ladder molecular weight marker (Invitrogen, UK) on either side of the test samples to assess the size of the cleaved amplicons. A positive and a negative control were also added. Electrophoresis was undertaken with a 1X tris-borate EDTA buffer (Invitrogen, UK). The gels were then stained with 10mg/mL ethidium bromide solution (Invitrogen, UK) in TBE buffer (concentration of 5μg / mL). DNA fragments were visualized using a short wave ultra-violet trans-illuminator and photographed using a photo printer. A diagram of genotype specific patterns found with RFLP is indicated in Figure 2.5.

Figure 2.4. Diagrammatic representation of most representative bands determining the digestion pattern observed with RFLP for HCV genotyping



*Based on Pohjanpelto et al. 1996. Numbers from 1a to 6 refer to genotypes and from 32 to 174 refer to base pairs. SM refers to the mixture of ScrfI and MvaI enzymes and HB to the mix of HinfI and BstUI enzymes for electrophoresis.

2.3.10. Purification of NS5b PCR products for sequencing (GFX Cleanup)

In order to prepare samples for sequencing, second-round PCR products were purified using a spin column based purification kit (Amersham Pharmacia, UK) following the manufacturer's instructions. Five hundred micro litres of capture buffer was added to a GFX column together with the second round PCR product. After mixing thoroughly by pipetting the sample up and down 6 times the GFX, the columns were placed in a microcentrifuge at full speed for 30 seconds. Following discard of the flow-through by empting the collection tube, 500 µL of wash buffer was added to the column and centrifuged at 8000 RPM for 30 seconds. The columns were transferred to a fresh microcentrifuge tube followed by the addition of 50 µL of elution buffer (nuclease free water). The samples were incubated at room temperature for 1 minute followed by centrifugation at 8000 RPM for 1 minute to recover the purified DNA.

Samples that did not sequence on the first attempt were cloned. These samples required purification of DNA from gel bands. The PCR product was run down a gel and the product cut out of the gel. The gel slice was weighted (maximum of 300 mg) and the same amount of capture buffer added to it in a 1.5 mL microcentrifuge tube, and incubated at 60°C until the agarose was completely dissolved (approximately 15 minutes). The sample was then transferred to the GFX column and incubated at room temperature for 1 minute. The procedure then followed that for the purification of DNA from a solution.

To confirm that the purification had been successful and to estimate the quantity of DNA recovered, 1 µL of the supernatant was run on a 2% agarose gel alongside a 1µg of 1-Kb ladder (Invitrogen, UK), stained and visualised before subsequent cloning or sequencing.

2.3.11. Cloning

Cloning was undertaken for some positive samples that were difficult to sequence. The TOPO-TA Cloning® kit (Invitrogen, UK) was used. TOPO-TA Cloning® reaction mix is indicated in Table 2.11.

Table 2.11. Mix for chemically competent E. coli reaction

Chemically competent E. *coli* reaction
1 µL of salt solution. 2 µL of PCR fresh product. Sterile water added to a final volume of 5 µL. 0.9 µL of vector. 5.9 µL of final volume.

E. coli was used to transform the cloning reaction. The constituents were incubated for 5 minutes at room temperature and then placed on ice for the addition of 2 µL of this reaction to separate vials of competent cells (OneShot® Top10 cells). The transformation mixture was placed in a thermostatic bath circulator at 42°C for 30 seconds and then left on ice for 2 minutes. Two hundred and fifty (250) µL of 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 cell / ligation tube. The tubes were left in an orbital incubator for 1 hour, at 200 RPM and at 37°C. Finally, 270 µL of the cell / ligation was spread on an L agar plate. The cloning reaction was left overnight on L agar (Luria agar: 1.0% tryptone, 5.0% yeast extract, 1.0% 0.17% MNaCl, 1.5% agar, pH 7.0) containing 50 mg/mL ampicillin at a 37°C incubation to allow for growth of the *E. coli* containing the HCV RNA amplicon. PCR using second-round primers was performed directly on the resulting colonies prior to sequencing. The cycling conditions were as follows: 94°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 40 seconds.

2.3.12. DNA sequencing

The samples were sequenced using the Beckman CEQ2000 automated capillary array sequencer.

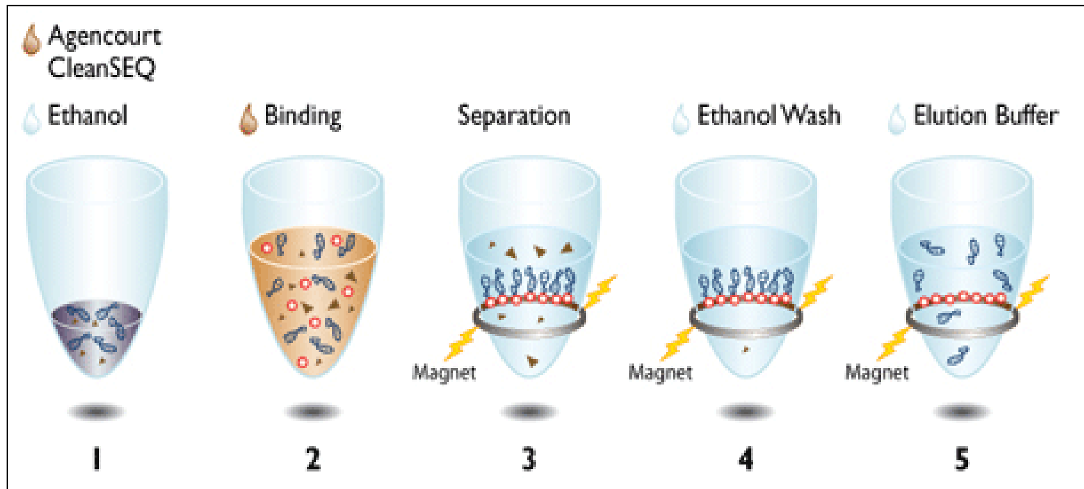
Purified DNA was added to the PCR sequencing reaction consisting of the following components: 2-8 µL of DNA (400 ng calculated for sequencing reaction), 3 µL of inner (P1204 or NS5bn2) PCR primers (2 pmoL), and 8 µL of Beckman dye terminator cycle quick start master mix (Beckman Coulter, UK), the volume adjusted with sterile water to a total volume of 20 µL. The cycling conditions were as follows: 30 cycles of 96°C for 20 sec, 50°C for 20 sec and 60°C for 4 minutes.

Sequencing PCR reactions were purified using the CleanSEQ® Sequencing Reaction Clean-Up System (Agencourt, USA). CleanSEQ® contains magnetic particles in an optimized binding buffer to selectively capture sequencing extension products.

The CleanSEQ® procedure (Figure 2.4) was performed in three stages. Selective binding of sequencing extension products to paramagnetic beads and separation of the beads with a magnetic field, followed by washing of the beads with 85% ethanol to remove unincorporated dyes, nucleotides, salts and other contaminants and finally the elution of the purified sequencing product from the paramagnetic beads using formamide.

The Agencourt CleanSEQ reagent and ethanol was added to the sequencing reaction and the sequencing products bind to the magnetic beads. The sequencing products were then separated from the contaminants with a magnetic plate. After the washing step with ethanol, the elution from magnetic particles was achieved with the addition of 40 µL of formamide. After the incubation of the plate for 5 minutes to elute, a drop of mineral oil was added to each sample.

Figure 2.5. Overview of the CleanSEQ® Process overview



http://www.agencourt.com/products/spri_reagents/cleanseq/

The sequencing products were then immediately loaded onto the automated sequencer. The Beckman CEQ-2000 uses a capillary system to electrophorese the samples through a polyacrylamide gel contained in a capillary tube. In the sequencing PCR mix, chain terminating nucleotide bases with different fluorescent tags are present and are incorporated into the DNA during PCR amplification. During electrophoresis a laser reads these fluorescent bases to determine the sequence of the sample.

Raw chromatograph data were analysed using the SeqMan sequence analysis software, and multiple alignments were made in Megalign. Both programs are from the LASERGENE sequence analysis package (DNAstar Inc.). A cluster V alignment was produced in Megalign to construct a guide tree using the UPGMA method of clustering analysis. Further analysis was undertaken using the blast search, using HCV databanks:

http://hcv.lanl.gov/content/hcv-db/BASIC_BLAST/basic_blast.html, http://www.ncbi.nlm.nih.gov/BLAST/.

2.3.13. Quantitative Real Time PCR

The quantitative Hepatitis C virus (HCV) assay was used for all the whole saliva, plasma, CD19, CD14, CD2 and CD45 samples. ABI Prism 7000 real-time PCR was used with QIAGEN TaqMan-PCR reagents (QIAGEN, UK). The quantification is based on a standard curve with a calibrated in house standard, calibrated by comparison to the NIBSC HCV standard 96/798. The assay used Brome mosaic virus (BMV) RNA as an internal control (IC), which was introduced at the extraction stage. BMV could not be added for the quantitative real time PCR of CD19, CD14, CD2 and CD45 samples, as they had been performed using extractions from the Qiagen QIAamp MinElute virus spin kit.

The multiplex real time PCR detects both HCV and BMV with differently labelled TaqMan probes. An in-house ‘run control’ was used as a monitor of performance (see Table 2.2 and section 2.3.2.2 for details). This assay is intended for detection of HCV RNA in serum or plasma, and quantification of HCV RNA between 100 and 10000000 IU/mL, and was adapted for use in the detection of HCV RNA in the whole saliva.

The master mix volume added into each well of an optical PCR plate consisted of 15 μL in total, and includes the Absolute quantitative RT-PCR mastermix, the HCV/BMV primer/probe mix, the enzyme and nuclease free

water. To this, 10 µL of RNA extraction of the sample was added. The tray was then sealed with an optical adhesive sealer (Applied Biosystems, UK). Tables 2.12, 2.13 and 2.14 indicate the mix of the real time PCR HCV assay and the primers and probes used, respectively.

Table 2.12. Mix for the real time PCR HCV assay

Reagent	Amount/sample
ABsolute quantitative RT-PCR mastermix	12.5 µL
HCV/BMV primer/probe mix	0.5 µL
Enzyme	0.25 µL
Nuclease free water	1.75 µL
RNA	10 µL
Total	25 µL

Table 2.13. Primers used for the HCV/BMV quantitative PCR (100 pMol/µL)

HCV Primer 1:	5'- GTCTAGCCATGGCGTTAGTA -3'
HCV Primer 2:	5'- GTACTCACCGGTTCCGC -3'
BMV Primer 1:	5’- GTTCACCGATAGACCGCTG-3’
BMV Primer 2:	5’- AAGAGCCCGGAATGTCAAGA-3’

Table 2.14. Probes used for the HCV/BMV quantitative PCR (100 pMol/µL)

HCV:	5'FAM- CCCTCCCGGGAGAGCCATAGTG-3'TAMRA
BMV:	5’-VIC- CCTCAAGCTGAAATGGCACGGATG -3'TAMRA

2.3.14. Pyruvate dehydrogenase (PDH) Quantitative Real Time PCR

Pyruvate dehydrogenase (PDH) was undertaken in order to quantify the average number of cells present in the extract in order to achieve also a viral number per cell on the top of the neat value.

The quantitative PDH PCR was used for all the PBMCs extracts (other than that of one patient) and for all saliva samples extracts (Koike *et al.*, 1990).

Quantification was made with a standard curve using a Human male DNA dilution. The human male DNA was diluted in a 1:10 with buffer AVE with carrier RNA (6 µL of carrier RNA added to 594 µL of buffer AVE, which is constituted of RNAse free water containing 0.04% of sodium azide). Ten fold dilutions of this standard are shown in Table 2.15.

Table 2.15. Ten fold dilution of human male DNA

Human male DNA	Composition
1666 copies	2µL Human male DNA (10ng/ µL) + 18 µL AVE*
166 copies	2µL 1666 copies/mL control + 18 µL AVE*
16.6 copies	2 µL 166 copies/mL control + 18 µL AVE*
1.6 copies	2 µL 16.6 copies/mL control + 18 µL AVE*
0.16 copies	2 µL 1.6 copies/mL control + 18 µL AVE*
Buffer AVE with carrier RNA	20 µL Buffer AVE with carrier RNA

The master mix volume added into each well of an optical PCR plate constituted 15 µL in total, and included the Absolute quantitative RT-PCR

mastermix, the PDH primer/probe mix, the enzyme and nuclease free water. To this, 10 µL of RNA extraction of the sample was added. The tray was then sealed with an optical adhesive sealer (Applied Biosystems, UK). Table 2.16 shows the mix for the real time PCR PDH assay. The primers and probe for PDH quantification via Real Time PCR are indicated in Tables 2.17 and 2.18, respectively.

Table 2.16. Mix for the real time PCR PDH assay

Reagent	Amount/sample
ABsolute quantitative RT-PCR mastermix	12.5 µL
PDH primer/probe mix	0.5 µL
Enzyme	0.25 µL
Nuclease free water	1.75 µL
RNA	10 µL
Total	25 µL

Table 2.17. Primers for the real time PCR PDH assay (concentration 25 pMol/µL)

PDH Taq 1:	5'-TGAAAGTTATACAAAATTGAGGTCACTGTT-3'
PDH Taq 2:	5'- TCCACAGCCCTCGACTAACC -3'

Table 2.18. Probe (concentration 100pMol/µL)

PDH:	5' VIC-CCCCCAGATACACTTAAGGGATCAACTCTTAATTGT-3'TAMRA

Table 2.19. Preparation of HCV Taqman primer/probe mix

Primer/probe	Concentration (pmol/ µL)	Half plate (48 wells)	Full plate (96 wells)
HCV primer 1	100	10 µL	20 µL
HCV primer 2	100	10 µL	20 µL
HCV probe	100	5 µL	10 µL
BMV primer 1	100	10 µL	20 µL
BMV primer 2	100	10 µL	20 µL
BMV probe	100	5 µL	10 µL

Table 2.20. Preparation of PDH Taqman primer/probe mix

Primer/probe	Concentration (pmol/ µL)	Half plate (48 wells)	Full plate (96 wells)
BMV primer 1	100	10 µL	20 µL
BMV primer 2	100	10 µL	20 µL
BMV probe	100	5 µL	10 µL
PDH primer 1	25	10 µL	20 µL
PDH primer 2	25	10 µL	20 µL
PDH probe	100	5 µL	10 µL

2.3.15. Statistical analysis

Data entry and analysis were performed using the SPSS for Windows (Statistical Package of Social Science) software, version 17.0. Frequency distributions and cross-tabulation tables were constructed in order to analyze the demographic, clinical and virological information. In an attempt to detect possible risk factors, both logistic regression and chi-squared testing (the *P*

value for statistical significance was set at <0.05) were used. The independent samples *t*-test, ANOVA, Mann Witney U and Bivariate correlation (Spearman's rank correlation) were used as appropriate.

Fisher's exact test was employed to compare the expected amino acid sequences within the dominant quasispecies of individual HCV genotypes between the different compartments detecting the presence of non-synonymous mutations within the NS5b region of the HCV genome.

Chapter 3

Prevalence of HCV RNA in Whole Saliva

Several studies have been published with regards to the aspects of the prevalence of HCV RNA in the saliva (Goncalves *et al.*, 2005, Diz *et al.*, 2005, Hermida *et al.*, 2002, Fabris *et al.*, 1999, Belec *et al.*, 2003b, Suzuki *et al.*, 2005, Roy *et al.*, 1998, Roy *et al.*, 1996, Arrieta *et al.*, 2001, Rey *et al.*, 2001, Harle *et al.*, 1993, Couzigou *et al.*, 1993, Jorgensen *et al.*, 1996, Sugimura *et al.*, 1995, Mariette *et al.*, 1995, Young *et al.*, 1993, Caldwell *et al.*, 1996, Komiyama *et al.*, 1995, van Doornum *et al.*, 2001, Liou *et al.*, 1992, Mastromatteo *et al.*, 2001, Eirea *et al.*, 2005, Wang *et al.*, 2006, Wang *et al.*, 1992b, Hsu *et al.*, 1991, Puchhammer-Stockl *et al.*, 1994, Takamatsu *et al.*, 1990, Wang *et al.*, 1991, Roy *et al.*, 1995, Taliani *et al.*, 1997, Becheur *et al.*, 2000, Ustundag *et al.*, 1997, Biasi *et al.*, 1995, Roy *et al.*, 1999, Pastore *et al.*, 2006, Nakano *et al.*, 1992, Kage *et al.*, 1997, Savoldi *et al.*, 2001, Fried *et al.*, 1992, Ogasawara *et al.*, 1993, Toussirot *et al.*, 2002, Chen *et al.*, 1995, Lins *et al.*, 2005, Maticic *et al.*, 2001). Nevertheless, only three of the cited papers involved the quantification of HCV RNA in saliva (Suzuki *et al.*, 2005, Rey *et al.*, 2001, Belec *et al.*, 2003b). The HCV RNA prevalence in the saliva ranges from 0% (van Doornum *et al.*, 2001, Hsu *et al.*, 1991, Fried *et al.*, 1992) to 100% (Lins *et al.*, 2005, Arrieta *et al.*, 2001, Harle *et al.*, 1993, Takamatsu *et al.*, 1990, Wang *et al.*, 1991, Biasi *et al.*, 1995).

The first study involving HCV RNA quantification was positive for 20 cases (62.5%) in the saliva and could only quantify 18 cases in plasma (56%) from all the 32 HCV seropositive patients. They claimed that the hybridization method used (”branched DNA” technique – Chiron, as opposed to RT-quantitative PCR in most other cases including our study) was probably unable to detect the virus in these two patients that were HCV RNA positive for the saliva and

not for plasma. The virus concentration of the only study that used branched DNA hybridization for HCV RNA quantification had an average of 38.8 ± 22.4 copies / mL in plasma and 67 ± 60.8 copies / mL in saliva (Savoldi *et al.*, 2001). Another study later at the same year performed HCV RNA quantification in the saliva showing a 37.3% rate of detection of the virus in the saliva. The mean viral RNA level was 1150000 copies / mL and the mean viral RNA level for plasma was 25200000 copies / mL, also using a branched DNA assay for HCV RNA quantification (Rey *et al.* 2001). Two years later, Belec *et al.* (2003) detected HCV RNA in 42% of the saliva pellet samples (cell associated whole saliva), 4% (only one patient) of saliva supernatant and in none of the cell free gingival crevicular fluid. Viral load was performed for the only positive patient with both whole saliva supernatant (1134 copies / mL) and cell associated whole saliva positivity (251 copies / mL). Viral load was also measurable in only 2 other patients from the 12 HCV RNA positives at the cell associated whole saliva. Measures were 198 copies / mL and 133 copies / mL (Belec *et al.* 2003a). Suzuki *et al.* (2005) found no significant association between viral RNA levels in plasma and viral RNA levels in the saliva or gingival crevicular fluid (GCF). HCV RNA was detected in 31% of the saliva samples and 85% of the GCF specimens using real-time RT-PCR and the mean viral RNA level was 19000 copies / mL for the saliva and 31000 copies / mL for the GCF (Suzuki *et al.*, 2005).

3.1. Aims

The aim of the present chapter is to determine the prevalence and viral load of HCV in whole saliva of patients with HCV disease attending clinics in Recife in Northeast Brazil. In addition, the influence of HCV in plasma and peripheral blood mononuclear cells (PBMCs) upon the salivary presence of this virus was examined.

3.2. Patients and samples

3.2.1. Study population

Samples were collected from two different locations: a general public hospital and a private dialysis clinic in Recife, Northeast Brazil (details in Section 2.1). All participants were of Brazilian nationality. Clinical data on all patients were obtained from standard medical records and documented onto standardized forms, which were linked to patient samples by numerical code. These forms were used during anamnesis undertaken before clinical examination. Oral findings and route of HCV acquisition can be found in Table 3.2.

3.2.2. Sample collection

Matched oral and blood samples were obtained from all patients as described in Sections 2.2.1 and 2.2.2.

3.2.3. Sample processing

After separation of plasma from the blood, the CD19+, CD14+, CD2+ and CD45+ cell subsets were immunomagnetically fractionated as described in Sections 2.2.3. WMS was not subjected to centrifugation and it was stored at -80°C until required. RNA was extracted from blood cell subsets and oral samples as described in Section 2.3.2. The presence of DNA in randomly selected extracts was verified by using the Microzone DNA OK kit as described in Section 2.3.3.

3.2.4. Molecular detection of HCV

3.2.4.1. RNA extraction

RNA extraction was performed both manually (Section 2.3.2.1) for all samples, but CD45 from patients 1 to 4 due to lack of blood for cell separation and automatically for whole saliva and plasma samples (Section 2.3.2.2).

3.2.4.2. Polymerase Chain Reaction (PCR)

Reverse transcription was carried out to generate cDNA for all samples (Section 2.3.4) followed by PCR of the 5'-noncoding region for plasma samples only (Section 2.3.5) and NS5b region for all samples (Section 2.3.6).

3.2.4.3. RFLP and direct sequencing

RFLP was performed for all plasma samples (Sections 2.3.8 and 2.3.9) and direct sequencing was performed for all samples (Section 2.3.12).

3.2.4.4. Quantitative real time PCR

Quantitative Hepatitis C virus (HCV) assay was used for all the whole saliva, plasma, CD19, CD14, CD2 and CD45 samples in order to obtain HCV viral load of the respective sites (Section 2.3.13).

3.3. Results

3.3.1. Hepatic function of patients

The Mann Witney U test established that there were no statistically different results in the levels of aspartate aminotransferase (P=0.563), alanine aminotransferase (P=0.367), alkaline phosphatase (P=0.212) and gamma-glutamyl transpeptidase (P=0.509) between patients treated and not treated for their HCV infection.

3.3.2. HCV prevalence in plasma

Seventy one (82.5%) of the total patient group of 86 patients had detectable HCV RNA in plasma. Within the untreated group 59 (88%) had detectable RNA while 6 (33%) of the treated group had detectable HCV RNA. Fisher's exact test established that there was no statistically significant difference in the prevalence of HCV RNA between these 2 groups (P=0.66).

3.3.3. HCV load in plasma

The median plasma HCV load in the total group was 483000 IU/ml (range 0 to 30800000). The median HCV loads in the treated and untreated patients was 93550 (range 0 to 19600000) and 562000 IU/ml (range 0 to 30800000) respectively however the Mann Witney U test revealed that there was no statistically significant difference in the plasma HCV loads between these two groups (P= 0.101).

3.3.4. HCV prevalence in whole saliva

Twenty seven (31.8%) of the 85 patients had detectable HCV RNA in whole saliva. HCV RNA was detected in 31.3% of the treated and 33.3% of the untreated patients, hence there were no statistically significant differences in the prevalence of saliva between these groups.

3.3.5. HCV load in whole saliva

The median salivary HCV load in the total group was 0 IU/mL (range 0 to 3910). The median HCV loads in the treated and untreated patients were 0 IU/mL (range 0 to 95) and 0 IU/mL (range 0 to 3910) respectively. Only 1 of the 27 HCV RNA positive samples was above the lowest level of quantification (100 IU/mL).

It should be noted that 5 of the positive salivary samples using block-based PCR were negative with real time PCR while 14 of the positives with block based PCR were negative with real time PCR. A positive result in one of the two assays was considered to indicate the presence of HCV RNA.

3.3.6. Correlation of prevalence of HCV RNA in whole saliva and plasma

Fisher's exact test revealed that there was a strong correlation (P=0.004) between the prevalence of HCV RNA in whole saliva and plasma of the total group. There were no instances of HCV being present in saliva and not plasma. These same trends were observed in the treated (P=0.049) and untreated groups (P= 0.054) separately.

3.3.7. Correlation of HCV load in whole saliva and plasma

In contrast to the above finding Spearman's test indicated that there was no correlation between the salivary and plasma HCV RNA loads in the total group and in the treated and untreated groups.

3.3.8. Influence of Oral Health

3.3.8.1. Presence of oral mucosal disease

Forty seven (47) out of 86 patients from this study had detectable oral mucosal disease during examination, only looking for gingival status with

probing depth measurement (Table 3.2). Twenty seven point seven per cent (27.7%) of patients with oral mucosal disease had detectable HCV RNA in saliva while 35.9% of those without oral mucosal disease had salivary HCV RNA. There were no statistically significant differences in the salivary HCV RNA load between the 2 groups (P=1.00). Lichen planus was not identified in any patient.

3.3.8.2. Presence of teeth

Thirty one point two per cent (31.2%) of 78 patients with teeth (i.e. dentate) and 37.5% of 8 patients without teeth (i.e. edentulous) had detectable HCV RNA in saliva respectively. There were no statistically significant differences in the salivary HCV RNA load between the 2 groups (P=0.705).

3.3.9. Correlation of route of possible acquisition of HCV and salivary carriage of HCV RNA

According to Pearson’s Chi Square test, there were no statistically significant differences in the prevalence of salivary HCV RNA between the probable routes of acquisition of HCV of all patients (P=0.712, Table 3.1)

Table 3.1. Prevalence of detectable HCV RNA in patients according to the possible route of acquisition

Possible route	No virus detected in saliva (%)	Virus detected in saliva (%)	Total
None listed	25 (67.6)	12 (34.2)	37 (100)
Sexual	2 (100)	0 (0)	2 (100)
Transfusion	20 (62.5)	12 (37.5)	32 (100)
IV drug user	2 (100)	0 (0)	2 (100)
Dialysis	8 (72.7)	3 (27.3)	11 (100)
Other*	1 (100)	0 (0)	1 (100)
Total	58 (68.2)	27 (31.8)	85 (100)

* Possible occupational acquisition.

Table 3.2. Details of likely route of acquisition of HCV and oral health status of 86 patients with known HCV infection

Patient ID	Route of HCV acquisition	Oral Health Status	Oral Lesions
1	Unknown	Healthy	None
2	Unknown	Healthy	None
3	Via contaminated glass syringe	Healthy	None
4	Sexually acquired with partner	Healthy	None
5	Unknown	Gingivitis	None
6	Blood transfusion	Gingivitis	Mucocele
7	Blood transfusion	Edentulous, xerostomia	None
8	Unknown	Periodontitis	None
9	Blood transfusion	Gingivitis	Aphthous ulcer
10	Blood transfusion	Xerostomia	Candidiasis
11	Unknown	Periodontitis	None
12	Dialysis	Periodontitis	None
13	Unknown	Periodontitis	None

14	Sexually acquired with partner	Periodontitis	Mucolece
15	Unknown	Periodontiti	None
16	Unknown	Periodontitis	None
17	Blood transfusion	Periodontitis	None
18	Injecting drug user	Periodontitis	None
19	Unknown	Periodontitis	Tonsilitis
20	Unknown	Edentulous	Denture-associated stomatitis
21	Blood transfusion	Periodontitis,xerostomia	Fibrous hyperplasia, xerostomia
22	Blood transfusion	Gingivitis	Fibrous hyperplasia
23	Unknown	Periodontitis, roots left	None
24	Blood transfusion	Gingivitis	None
25	Unknown	Edentulous	None
26	Unknown	Healthy	None
27	Unknown	Caries, periodontitis	None
28	Unknown	Healthy	Aphthous ulcer
29	Unknown	Edentulous, xerostomia	Hairy tongue
30	Blood transfusion	Healthy	Fibrous hyperplasia, caries

31	Blood transfusion	Caries, gingivitis	None
32	Dialysis	Gengivitis, xerostomia	Hairy tongue
33	Unknown	Edentulous	Denture-associated stomatitis
34	Blood transfusion	Healthy	None
35	N/A	Periodontitis, caries	Hairy tongue
36	Blood transfusion	Healthy	Frictional keratosis
37	Blood transfusion	Healthy	Denture-associated stomatitis
38	Dialysis	Periodontitis, caries	Hairy tongue
39	Blood transfusion	Healthy	Aphthous ulcer
40	Unknown	Healthy	Bilateral morsicatium buccarum
41	Unknown	Healthy	Hiperplasia of stenon ducts
42	Unknown	Healthy	Denture-associated stomatitis
43	Unknown	Healthy	None
44	Dialysis	Healthy	None
45	Unknown	Xerostomia	Erythroplakia
46	Blood transfusion	Healthy	Morsicatio linguarum
47	Unknown	Periodontitis	None

48	Unknown	Periodontitis	Hairy tongue
49	Blood transfusion, cocaine	Healthy	Leuco/erythroplakia
50	Blood transfusion	Healthy	None
51	Blood transfusion	Edentulous	Denture-associated stomatitis
52	Unknown	Health	Frictional keratosis, hyperplasia of stenon duct (erythematous)
53	Unknown	Healthy	Denture-associated stomatitis, mucocele
54	Unknown	Healthy	Epulis suggestive of peripheral giant cell granuloma (purple coloured)
55	Unknown	Healthy	Aphthous ulcers
56	Unknown	Healthy	None
57	Unknown	Roots left to be pulled	None
58	Blood transfusion	Gengivitis, caries	None
59	Blood transfusion	Caries	Geographic tongue
60	Blood transfusion	Caries, periodontitis	Denture-associated stomatitis
61	Unknown	Caries	Herpes simplex
62	Unknown	Gengivitis, caries	None

63	Unknown	Edentulous	BMS
64	Unknown	Periodontitis	Hemorrhagic petechias
65	Unknown	Healthy	Denture-associated stomatitis
66	Unknown	Caries, periodontitis	Hairy tongue
67	Blood transfusion	Healthy	Hairy tongue
68	Blood transfusion	Gingivitis	None
N1	Dialysis	Healthy	None
N2	Blood transfusion	Caries, gingivitis	Hairy tongue
N3	Blood transfusion	Caries, periodontitis	Hairy tongue
N4	Dialysis	Healthy	Denture-associated stomatitis
N5	Blood transfusion	Healthy	Linea alba
N6	Blood transfusion	Xerostomia, edentulous	None
N7	Blood transfusion	Healthy	None
N8	Blood transfusion, Dialysis	Periodontitis	Hairy tongue
N9	Blood transfusion, Dialysis	Healthy	None
N10	Dialysis	Gingivitis	None
N11	Blood transfusion	Healthy	Linea alba, traumatic ulcer

N12	Dialysis	Healthy	Aphthous ulcers
N13	Blood transfusion, Dialysis	Healthy	None
N14	Dialysis	Periodontitis	Hairy tongue
N15	Injecting drug user, Dialysis	Edentulous with just one root to be pulled out	None
N16	Blood transfusion	Caries, periodontitis and roots to be pulled out	None
N17	Dialysis	Healthy	Traumatic ulcer, linea alba
N18	Dialysis	Gingivitis	None

* N denotes patients from the renal unit at Nefroclínica private dialysis centre.

3.3.10. Correlation of HCV RNA detection in saliva versus treatment status

There was no statistically significant difference in the prevalence of HCV in whole saliva of patients who had received HCV therapy when compared with those who had not received such therapy (P=1.00, Table 3.3).

Table 3.3. Prevalence of detectable HCV RNA in patients according to whether or not they have received therapy

Treatment status	No virus detected in saliva (%)	Virus detected in saliva (%)	Total
Untreated	46 (68.7)	21 (31.3)	67 (100)
Treated	12 (66.7)	6 (33.3)	18 (100)
Total	58 (68.2)	27 (31.8)	85 (100)

The salivary load ranged from 0 to 3940 IU per mL (median 0 IU / mL, mean 54 IU / mL) and only 1 out of the 27 HCV RNA positive saliva samples was above the lowest level of quantification (100 IU / mL) with 3940 IU / mL. Three of the 27 HCR RNA saliva positive patients are edentulous. All of them were below the level of quantification for the quantitative real time PCR assay. Five of the positives for the block based PCR came up as negatives at the real time PCR assay and fourteen of them came up as positives at the real time PCR assay despite negative results at the block based PCR.

3.3.11. Correlation of HCV RNA detection between whole saliva and plasma.

The relative risk for the association between whole saliva HCV RNA and plasma HCV RNA detection was 1.614 (95% CI 1.345-1.936). The relative risk for these parameters was 2 (95% CI 1.135-3.522) for treated and 1.553 (95% CI 1.284-1.877) for untreated patients (Table 3.4).

Table 3.4. Relative risk for detectable HCV RNA in plasma according to therapy status

Therapy group	Plasma virus status	Virus not detected in saliva	Virus detected in saliva	RR (95%CI)
Untreated	No virus detected	8	0	
	Virus detected	38	21	1.553 (1.284, 1.877)
Treated	No virus detected	6	0	
	Virus detected	6	6	2.000 (1.136, 3.522)
Combined	No virus detected	14	0	
	Virus detected	44	27	1.614 (1.345, 1.936)

3.3.12. Relationship to Cellular Viral Load (CD19, CD14, CD2, CD45)

Spearman's nonparametric correlations have shown that there is a strong correlation of the HCV salivary detection in relation to all the studied cell compartments (CD19, CD14, CD2 and CD45). The correlation was very similar when using the same statistics test for the salivary viral load per 1000

cells as opposed to compartments loads (CD19, CD14, CD2 and CD45) per 1000 cells.

3.4. Discussion

To date, only four studies analysing the quantitative detection of HCV RNA in oral fluids in comparison to plasma could be found after a literature search on the subject (Rey *et al.*, 2001, Suzuki *et al.*, 2005, Belec *et al.*, 2003b, Savoldi *et al.*, 2001). This would thus appear to be the first study to address the HCV RNA viral load in oral fluids, plasma and PBMCs and it's aspects with regards to compartmentalization of HCV.

In the present study whole saliva has been utilized as preliminary studies did not verify any significant difference between HCV presence in the saliva supernatant and saliva pellet after separation by centrifugation (data not shown). Similarly, it has been shown in a previous study that centrifugation did not modify HCV-RNA concentration in saliva (Savoldi *et al.*, 2001). GCF was not collected as the present study was focused on the presence of HCV in whole saliva, as the likely route of any non-sexual and non-parenteral transmission. Peripheral blood mononuclear cells (PBMCs), which is one of the likely sources for the virus presence at the GCF, was also also examined for the presence of HCV RNA (refer to Chapter 4).

In the present study approximately one third of all patients had detectable HCV RNA in saliva, there being no statistically significant difference between those treated and not treated with anti-HCV antivirals. The present HCV

genoprevalence in whole saliva is in general agreement with the mean prevalence of HCV RNA of all previous studies of untreated patients and certainly indicates that HCV can be present in oral fluids regardless of the treatment status of patients. The present HCV genoprevalence of untreated patients (31.3%) is higher than that of a similar study of patients from the Espírito Santo region of Brazil, where only 20% of HCV seropositivy patients had detectable HCV RNA in saliva (Goncalves *et al.*, 2005). The reasons for the different genoprevalences of the two studies are unclear as both employed essentially similar methods of collection although the present study may have employed a much more sensitive method of detection of HCV RNA than that of the Espírito Santo patients.

Maticic *et al.* (2001) have mentioned that the HCV RNA presence in whole saliva was significantly related to the presence of blood in the cited compartment (Maticic *et al.*, 2001), although it had been previously demonstrated that the presence of blood in the saliva samples does not correlate with the presence of HCV RNA in the respective fluids (Komiyama *et al.*, 1995, Liou *et al.*, 1992). Traces of blood within saliva samples have been detected in around 10% (Caldwell *et al.*, 1996) or 20% of cases from all studies involving HCV RNA detection in the saliva (Belec *et al.*, 2003b, Hsu *et al.*, 1991, Komiyama *et al.*, 1995, Liou *et al.*, 1992, Maticic *et al.*, 2001, Sugimura *et al.*, 1995) with the HCV RNA detection ranging from 0% (Hsu *et al.*, 1991) to 66% (Komiyama *et al.*, 1995) with an average of approximately 31%. This average percentage is much lower when compared to studies that have excluded saliva samples with traces of haemoglobin (Diz *et al.*, 2005, Eirea *et al.*, 2005, Hermida *et al.*, 2002) or much lower (Lins *et al.*, 2005) and

exactly the same (Suzuki *et al.*, 2005) when all saliva samples were macroscopically negative for the presence of blood. Thus, we have not tested our samples for blood presence in our study, but only macroscopically checked for the presence of blood.

In the present study the discrepancies on the detection of HCV in the saliva via block-based PCR and real time PCR are likely to be due to the low level of the virus that was detected within the saliva samples. As mentioned before, just one of the HCV positive patients for the saliva had a viral load above the lower level of quantification, although, it was still below 5000 IU / mL.

As it was previously discussed in Chapter 1, HCV is typically transmitted via parenteral routes (Shepard *et al.*, 2005), however while the majority of infections have arisen as a consequence of injecting recreational drug use, receipt of blood or blood products or other iatrogenically associated exposures, 20 – 40% of HCV infected persons have no identifiable route of acquisition of the virus. It is thus possible that body fluids other than blood may be vehicles for the transmission of HCV (Belec *et al.*, 2003a, Belec *et al.*, 2003b, Briat *et al.*, 2005, Minosse *et al.*, 2006, Nowicki *et al.*, 2005, Pekler *et al.*, 2003).

Saliva is a potential vehicle for non-parenteral transmission of HCV but the supporting evidence remains inconsistent. Prior to the discovery of HCV, experimental transmission of non-A non-B Hepatitis via saliva was achieved in chimpanzees (Abe *et al.*, 1987) with later demonstration of HCV transmission via inoculation of HCV-infected saliva in non-human primates (Abe *et al.*,

1991). HCV transmission via human bites has been reported, all be it very rarely (Figueiredo *et al.*, 1994, Dusheiko *et al.*, 1990). Vertical transmission, potentially as a result of exposure to oral fluids has apparently never been reported, but although HCV is rarely observed or not detected at all in breast milk (Kurauchi *et al.*, 1993) it may be present in the whole saliva in 36% (Kage *et al.*, 1997) to 50% (Ogasawara *et al.*, 1993) of breast-feeding HCV infected mothers. Nevertheless, it is important to consider that both studies on whole saliva detection in pregnant women had a low number of patients enrolled – 11 and 10 patients, respectively. Similarly, children with malignancy may have acquired HCV as a consequence of patient-to-patient non-parenteral routes (Fink *et al.*, 1993). Intrafamilial transmission (e.g. via kissing shared utensils, toothbrushes and razors) is not common, the prevalence of HCV infection of household contacts of HCV-infected individuals being approximately 3.6% (Everhart *et al.*, 1990, Hou *et al.*, 1995, Mastromatteo *et al.*, 2001). Evidence for the sexual transmission of HCV has been shown since the early nineties (Tedder *et al.*, 1991). Vaginal shedding of HCV has been demonstrated, where the RNA detection ranged from ~30% in HIV co-infected women (Belec *et al.*, 2003b, Belec *et al.*, 2003a, Minosse *et al.*, 2006, Nowicki *et al.*, 2005) to 50% of HCV mono-infected women (Gameiro *et al.*, 2001).

The HCV seroprevalence of Dental health care workers is not consistently higher than that of appropriate control populations although early reports did suggest that those who frequently undertook surgical procedures may be at increased risk of nosocomial acquisition of HCV (Klein *et al.*, 1991), with the higher risk of infection extending to the health-care workers in general (Jochen, 1992, Cummins *et al.*, 1992), when a case presentation of an acute

hepatitis C infection due to a needlestick after occupational exposure highlights this increased risk (Sulkowski *et al.*, 2002). On the other hand, other authors described a higher (twice as high antibody presence than in health care workers) risk related to socioeconomic variables after a comparative study with factory workers as controls (De Luca *et al.*, 1992).

The results of previous studies of the prevalence of HCV in oral fluids add weight to the notion that HCV transmission via saliva may be possible, although not frequent. There have been a substantial number of relevant studies with increasingly larger groups of study patients indicating that the prevalence of detection of HCV RNA in saliva may from 0 to 100% (Table 3.5). The study groups have predominantly comprised individuals with untreated HCV-induced chronic hepatic disease. The varying prevalence of difference in the prevalence of salivary carriage of HCV of previous studies has been suggested to reflect variable methods of collection of saliva (e.g. oropharyngeal washes, stimulated versus non-stimulated saliva, use of sponges) but it is striking that despite these different methods only when sponges have been used (van Doornum *et al.*, 2001) HCV has almost always been detected, all be it with different prevalences. The virological methods for detection of HCV have become more sensitive in recent years although this has not lead to a consistent increase in the detected salivary HCV genoprevalence.

The present study represents the most extensive of HCV genoprevalence in oral fluids. In particular with the exclusive use of PDH real time PCR for cell

quantification, thus, providing us with a viral load per 1000 cells present in each saliva extract, together with the absolute viral load.

Table 3.5. All studies regarding the prevalence of HCV RNA in the saliva

First author & year	N	Clinical data	Methods	Type of saliva / GCF	HCV-RNA in plasma N (%)	HCV-RNA in saliva N (%)
(Takamatsu *et al.*, 1990)	5	Chronic hepatitis C (1); liver cirrhosis (2) ; liver cirrhosis & hepatocellular carcinoma (2)	NS3 nested PCR	Saliva supernatant	N/A	5 (100)
(Hsu *et al.*, 1991)	19	Chronic hepatitis C	5'-NCR nested PCR	Whole saliva	19 (100)	0 (0)
(Wang *et al.*, 1991)	3	Post-transfusion chronic hepatitis C	NS3 and 5'-NCR nested PCR	Whole saliva	3 (100)	3 (100)
(Wang *et al.*, 1992b)	14	Post-transfusion chronic hepatitis	5'-NCR nested PCR	Whole saliva	10 (71.4)	7 (50)
(Liou *et al.*, 1992)	31	Chronic hepatitis C	5'-NCR nested PCR	Whole saliva	31 (100)	15 (48.3)
(Nakano *et al.*, 1992)	10	Chronic hepatitis C	5'-NCR nested PCR	Whole saliva	10 (100)	6 (60)

(Fried *et al.*, 1992)	14	Chronic hepatitis C males	5'-NCR nested PCR	Saliva supernatant	14 (100)	0 (0)
(Ogasawara *et al.*, 1993)	10	Pregnant HCV RNA positive patients	5'-NCR nested PCR	Whole saliva	10 (100)	5 (50)
(Young *et al.*, 1993)	50	Chronic hepatitis C	5'-NCR nested PCR	Whole saliva	41 (82)	25 (50)
(Harle *et al.*, 1993)	30	Patients suspected to be infected by HCV but seronegative for the virus	PCR (no details)	Whole saliva	5 (16.7)	14 (46.7)
(Couzigou *et al.*, 1993)	37	Chronic hepatitis C	5'-NCR nested PCR	Whole saliva	N/A	23 (62)
(Numata *et al.*, 1993)	23	HCV seropositive patients	Single round 5'-NCR PCR	Whole saliva	23 (100)	8 (34.8)

(Puchhammer-Stockl *et al.*, 1994)	46 / 35	Chronic hepatitis C children with malignant diseases who received blood	5'-NCR nested PCR and line probe hybridization for some amplified samples	Throat wash supernatant	35/46 (73)	7/35 (20)
(Roy *et al.*, 1995)	14	Chronic hepatitis C	5'-NCR nested PCR and quantitative branched DNA on plasma	1 – Whole saliva; 2 – Saliva pellet; 3 – Saliva supernatant; 4 – Salivette (sponges)	13 (93)	1 – 11 (78.6) 2 – 7 (50) 3 – 7 (50) 4 – 10 (71.4)
(Komiyama *et al.*, 1995)	32	Chronic hepatitis C with liver cirrhosis or hepatoma, patients HCV seropositive	Single round 5'-NCR PCR followed by southern blot hybridization	Whole saliva	N/A	21 (66)
(Mariette *et al.*, 1995)	28	Chronic hepatitis C, 13 HIV coinfected	Amplicor HCV test (Roche) and	Whole saliva	28 (100)	17 (61)

			branched DNA on plasma			
(Sugimura *et al.*, 1995)	76	Elderly Chronic hepatitis C patients	5'-NCR and envelope (E1) nested PCR	Whole saliva	24 (44)	27 (35.5)
(Chen *et al.*, 1995)	26	Anti-HCV positive patients, 11 of them co-infected with HIV-1	5'-NCR nested PCR	1 – Saliva pellet; 2 – Saliva supernatant; 3 – Saliva pellet 4 – supernatant of submaxillary glands	23 (88)	1 – 4/23 (17.4); 2 – 0 (0); 3 – 0 (0); 4 – 0 (0)
(Biasi *et al.*, 1995)	1	HCV seropositive, chronic sialadenitis	5'-NCR nested PCR	Saliva supernatant	0 (0)	1 (100)

(Caldwell *et al.*, 1996)	33	Chronic hepatitis C, 21 of them undergone liver transplantation	5'-NCR nested PCR and quantitative branched DNA on plasma	Whole saliva	33 (100)	4/21 (19); 0/12 (0)
(Jorgensen *et al.*, 1996)	16	Chronic hepatitis C and Sjögren Syndrome	Single round 5'-NCR PCR followed by southern blot hybridization	Whole saliva	15 (93)	13 (83)
(Roy *et al.*, 1996)	21	Haemophiliac HCV-seropositive patients	5'-NCR nested PCR	1 – Whole saliva; 2 – Saliva pellet; 3 – Saliva supernatant; 4 – Salivette (sponges)	21 (100)	1 – 5 (23); 2 – 5 (23); 3 – 4 (19); 5 – 5 (23%)
(Taliani *et al.*, 1997)	20	Chronic hepatitis C (11) and cirrhosis (9)	5'-NCR nested PCR and	Whole saliva	20 (100)	3 (15)

			quantitative branched DNA on plasma			
(Ustundag *et al.*, 1997)	10	HGBV-C and HCV co-infected, haemodialysis	5'-NCR nested PCR	Whole saliva	10 (100)	3 (30)
(Kage *et al.*, 1997)	11	Hepatitis C carriers (HCV RNA and antibody positive), pregnant patients	5'-NCR nested PCR	Whole saliva	11 (100)	4 (36)
(Roy *et al.*, 1998)	50	HCV seropositive intravenous drug users, 27 of them also HIV Seropositive	5'-NCR nested PCR, quantitative branched DNA on plasma	1 – Whole saliva; 2 – Salivette (sponges)	33 (66)	1 and/or 2 – 19/33 (57.6)
(Fabris *et al.*, 1999)	45	Chronic hepatitis C	5'-NCR nested PCR	1 – Whole saliva; 2 – Saliva pellet; 3 – Saliva supernatant	39 (81.2)	22/39 (56.4)

(Roy *et al.*, 1999)	50	HCV seropositive intravenous drug users	5'-NCR nested PCR	1 – Whole saliva; 2 – Salivette (sponges); 3 – Omni-Sal (sponges)	33 (66)	1 – 17 (34); 2 – 9 (18); 3 – 13 (26)
(Becheur *et al.*, 2000)	15	Untreated chronic hepatitis C	Amplicor HCV test (Roche)	Whole saliva	15 (100)	4 (26.6)
(Arrieta *et al.*, 2001)	9	HCV seropositive (4) and seronegative (5), xerostomia	Amplicor HCV test (Roche)	Whole saliva	4/4 (100); 0/5 (0)	4/4 (100); 0/5 (0)
(Mastromatteo *et al.*, 2001)	45 / 138	Chronic hepatitis C (45) and cohabitants (138)	5'-NCR nested PCR	Whole saliva	23/45 (52.6); 7/138 (5.1)	13/45 (28.8); 5/138 (3.6)
(van Doornum *et al.*, 2001)	102	HCV seropositive, HIV infected illicit drug users	Amplicor HCV test (Roche)	Salivette and Omni-Sal (sponges)	76 (74.5)	0 (0)
(Maticic *et al.*,	50	Chronic hepatitis C	Amplicor HCV	1 – Whole saliva;	50 (100)	1 – 17/48

2001)			test (Roche)	2 – Gingival crevicular fluid		(35.4); 2 – 29/49 (59.2)
(Savoldi *et al.*, 2001)	32	HCV seropositive geriatric patients with odontostomatologic problems	Quantitative branched DNA on plasma and saliva	Whole saliva	18 (56.3)	20 (62.5)
(Rey *et al.*, 2001)	59	HIV-HCV co-infected patients	5'-NCR nested PCR and quantitative branched DNA on saliva and plasma positives	Whole saliva	45/56 (80.4)	22/59 (37.3)
(Hermida *et al.*, 2002)	61	Chronic hepatitis C	5'-NCR nested PCR	Whole saliva	61 (100)	32 (52.4)

(Toussirot *et al.*, 2002)	5	Chronic hepatitis C and Sjögren Syndrome	Amplicor HCV test (Roche), quantitative branched DNA on plasma	Whole saliva	5 (100)	5 (100)
(Belec *et al.*, 2003b)	28	Hospitalized HCV seropositive patients	Quantitative amplicor HCV test (Roche), Single round 5'-NCR PCR followed by southern blot hybridization	1 – Saliva pellet; 2 – Saliva supernatant; 3 – cell free gingival crevicular fluid	28 (100)	1 – 12 (42.9); 2 – 1 (3.6); 3 – 0 (0)
(Eirea *et al.*, 2005)	75	Chronic hepatitis C co-infected with HIV	5'-NCR nested PCR	Whole saliva	75 (100)	49 (65.3)
(Diz *et al.*, 2005)	44	Chronic hepatitis C	Quantitative amplicor HCV test	Whole saliva	44 (100)	26 (59.1)

			(Roche) for plasma and 5'-NCR nested PCR for saliva			
(Goncalves *et al.*, 2005)	39	HCV seropositive	5'-NCR nested PCR	Saliva supernatant	32 (82.5)	8 (20.5)
(Lins *et al.*, 2005)	50	HCV seropositive	Quantitative amplicor HCV test (Roche), 5'-NCR nested PCR	Whole saliva	50 (100)	50 (100)
(Suzuki *et al.*, 2005)	26	HCV seropositive	Quantitative 5'-NCR core reagents HCV test (Applied Biosystems)	1 – Whole saliva; 2 – Gingival crevicular fluid	20 (76.9)	1 – 8 (30.8); 2 – 22 (84.6)

(Wang *et al.*, 2006)	23	Chronic hepatitis C	Quantitative amplicor HCV test (Roche) for plasma and transcription-mediated amplification Versant HCV test (Bayer)	Whole saliva	23 (100)	22 (95.7) at least in 1 day of 21 consecutive days tested
(Pastore *et al.*, 2006)	46	Chonic hepatitis C	Quantitative amplicor HCV test for plasma and Cobas amplicor HCV test for saliva (Roche)	Saliva supernatant	46 (100)	18 (39.1)

A striking feature of the present study is the absence of any significant difference in the salivary HCV genoprevalence rates between treated and non-treaded patients. While this is a cross-sectional study, and thus did not examine the HCV genoprevalance longitudinally, the present absence of any significant difference in the prevalence or load of HCV in saliva between the two patient groups is of clinicopathological significance. One previous longitudinal study of HCV-infected patients in Spain reported that approximately 10% of patients may have persistence of HCV in saliva for at least 6 months despite clearance of HCV from blood following interferon and ribavirin (Diz *et al.*, 2005). The present results, together with those of Diz Dios and co-workers (2005) raise the possibility that there is some compartmentalized extrahepatic HCV replication, e.g., in the salivary glands (Arrieta *et al.*, 2001), that is resistant to anti-HCV therapy.

In the present study there was no correlation between the salivary prevalence or load of HCV with those of plasma. HCV loads have been found in some (Fabris *et al.*, 1999, Hermida *et al.*, 2002, Mariette *et al.*, 1995, Numata *et al.*, 1993) but not all (Rey *et al.*, 2001, Roy *et al.*, 1995) similar studies. This lack of correlation may point towards the mouth or salivary glands being a site of extra-hepatic HCV replication. Indeed in some instances HCV has been more frequent in saliva than in blood (Biasi *et al.*, 1995, Harle *et al.*, 1993, Savoldi *et al.*, 2001). Adding weight to this argument, no association was observed between the dental status (i.e., dentate versus edentate) in the present group of patients. Previous studies have not found consistent correlations between dental disease (e.g. gingival and periodontal disease markers, the presence of

oral mucosal disease and/or number of teeth) and the presence or load of HCV RNA in saliva of HCV infected patients (Lins *et al.*, 2005). Similarly, although gingival crevicular fluid may have higher frequencies and levels of detectable HCV RNA than whole saliva, no significant association between GCF levels of HCV RNA and gingival disease has been observed (Maticic *et al.*, 2001, Suzuki *et al.*, 2005).

Likewise HCV has been detected in the saliva of edentulous individuals in the present and previous studies. Eleven per cent (11%) of the HCV RNA saliva positive patients were edentulous, making it more questionable the possible leakage of the virus via the GCF and rising again the possibility that the virus could indeed replicate within the oral mucosa (Carrozzo *et al.*, 2002) or maybe in salivary glands, where both positive and negative sense RNA were detected by *in situ* hybridization at the epithelial cells of the acini structures (Arrieta *et al.*, 2001). Nevertheless, both studies have detected the virus in patients affected by lichen planus and Sjögren's syndrome or chronic sialadenitis, respectively. Lins *et al.* (2005) have also detected HCV RNA in the saliva of 2 edentulous patients with no oral lesions (Lins *et al.*, 2005). Elderly persons were the subject of one study, some of whom would be expected to be edentulous (Sugimura *et al.*, 1995), hence, suggesting that while HCV-infected peripheral blood mononuclear cells may allow HCV to infiltrate the GCF and saliva (as a consequence of transmigration from blood to the gingival crevice) it is unlikely that these are a major source of HCV in oral fluids (Suzuki *et al.*, 2005).

There was no significant difference in the prevalence of salivary HCV RNA of patients with different routes of possible HCV acquisition. This accord with the findings of a previous study of patients from another region of Brazil (Goncalves *et al.*, 2005). It is possible that the carriage of HCV in oral fluids might be influenced by sexually- or parenterally-acquired factors although HIV-related immunodeficiency does not influence salivary carriage of HCV (Rey *et al.*, 2001) and unless a particular route of acquisition was to favour infection by an HCV variant that is notably tropic for salivary gland tissue or oral epithelium it seems most unlikely that the route of acquisition is a predictor of oral carriage in the mouth.

The present results confirm that HCV RNA is present in saliva of approximately one third of all patients with HCV infection and that salivary genoprevalence is not influenced by local factors such as dental status nor recent anti-HCV therapy. Oral carriage of HCV may however be influenced by genetic variation of the infecting virus.

There was no correlation of IgG levels in saliva and high viral load in plasma of the respective patients. That is another indication of no viral leakage from the gingival crevicular fluid. The other finding that reinforces this hypothesis is that we could detect HCV RNA in the saliva of edentulous patients.

Whatever the mechanism is, the presence of HCV RNA in saliva, regardless of the viral load levels detected, could have important implications for hepatitis C epidemiology, as the origin of infection remains unknown in up to 40% of cases. (Rey *et al.*, 2001) In the other hand, our study is only the fourth study

to evaluate the quantitative presence of HCV RNA in the saliva, and we have analysed a higher number of patients, showing a much lower HCV RNA titration in the saliva in comparison to the other studies. This could mean that chance of transmission of the virus via the saliva could be lower than previously expected. Nevertheless, further studies are needed with regards to HCV RNA quantification in the oral fluids.

Chapter 4

Compartmentalization of HCV in Saliva and Blood

4. 1. Introduction

The presence of Hepatitis C virus (HCV) within oral fluids may be the consequence of local carriage or replication within leukocytes that are trafficking to the oral mucosa, gingivae or salivary glands. Alternatively there may be local replication of HCV within oral mucosal and gingival keratinocytes or salivary gland acinar cells (see Chapter 1).

Compartmentalisation of HCV within extra-hepatic sites certainly does occur as HCV RNA has been detected in a number of extra-hepatic sites including peripheral blood mononuclear cells (PBMC; see below), dendritic cells, CNS (Morsica *et al.*, 1997, Bagaglio *et al.*, 2005, Laskus *et al.*, 2002), salivary glands (Arrieta *et al.*, 2001, Takamatsu *et al.*, 1992) – see Chapter 1, oral mucosa (Carrozzo *et al.*, 2002) – see also chapter 1, female genital tract (Nowicki *et al.* 2005), lymph nodes (Sansonno *et al.* 1996) and kidneys (Widell *et al.*, 1995, Rodriguez-Inigo *et al.*, 2000). The detection of negative-stranded RNA (intermediary of HCV replication) and demonstration of quasispecies compartmentalisation additionally support the concept of compartmentalisation and extra-hepatic HCV replication (Blackard *et al.* 2006; Laskus *et al.* 2007; Roque Afonso *et al.* 1999).

Some of the studies that have demonstrated compartmentalisation of HCV have involved patients who are immunocompromised, e.g. with HIV co-infected patients (Morsica *et al.*, 1997, Nowicki *et al.*, 2005) perhaps suggesting that there is simply non-permissive HCV infection, however many

of the studies that have found HCV RNA within PBMC have been in immunocompetent individuals suggesting strongly the existence of extra-hepatic HCV replication, even in occult infection, when HCV RNA is found only within PBMC (Castillo *et al.* 2005; Cavalheiro *et al.* 2007).

Hepatitis C virus RNA has been detected in the PBMC of up to 79% of examined patients with chronic hepatitis (Table 4.1). HCV RNA in PBMC has been detected in different groups liable to HCV acquisition and is thus independent of any route of acquisition of HCV (Azzari *et al.* 2008). The presence of HCV RNA within PBMC is not consistently correlated with plasma or serum levels of HCV RNA (Lerat *et al.* 1998; Meier *et al.* 2001), indeed as patients with occult disease have no serum HCV yet HCV RNA can be detected in PBMC. HCV RNA is not consistently influenced by HCV genotype (Kao *et al.* 1997; Mazur *et al.* 2001). Significantly HCV RNA can be detected in PBMC for years after complete clinical and virological resolution of clinical disease suggesting that PBMC may be a natural reservoir of HCV (Radkowski *et al.* 2005; Pham *et al.* 2005).

There is limited evidence that HCV replicates within keratinocytes nor salivary gland acinar cells (see Chapter 1) and as noted in Chapter 3 HCV is not consistently present in oral fluids and when present is in low concentration. However the consistent finding of HCV RNA within PBMC does suggest that any extra-hepatic compartmentalisation of HCV within circulating PBMC is likely to influence the carriage of HCV within oral fluids. To date there have been no studies that have examined the association of salivary carriage with

HCV compartmentalisation in PBMC and plasma. Knowledge of the likely influence of HCV compartmentalisation of HCV between blood and oral fluids would provide insight into source of HCV in oral fluid and the likely impact of HCV therapy upon clearance of HCV from saliva.

Table 4.1. Results of previous studies of the prevalence of HCV RNA in peripheral blood mononuclear cells

First author & year	N	Clinical data	Methods	Type of PBMC	HCV-RNA in plasma N (%)	HCV-RNA in PBMC N (%)
(Bouffard *et al.*, 1992)	24	HCV-infected patients (all HCV antibody positive, 8 chronic active hepatitis, 3 chronic persistent hepatitis, 3 cirrhosis with chronic active hepatitis, 10 without biopsy)	5'-NCR nested PCR	Total PBMCs	"+" strand – 19 (79.2); "-" strand – 0/5 (0)	"+" strand – 17 (70.8); "-" strand – 1/5 (20)
(Takehara *et al.*, 1992)	9	Chronic hepatitis C, 6 with chronic active hepatitis and 3 with cirrhosis	5'-NCR single round PCR	Total PBMCs	"+" strand – 9 (100); "-" strand – 0 (0)	"+" strand – 7 (77.8); "-" strand – 0 (0)

(Wang *et al.*, 1992a)	7	Posttransfusion hepatitis C	5'-NCR nested PCR	Total PBMCs	"+" strand – 7 (100); "-" strand – 0 (0)	"+" strand – 7 (100); "-" strand – 3 (42.9)
(Zignego *et al.*, 1992)	5	Chronic active hepatitis anti-HCV positive drug addicts	5'-NCR and NS5 regions nested PCR	1 – Total PBMCs; 2 – T-lymphocytes; 3 – B-lymphocytes; 4 – Macrophage monocytes	"+" strand - 4 (80); "-" strand – 3 (60)	"+" and "-" strands (same results) – 1 – 5 (100); 2 – 3/3 (100); 3 – 3/3 (100); 4; 2/3 (66.7);

(Gil *et al.*, 1993)	20	Histologically proven chronic hepatitis and positive and negative strand of HCV RNA present in plasma of all patients; samples analysed after 12 months of therapy (6 untreated patients)	5'-NCR nested PCR; semi-quantitative titration of HCV RNA	Total PBMCs	"+" strand – 6/14 (42.9); "-" strand – 5/14 (35.7)	"+" strand – 12/18 (66.6); "-" strand – 8/18 (44.4)
(Muller *et al.*, 1993)	5	Two post-transfusional hepatitis C and three orthotopic liver transplantation due to chronic hepatitis C	5'-NCR and NS3/NS4 region nested PCRs	1 – B lymphocytes; 2 – T lymphocytes; 3 – NK cells	5 (100)	"+" strand – 1 – 3 (60); 2 – (0); 3 – 0 (0); "-" strand (southern blot) – 1 – 4 (80); 2 – (0); 3 – 0 (0);
(Gabrielli *et*		Type II (10) and type	5'-NCR	Total PBMCs	A – 7 (63.6); B – 10	A – 6 (54.5); B – 1

***al.*, 1994)**		**III** (1) mixed cryoglobulinemia - MC (A) – 6 under interferon-alpha therapy and 11 chronic hepatitis without MC (B) – 3 under interferon-alpha therapy	single round PCR		(90.9)	(9.1)
(Willems *et al.*, 1994)	47	Hemophiliacs with chronic hepatitis C	5'-NCR nested PCR	Total PBMCs	"+" strand – 35 (74.5); "-" strand – 16/29 (55.2)	"+" strand – 36 (76.6); "-" strand – 22/29 (75.9)
(Berenguer *et al.*, 1995)	1– 5; 2 – 16	1 – non-responders to anti-HCV therapy; 2 – responders to HCV therapy	5'-NCR nested PCR	Total PBMCs	1 – 5 (100); 2 – 0 (0)	1 – Not tested; 2 – 6/13 (46.2)

(Biasi *et al.*, 1995)	1	HCV seropositive, chronic sialadenitis	5'-NCR nested PCR	Lympho-monocytes	0 (0)	1 (100)
(De Maddalena *et al.*, 1995)	1 – 39; 2 – 11	Type **II** (24) and type **III** (15) mixed cryoglobulinemia - MC (1) and 11 chronic hepatitis without MC (2)	5'-NCR nested PCR and 5'-NCR Amplicor (Roche)	Total PBMCs	34/37 (91.9)	1 – 14/20 (70); 2 – 8/11 (72.7)
(Lanford *et al.*, 1995)	1 – 5; 2 – 10	1 – 2 acutely infected chimpanzees and 3 chronically infected chimpanzees; 2 – HCV-infected humans	5'-NCR single round rTth PCR followed by southern blot hybridization	Total PBMCs	"+" strand – 1 – 5 (100); 2 – 2/2 (100); "-" strand – 1 – 5 (100); 2/2 (100)	"+" strand – 1 – 4 (80); 2 – 7 (70); "-" strand – 1 – 0 (0); 2 – 0 (0)
(Oesterreiche	67	Chronic	5'-NCR	Total PBMCs	10 (14.9)	6 (8.9)

r *et al.*, 1995)		hemodialysis	nested PCR			
(Taliani *et al.*, 1995)	20	Histologically proven chronic hepatitis C	5'-NCR nested PCR	Total PBMCs	"+" strand – 18 (90); "-" strand – 0 (0)	"+" strand – 15 (75); "-" strand – 13 (65)
(Zignego *et al.*, 1995)	34	HCV seropositive for more than 1 year	5'-NCR nested PCR	Fresh (1) and mitogen-stimulated (2) total PBMC	22 (64.7)	"+" strand – 1 – 20 (58.8); 2 – 22 (64.7); "-" strand – 1– 12 (35.3); 2 – 19 (55.9)
(Chang *et al.*, 1996)	1 – 11; 2 – 48	1 – Acute posttrans-fusion hepatitis C; 2 – Chronic Hepatitis C	5'-NCR nested PCR; quantitative branched DNA on plasma	Total PBMCs	1 – 10 (90.9); 2 – 41 (85.4)	1 – "+" strand – 0 (0); "-" strand – 0 (0); 2 – "+" strand – 12 (25); "-" strand – 6/12 (50)

(Lerat *et al.*, 1996)	32	Chronic hepatitis C (A) and liver transplant-tation (B)	“+” strand – 5’NCR nested PCR; “-“ strand – 5’NCR, 5’ tagged NCR and nucleocap-sid nested PCRs	Total PBMCs	“+” strand – 32 (100); “-“ strand – 0 (0)	“+” strand – A – 15/23 (652); B – 3/3 (100); “-“ strand – A – 1/23 (4.3); B – 3/3 (33.3);
(Zehender *et al.*, 1997)	15	Chronic hepatitis C, 4 with mixed cryoglobu-linemia	5’-NCR nested PCR; semi-quantitative titration of HCV cDNA	1 – B cells CD19; 2 – T cells CD3; 3 – T cells CD14	15 (100)	1 – 15(100); 2 – 5/14 (35.7); 3 – 5/12 (41.6)
(Kao *et al.*,	106	Chronic hepatitis C	5’-NCR	Total PBMCs	“+” strand – 89 (84.0);	“+” strand – 83 (78.3);

1997)			nested PCR		"-" strand – 0 (0)	"-" strand – 33 (31.1)
(Laskus *et al.*, 1997)	27	Chronic hepatitis C	5'-NCR (1) and NS5 region (2) single round PCR followed by southern blot hybridization	Total PBMCs	1 – 27 (100); 2 – 20 (74.1); all "-" strand RNA negative	1 – 17 (63); 2 – 12 (44.4); all "-" strand RNA negative

(Lerat *et al.*, 1998)	38	HCV RNA positive and seropositive	INNO-LIPA (Inno-genetics) 5'-NCR (A) and nucleo-capsid nested PCRs (B) and quantitative branched DNA on plasma	1 – Monocytes; 2 – B lymphocytes; 3 – granulocytes	38 (100)	(A) "+" strand – 1 – 21 (56); 2 – 24 (63); 3 – 34 (89); (B) "-" strand – 1 – 1/10 (10); 2 – 2/9 (22.2); 3 – 3/10 (30)
(Morsica *et al.*, 1999)	4	Chronic hepatitis C	5'-NCR nested PCR	1 – Total PBMCs; 2 – B cells CD19; 3 – T cells CD4; 4 – T cells CD8	4 (100); all "-" strand RNA negative	1 – 3 (75); 2 – 3 (75); 3 – 1 (25); 4 – 1 (25); all "-" strand RNA negative
(Roque	9	HCV antibody positive	HVR1 nested	1 – B cells CD19;	9 (100)	1 – 7 (77.7); 2 – 3/8

Afonso *et al.*, 1999)			PCR; quantitative Amplicor for plasma (Roche)	2 – T cells CD8; 3 – T cells CD4; 4 – Negatively selected cell fraction (NF)		(37.5); 3 – 1 (11.1); 4 – 7/8 (87.5)
(Azzari *et al.*, 2000)	1 – 13; 2 – 53	1 – Mothers whose babies became infected; 2 – Mothers whose babies did not become infected	5'-NCR nested PCR	Lymphocytes	1 – 13 (100); 2 – 53 (100); ; all "-" strand RNA negative	"+" strand – 1 – 13 (100); 2 – 13 (24.5) ; "-" strand – 1 – 5 (38.5); 2 – 0 (0)
(Garcia *et al.*, 2000)	22	Chronic hepatitis C	NS3 region nested PCR	Total PBMCs	22 (100)	"+" strand – 8/21 (38.1); "-" strand – 2/8 (25)

(**Takyar** ***et al.*****, 2000)**	8	HCV carriers	5'-NCR nested PCR; quantitative Amplicor for plasma (Roche)	Total PBMCs	8 (100)	"-" strand – 4 (50)
(**Gameiro** ***et al.*****, 2001)**	14	HCV infected women	5'-NCR PCR and 9 cases quantitative Amplicor for plasma (Roche)	Total PBMCs	14 (100)	8 (57.1)
(**Mazur** ***et al.*****, 2001)**	45	8 anti-HCV positive haemodialysis patients (1) and 37 anti-HCV negative (2)	5'-NCR nested PCR	Total PBMCs	1 – 2 (4.4); 2 – 2 (4.4)	"+" strand – 1 – 4 (8.8); 2 – 3 (6.6); "-" strand – 1 – 4 (8.8); 2 – 1 (2.2)
(**Meier** ***et***	15	Chronic hepatitis C	5'-NCR	Total PBMCs	1 – 15 (100); 2 – 14/14	1 – 15 (100); 2 – 0/14

***al*., 2001)**		before (1), during (2) and after interferon alfa therapy examined when it was present in both sites (3), and present in plasma but not in PBMC (4) later	nested PCR; quantitative Amplicor for plasma (Roche)		(100); 3 – 8 (53.3); 4 – 4/11 (36.4)	(0); 3 – 8 (53.3); 4 – 0/11 (0)
(Ye *et al*., 2001)	583	Chronic hepatitis C	Nested PCR	Total PBMCs	112 (19.2)	143 (24.5)
(Laskus *et al*., 2002)	13	HCV seropositive undergoing nontrau-matic lumbar puncture for diagnostic purposes	5'-NCR nested PCR	Total PBMCs	13	12 (92.3)

(Mazurek *et al.*, 2002)	1 – 154; 2- 54	Chronic liver disease with hepatitis C infection (1) and HCV/HBV coinfected (2)	5'-NCR nested PCR	Lymphocytes	"+" strand – 1 – 98 (63.6); 2 – 49 (90.7); "- " strand – 1 – 70 (45.5); 2 – 16 (29.6)	"+" strand – 1 – 126 (81.8); 2 – 21 (38.8); "- " strand – 1 – 98 (63.6); 2 – 14 (25.9)
(Ducoulombier *et al.*, 2004)	14	Chronic hepatitis C	HVR1 nested PCR and quantitative real time PCR (Roche)	1 – Total PBMCs; 2 – CD19; 3 – CD8; 4 – CD4; 5 – CD14	14 (100)	1 – 3/3 (100); 2 – 11/11 (100); 3 – 10/11 (90.9); 4 – 4/11 (36.4); 5 – 0/11 (0)
(Fernandez *et*	10	HCV RNA positive	5'-NCR	Total PBMCs	1 – 10 (100); 2 – 10	1 – 7 (70); 2 – 7 (70); 3

***al.*, 2004)**		under hemodialysis; samples collected before Dialysis (1), 5 minutes after the session (2) and 2 days later – before next Dialysis session (3)	nested PCR; quantitative branched DNA on plasma		(100); 3 – 10 (100)	– 7 (70)
(Castillo *et al.*, 2005)	18	Occult hepatitis C, PBMC RNA positive	5'-NCR nested PCR detected by fluorescent in situ hybridisation	Total PBMCs	0 (0)	"+" strand – 18 (100); "-" strand – 11 (61)
(Blackard *et*	48	HCV seropositive	5'-NCR	Total PBMCs	1 – 42/47 (89); A – 23	"+" strand – 1 – 20

***al.*, 2005)**		women (IDU as main risk factor) 28 HCV monoinfected (A) and 20 HCV-HIV coinfected (B) before (1) and after ART treatment for HIV started (2).	single round PCR		(82); B – 19/19 (100); 2 – 43/47 (91); A – 26 (93); B – 17/19 (89)	(42); A – 9 (32); B – 11 (55); 2 – 20/45 (44); A – 8 (29); B – 12/17 (71); "-" strand – 1 – 17 (35); A – 9 (32); B – 8 (40); 2 – 17/45 (38); A – 7 (25); B – 10/17 (59)
(Cavalheiro *et al.*, 2007)	54	Chronic hepatitis C after combined therapy	5'-NCR single round PCR Amplicor (Roche)	Total PBMCs	35 (65)	38 (70.6)
(Di Liberto *et al.*, 2006)	119	Chronic hepatitis C	5'-NCR nested PCR	Total PBMCs	113 (94)	113 (94)
(Carreno *et*	12	HCV antibody positive,	Amplicor	Total PBMCs	0 (0)	"+" strand – 6 (50);

***al.*, 2006)**		normal ALT levels and serum HCV RNA negative for at least 12 months	(Roche), 5'-NCR single round PCR followed by in situ hybridizati-on, real time PCR			"-" strand – 5 (41.7)
(Azzari *et al.*, 2008)	1 – 48; 2 – 122	1 – mothers whose babies became infected; 2 – mothers whose babies did not become infected	5'-NCR nested PCR, quantitative Amplicor for plasma (Roche)	Lymphocytes	1 – 48 (100) 2 – 122 (100)	1 – 48 (100) 2 – 66 (54.1)
(Barria *et al.*, 2008)	47	HCV seropositi-ve for more than 6 months	Single round 5'-NCR and NS5b PCR	Total PBMCs	47 (100)	34 (72.3)
(Bernardin *et*	56	Blood donors viremic	Cell	Total PBMCs	56 (100)	43 (76.8)

***al.*, 2008)**		patients	associated – transcrip-tion mediated amplificati-on (GenPro-be), Core region nested PCR			
(Thongsawat *et al.*, 2008)	11	Individuals who had transitioned from plasma RNA positive to plasma RNA negative	Core/E1 nested PCR	Total PBMCs	11 (100), then 0 (0), between 24 and 36 month follow up	2 (18.2), from the 30th month follow up

(Gallegos-Orozco *et al.*, 2008)	25	Chronic hepatitis C after SVR to combined therapy	Qualitative real-time nested 5’NCR PCR	Total PBMCs	0 (0)	5 (20)
(Inokuchi *et al.*, 2009)	75	HCV antibody positive and HCV RNA positive	“+” strand – Quantitative real-time single round 5’NCR PCR; “-” strand – NS5b single round PCR	1 – B cells; 2 – T cells CD8; 3 – T cells CD4; 4 – remaining PBMCs	75 (100)	“+” strand – 1 – 47 (63); 2 – 12 (16); 3 – 11 (15); 4 – 17 (23); “-” strand – 1 – 4 (5.3)
(Vera-Otarola *et al.*, 2009)	45	Chronic hepatitis C (HCV antibody positive and HCV RNA positive for more than 6 months)	Qualitative single round 5’NCR PCR; added tag sequence for “-” strand detection	Lymphocytes	45 (100)	“+” strand – 33 (73); “-” strand – 0/33 (0)

4.2. Aims

The aim of the present chapter is to determine the prevalence and viral load of HCV in peripheral blood mononuclear cells (PMBCs) of patients with HCV disease attending clinics in Recife in Northeast Brazil. In addition, the influence of HCV in plasma upon the PMBC presence of this virus was examined.

4.3. Patients and samples

4.3.1. Study population

Samples were collected from two different locations: a general public hospital and a private dialysis clinic in Recife, Northeast Brazil (details in Section 2.1). All participants were of Brazilian nationality. Clinical data on all patients were obtained from standard medical records and documented onto standardized forms, which were linked to patient samples by numerical code. These forms were used during anamnesis undertaken before clinical examination. Oral findings and route of HCV acquisition can be found in Table 3.1.

4.3.2. Sample collection

Matched oral and blood samples were obtained from all patients as described in Sections 2.2.1 and 2.2.2.

4.3.3. Sample processing

After separation of plasma from the blood, the CD19+, CD14+, CD2+ and CD45+ cell subsets were immunomagnetically fractionated as described in Sections 2.2.3. WMS was not subjected to centrifugation and it was stored at -80°C until required. RNA was extracted from blood cell subsets and oral samples as described in Section 2.3.2. The presence of DNA in randomly

selected extracts was verified by using the Microzone DNA OK kit as described in Section 2.3.3.

4.3.4. Molecular detection of HCV

4.3.4.1. RNA extraction

RNA extraction was performed both manually (Section 2.3.2.1) for all samples, but CD45 from patients 1 to 4 due to lack of blood for cell separation and automatically for whole saliva and plasma samples (Section 2.3.2.2).

4.3.4.2. Polymerase Chain Reaction (PCR)

Reverse transcription was carried out to generate cDNA for all samples (Section 2.3.4) followed by PCR of the 5'-noncoding region for plasma samples only (Section 2.3.5) and NS5b region for all samples (Section 2.3.6) with the exception of patients 1, 2, 3 and 4 for CD45 (not available).

4.3.4.3. RFLP and direct sequencing

RFLP was performed for all plasma samples (Sections 2.3.8 and 2.3.9) and direct sequencing was performed for all samples (Section 2.3.12).

4.3.4.4. Quantitative real time PCR

Quantitative Hepatitis C virus (HCV) assay was used for all the whole saliva, plasma, CD19, CD14, CD2 and CD45 samples (with the exception of patients 1, 2, 3 and 4 for CD45) in order to obtain HCV viral load of the respective sites (Section 2.3.13).

4.4. Results

4.4.1. HCV RNA in CD19 leukocytes

4.4.1.1. Prevalence of HCV RNA in CD19 leukocytes

All patients were tested for HCV RNA detection in CD19 cells. The prevalence of HCV RNA in CD19 leukocytes is summarized in Table 4.2. HCV RNA was detected in 81.2% of the CD 19 cells of the total group, and was present in 86.6% of the untreated group and 61% of the treated group. Fisher's exact test revealed that the prevalence of HCV RNA in CD19 cells was significantly greater in the untreated group compared with the treated group (P=0.036).

Table 4.2. Prevalence of HCV RNA in CD19 leukocytes

Group		CD19		Total
		No HCV RNA detected	HCV RNA detected	
Untreated	Number	9	58	67
	%	13.4%	86.6%	100.0%
Treated	Number	7	11	18
	%	38.9%	61.1%	100.0%
Total	Number	16	69	85
	%	18.8%	81.2%	100.0%

4.4.2. Total HCV load and load per 1000 cells in CD 19 cells

All patients were tested for HCV RNA viral load per 1000 cells in CD 19 leukocytes. The total HCV load and load per 1000 cells in CD19 leukocytes in the total patient group and the untreated and treated groups are summarized in Table 4.3. The HCV load in the total group ranged from 0 to 17770000/IU/ml while the load per 1000 cells ranged form 0 to 15807.1.

The Mann Witney U test revealed that there no statistically significant difference in the total HCV load (P=0.186) or HCV load per 1000 CD 19 cells (P=0.278 between the treated and untreated patients).

Table 4.3. Total HCV load in CD19 cells and load per 1000 HCV cells

Group		CD19_Load	CD19_1000
Untreated	Mean	26182.87	19048.158
	N	67	67
	Std. Deviation	71224.949	118319.5830
	Minimum	0	.0
	Maximum	412000	967528.0
	Median	2573.00	458.400
Treated	Mean	114027.22	3743.267
	N	18	18
	Std. Deviation	416850.595	7107.6069
	Minimum	0	.0
	Maximum	1770000	23766.5
	Median	977.00	101.800
Total	Mean	44785.20	15807.122
	N	85	85
	Std. Deviation	201136.593	105116.1954
	Minimum	0	.0
	Maximum	1770000	967528.0
	Median	2370.00	432.300

4.4.3. HCV RNA in CD14 leukocytes

4.4.3.1. Prevalence of HCV RNA in CD14 leukocytes

All patients were tested for HCV RNA detection in CD 14 leukocytes. The prevalence of HCV RNA in CD14 leukocytes is summarized in Table 4.4. HCV RNA was detected in 76.5% of the CD 14 cells of the total group, and was present in 82.1% of the untreated group and 55.6% of the treated group. Fisher’s exact test revealed that the prevalence of HCV RNA in CD14 cells

was significantly greater in the untreated group compared with the treated group (P=0.028).

Table 4.4. Prevalence of HCV RNA in CD14 leukocytes

Group		CD14		Total
		No HCV RNA detected	HCV RNA detected	
Untreated	Number	12	55	67
	%	17.9%	82.1%	100.0%
Treated	Number	8	10	18
	%	44.4%	55.6%	100.0%
Total	Number	20	65	85
	%	23.5%	76.5%	100.0%

4.4.4. Total HCV load and load per 1000 cells in CD 14 cells

All patients were tested for HCV RNA viral load and viral load per 1000 cells in CD 14 leukocytes. The total HCV load and load per 1000 cells in CD14 leukocytes in the total patient group and the untreated and treated groups are summarized in Table 4.5. The HCV load in the total group ranged from 0 to 523000 while the load per 1000 cells ranged form 0 to 46283.2. The Mann Witney U test revealed that there was no statistically significant difference in the total HCV load (P=0.098) or HCV load per 1000 CD 14 cells (P=0.255) between the treated and untreated patients.

Table 4.5. Total HCV load and load per 1000 cells in CD 14 cells

Group		CD14_Load	CD14_1000
Untreated	Mean	11398.49	1049.918
	N	67	67
	Std. Deviation	64616.829	5763.6263
	Minimum	0	.0
	Maximum	523000	46283.2
	Median	594.00	27.000
Treated	Mean	15203.22	1630.961
	N	18	18
	Std. Deviation	51008.829	5322.5500
	Minimum	0	.0
	Maximum	215000	22751.3
	Median	1.00	.650
Total	Mean	12204.20	1172.962
	N	85	85
	Std. Deviation	61722.283	5647.2411
	Minimum	0	.0
	Maximum	523000	46283.2
	Median	456.00	21.100

4.4.5. HCV RNA in CD2 leukocytes

4.4.5.1. Prevalence of HCV RNA in CD2 leukocytes

All patients were tested for HCV RNA detection in CD2 leukocytes. The prevalence of HCV RNA in CD2 leukocytes is summarized in Table 4.6. HCV RNA was detected in 77.6% of the CD2 cells of the total group, and was present in 82.1% of the untreated group and 61.1% of the treated group. Fisher's exact test revealed that there was no statistically significant difference in the prevalence of HCV RNA between the CD2 cells of the two groups (P = 0.107).

Table 4.6. Prevalence of HCV RNA in CD2 leukocytes

Group		No HCV RNA detected	HCV RNA detected	Total
Untreated	Number	12	55	67
	%	17.9%	82.1%	100.0%
Treated	Number	7	11	18
	%	38.9%	61.1%	100.0%
Total	Number	19	66	85
	%	22.4%	77.6%	100.0%

4.4.5.2. Total HCV load and load per 1000 cells in CD 2 cells

All patients were tested for HCV RNA viral load and viral load per 1000 cells in CD2 cells. The total HCV load and load per 1000 cells in CD2 leukocytes in the total patient group and the untreated and treated groups are summarized in Table 4.7. The HCV load in the total group ranged from 0 to 452000 while the load per 1000 cells ranged form 0 to 5415.6049. The Mann Witney U test revealed that there was no statistically significant difference in the total HCV load (P=0.245) or HCV load per 1000 CD 2 cells (P=0.258) between the treated and untreated patients.

Table 4.7. Total HCV load and load per 1000 cells in CD2 cells

Group		CD2_Load	CD2_1000
Untreated	Mean	11097.49	636.781
	N	67	67
	Std. Deviation	58375.167	2344.4382
	Minimum	0	.0
	Maximum	452000	14675.3
	Median	127.00	2.800
Treated	Mean	10182.22	2631.329
	N	18	18
	Std. Deviation	31447.175	10966.2891
	Minimum	0	.0
	Maximum	129000	46570.4
	Median	4.00	.350
Total	Mean	10903.67	1059.156
	N	85	85
	Std. Deviation	53644.462	5415.6049
	Minimum	0	.0
	Maximum	452000	46570.4
	Median	100.00	2.800

4.4.6. HCV RNA in CD45 leukocytes

4.4.6.1. Prevalence of HCV RNA in CD45 leukocytes

All patients, with the exceptions of patients 1, 2, 3 and 4, were tested for prevalence of HCV RNA in CD 45 leukocytes. The prevalence of HCV RNA in CD45 leukocytes is summarized in Table 4.8. HCV RNA was detected in 76.5% of the CD45 cells of the total group, and was present in 81.5% of the untreated group and 56.3% of the treated group. Fisher’s exact test revealed that the prevalence of HCV RNA in CD45 cells was just significantly greater in the untreated group compared with the treated group (P=0.048).

Table 4.8. Prevalence of HCV RNA in CD45 leukocytes

Group		No virus detected	Virus detected	Total
Untreated	Number	12	53	65
	%	18.5%	81.5%	100.0%
Treated	Number	7	9	16
	%	43.8%	56.3%	100.0%
Total	Number	19	62	81
	%	23.5%	76.5%	100.0%

4.4.6.2. Total HCV load and load per 1000 cells in CD45 cells

All patients, with the exceptions of patients 1, 2, 3 and 4, were tested for HCV RNA viral load and viral load per 1000 cells in CD 45 leukocytes. The total HCV load and load per 1000 cells in CD45 leukocytes in the total patient group and the untreated and treated groups are summarized in Table 4.9. The HCV load in the total group ranged from 0 to 747000 while the load per 1000 cells ranged form 0 to 41270.7. Spearman's test revealed that there was no statistically significant difference in the total HCV load (P=0.559) or HCV load per 1000 CD 2 cells (P=0.719) between the treated and untreated patients.

Table 4.9. Total HCV load and load per 1000 cells in CD45 cells

Group		CD45_Load	CD45_1000
Untreated	Mean	20638.03	593.179
	N	65	65
	Std. Deviation	94946.913	2648.2927
	Minimum	0	.0
	Maximum	719000	19861.9
	Median	372.00	7.000
Treated	Mean	54390.75	2674.800
	N	16	16
	Std. Deviation	186288.636	10295.2111
	Minimum	0	.0
	Maximum	747000	41270.7
	Median	334.00	7.050
Total	Mean	27305.23	1004.364
	N	81	81
	Std. Deviation	117905.356	5116.5993
	Minimum	0	.0
	Maximum	747000	41270.7
	Median	372.00	7.000

4.4.7. Correlations of plasma load versus total HCV load in the 4 different leukocytes – total patient group

Correlations between the log plasma load and log total HCV load of the 4 examined leukocytes in the total group and the untreated and treated groups are summarized in Tables 4.10, 4.11 and 4.12. There were strong correlations between plasma load and the load within each cell type, these all being significant at the 0.01 level (as determined by Spearman’s test). In addition it was evident that there were similarly strong correlations between the HCV loads of each of the examined cell types.

Table 4.10. Correlations of plasma load versus total HCV load in the 4 different leukocytes – total patient group

		Log plasma load	Log CD19 load	Log CD14 load	Log CD2 load	Log CD45 load
Log plasma load	Correlation Coefficient	1.000	.595**	.534**	.523**	.554**
	Sig. (2-tailed)	.	.000	.000	.000	.000
	N	71	71	71	71	68
Log CD19 load	Correlation Coefficient	.595**	1.000	.631**	.553**	.663**
	Sig. (2-tailed)	.000	.	.000	.000	.000
	N	71	71	71	71	68
Log CD14 load	Correlation Coefficient	.534**	.631**	1.000	.683**	.600**
	Sig. (2-tailed)	.000	.000	.	.000	.000
	N	71	71	71	71	68
Log CD2 load	Correlation Coefficient	.523**	.553**	.683**	1.000	.644**
	Sig. (2-tailed)	.000	.000	.000	.	.000
	N	71	71	71	71	68
Log CD45 load	Correlation Coefficient	.554**	.663**	.600**	.644**	1.000
	Sig. (2-tailed)	.000	.000	.000	.000	.
	N	68	68	68	68	68

**. Correlation is significant at the 0.01 level (2-tailed)

*. Correlation is significant at the 0.05 level (2-tailed)

Table 4.11. Correlations of plasma HCV load versus total HCV load in the 4 different leukocytes – untreated patient group

		log_plasma_load	log_CD19_load	log_CD14_load	log_CD2_load	log_CD45_load
log_plasma_load	Correlation Coefficient	1.000	.542**	.429**	.475**	.505**
	Sig. (2-tailed)	.	.000	.001	.000	.000
	N	59	59	59	59	57
log_CD19_load	Correlation Coefficient	.542**	1.000	.611**	.507**	.624**
	Sig. (2-tailed)	.000	.	.000	.000	.000
	N	59	59	59	59	57
log_CD14_load	Correlation Coefficient	.429**	.611**	1.000	.662**	.559**
	Sig. (2-tailed)	.001	.000	.	.000	.000
	N	59	59	59	59	57
log_CD2_load	Correlation Coefficient	.475**	.507**	.662**	1.000	.614**
	Sig. (2-tailed)	.000	.000	.000	.	.000
	N	59	59	59	59	57
log_CD45_load	Correlation Coefficient	.505**	.624**	.559**	.614**	1.000
	Sig. (2-tailed)	.000	.000	.000	.000	.
	N	57	57	57	57	57

**. Correlation is significant at the 0.01 level (2-tailed)

*. Correlation is significant at the 0.05 level (2-tailed)

Table 4.12. Correlations of plasma HCV load versus total HCV load in the 4 different leukocytes – treated patient group

		Log plasma load	Log CD19 load	Log CD14 load	Log CD2 load	Log CD45 load
Log plasma load	Correlation Coefficient	1.000	.755**	.852**	.701*	.633*
	Sig. (2-tailed)	.	.005	.000	.011	.036
	N	12	12	12	12	11
Log CD19 load	Correlation Coefficient	.755**	1.000	.669*	.746**	.770**
	Sig. (2-tailed)	.005	.	.017	.005	.006
	N	12	12	12	12	11
Log CD14 load	Correlation Coefficient	.852**	.669*	1.000	.727**	.715*
	Sig. (2-tailed)	.000	.017	.	.007	.013
	N	12	12	12	12	11
Log CD2 load	Correlation Coefficient	.701*	.746**	.727**	1.000	.779**
	Sig. (2-tailed)	.011	.005	.007	.	.005
	N	12	12	12	12	11
Log CD45 load	Correlation Coefficient	.633*	.770**	.715*	.779**	1.000
	Sig. (2-tailed)	.036	.006	.013	.005	.
	N	11	11	11	11	11

**. Correlation is significant at the 0.01 level (2-tailed)

*. Correlation is significant at the 0.05 level (2-tailed)

4.7.8. Correlations of the prevalence of HCV RNA in saliva and 4 different leukocytes

Spearman's non-parametric test established that there were significant associations between the prevalence of HCV RNA in saliva and all the 4

studied cell compartments CD19, CD14, CD2 and CD45 (Table 4.13). In only 3 instances was HCV not in saliva yet present in the circulating CD 14 (one instance) or CD 2 (two instances) cells.

Table 4.13. Relative risk for detectable HCV RNA in saliva according to cell type

Cell type	Cell virus status	Virus not detected in saliva	Virus detected in saliva	RR (95%CI)
CD19	No virus detected	16	0	
	Virus detected	42	27	1.643 (1.360, 1.985)
CD14	No virus detected	19	1	
	Virus detected	39	26	12.667 (1.596, 100.502)
CD2	No virus detected	17	2	
	Virus detected	41	25	5.183 (1.103, 24.353)
CD45	No virus detected	19	0	
	Virus detected	35	27	1.771 (1.424, 2.204)

4.7.9. Correlations of salivary HCV load versus total HCV load in the 4 different leukocytes

No significant correlations between salivary HCV load and the 4 examined leukocytes in the total group and the untreated and treated groups were found when using Spearman’s test. There were strong correlations between plasma load and the load within each cell type, these all being significant at the 0.01 level (as determined by Spearman’s test). In addition it was evident that there were similarly strong correlations between the HCV loads of each of the examined cell types.

4.5. Discussion

The present investigation is the first to examine potential compartmentalisation of HCV between blood and salivary compartments by determining the load as well as the prevalence of HCV RNA within oral and blood compartments. An understanding of this is essential to determine factors that may influence potential oral carriage of the virus. In addition by estimation of HCV load within cells it is possible to clearly determine the cells that truly harbour HCV and thus are potential sites of HCV replication and reservoirs capable of leading to nosocomial transmission and/or rekindling systemic infection (Radkowski *et al.*, 2005, Pham *et al.*, 2005).

In the present study HCV RNA was present in up to 85% of examined PBMCs. Although previous studies of HCV RNA within PBMC examined a range of different patient groups, patients with treated and untreated HCV disease or had had variable responses to therapy or different manifestations of disease, the present high frequency of HCV in PBMC is in general agreement with the results studies that examined patients with chronic HCV disease. The present investigation utilised real time PCR as opposed to most studies that have not even quantified HCV RNA. Some have used branched DNA assays for HCV quantification – which is an old technique that is not as reliable as the assay presently employed – or only the automated Amplicor without quantification, as used in many studies (Azzari *et al.*, 2008, Cavalheiro *et al.*, 2007, De Maddalena *et al.*, 1995, Gameiro *et al.*, 2001, Meier *et al.*, 2001, Roque-Afonso *et al.*, 2005, Takyar *et al.*, 2000).

Previous studies have found HCV RNA within macrophage/monocytes, CD2+, CD3+, CD4+, CD8+, CD14+, CD19+ and CD45+ cells (Bouffard *et al*., 1992, Ducoulombier *et al*., 2004, Gabrielli *et al*., 1994, Muller *et al*., 1993, Zehender *et al*., 1997). However HCV RNA has been more frequently detected in CD19+ B lymphocytes and CD14+ monocytes than T lymphocytes such as CD4+ and CD8+ (Ducoulombier *et al*., 2004). In the present study HCV RNA was detected in similar frequencies and had similar loads in cells of monocyte/macrophage lineage (CD14+), B lymphocytes (CD19+) as well as CD2+ T lymphocytes and CD45+ pan leukocytes. Additionally as there was no difference in the frequency or load of HCV within the PBMCs between patients with or without treatment for HCV disease (Tables 4.3 and 4.4) the present observations for the cellular distribution of HCV RNA are consistent. In agreement with previous studies the presence of HCV RNA within PBMC was not influenced by the route of acquisition or genotype of HCV (data not shown).

In the present study HCV RNA was more frequent in plasma than in PBMC (Table 4.2), an observation reported by many others (Table 4.1). This could suggest that PBMC acquire HCV from plasma or that the PBMC are a site of replication of HCV. The notion that PBMC may be a potential reservoir of infection (and hence source of plasma HCV) is supported by knowledge that primary lymphoid cells are susceptible to *ex-vivo* infection and the virus derived from these *ex-vivo* cultures are infectious (Bare *et al*., 2005, Kato *et al*., 1995, Bronowicki *et al*., 1998, Cribier *et al*., 1995, Macparland *et al*.,

2006, Sung *et al.*, 2003). In support of the contrasting argument that PBMC secondarily acquire HCV from plasma, monocytes may acquire HCV via phagocytosis or infection of antibody-bound HCV particles (Bouffard *et al.*, 1992) while HCV enter CD19+ B lymphocytes via HCV E2 envelope protein binding to CD81, a tetraspanning receptor on B lymphocytes (Pileri *et al.* 1998).

Regardless of the mechanism that underlies any interplay of HCV between plasma and PBMC there remains the concern as to whether HCV RNA within any extrahepatic compartment (e.g. blood or oral fluids) is infectious and capable of replication. Extra-hepatic replication of infectious virus remains controversial. HCV replicates by the synthesis of a complimentary negative RNA strand that serves as a template for the positive strand, hence the presence of a negative strand could be considered to be the most reliable marker of ongoing viral replication. This is supported by a rise in levels of the negative strand of HCV within PBMC following mitogenic stimulation (Muller *et al.*, 1993). The rate of detection of the negative strand in PBMC has ranged from 0 to 61%, in comparison with the liver where the rate may be of the order of over 75% (Castillo *et al.*, 2005, Laskus *et al.*, 1997, Lerat *et al.*, 1996, Lerat *et al.*, 1998, Mazur *et al.*, 2001, Wang *et al.*, 1992a) and is thus less than that of the positive RNA strand. The present study did not examine the presence of the negative strand hence it is not possible to truly determine if HCV RNA in blood or saliva is a potential source of sustained HCV infection. However the presence of HCV RNA (not the negative strand) within PBMC may be associated with accelerated progress of hepatic cirrhosis

(Barria *et al.*, 2008), and patients with HCV RNA within PBMC have greater hepatic dysfunction than those without HCV RNA in PBMC (Bernardin *et al.*, 2008), thus HCV RNA within PBMC is at least a marker of probable sustained HCV replication and disease.

As noted in the previous chapter although HCV RNA was infrequently detected in saliva, there was however a wide range in the HCV load, thus allowing analysis of the possible impact of HCV compartmentalisation within blood upon oral carriage. As noted in Table 4.5 there was strong correlation between HCV RNA and each of the 4 cellular compartments (or plasma), the same happening when the rank correlation was done using HCV RNA per 1000 cells (table 4.6) and HCV RNA in whole saliva (see chapter 3, table 3.2).

The strong correlation between salivary HCV RNA and that of plasma and cellular HCV RNA point towards likely routes by which HCV passes into saliva. HCV may passively migrate from plasma via gingival crevicular fluid (GCF) into saliva as GCF represents a transudate of serum (Lamster *et al.*, 2007). Additionally it has previously been found that HCV load is higher in GCF than whole saliva and that there is a (non-significant) trend for salivary HCV levels to reflect those of serum (Suzuki *et al.*, 2005). Of perhaps greater significance is the present finding that there was a significant correlation between the presence of HCV in PBMC and whole saliva thus raising the possibility that HCV is carried into the mouth via PBMC. It is known that PBMC, particularly neutrophils and monocytes, traffic into GCF (Lamster *et*

al., 2007) hence it might be expected that HCV within such cells will eventually pass into whole saliva. Although some groups have not found any association between gingival health and the presence of HCV in saliva (Lins *et al.*, 2005), others have found that the likelihood of HCV RNA being present in saliva correlates with the presence of GCF HCV (Maticic *et al.* 2001) and that levels of HCV RNA in GCF are higher than those of saliva – pointing towards a dilution effect (Suzuki *et al.*, 2005). The striking observation of the present study is the very high correlation between HCV in saliva and CD14+ cells. These are circulating monocytes capable of differentiation into dendritic cells and macrophages within tissues (Geissmann *et al.*, 2010), where they could be fagocyting HCV particles. Although there are no studies of their precise presence within the GCF in health, they constitute up to 2% of the cellular component of GCF (Suzuki *et al.*, 1997) and it would be expected that monocytes will easily migrate from blood into GCF (Attstrom *et al.*, 1970). In addition HIV RNA in GCF has been found within CD14+ macrophages (Suzuki *et al.*, 1997) suggesting that they are a haematological source of at least HIV, although loss of CD14 occurs rapidly during migration through the vascular endothelium or within the gingival tissues.

The relatively low correlation between CD19+ PBMC and salivary HCV could be intriguing as the levels of B lymphocytes in GCF are considered to be slightly greater than that of monocytes. This can be understood by the low sampling number used at the referred statistical analysis. Even if levels of correlation differ widely between both compartments and there is presence or absence of saliva, the correlation of B lymphocytes and saliva have a very

narrow confidence interval, whereas the correlation between macrophages and monocytes has a very wide confidence interval.

CD19 is associated with CD81, a putative receptor for HCV, which is considered to be a possible strategy to help HCV achieve persistent infection (Roque Afonso *et al.* 1999). Neverthless consistent HCV replication in B cells could not be achieved *in vitro*, even if the highest viral load found in PBMCs was found in these respective cells (Boisvert *et al.*, 2001). Another study also reported B cells to present higher viral load than other PBMCs (Inokuchi *et al.*, 2009).

Azzari *et al.* have shown evidence that the HCV infection of PBMCs (Azzari *et al.*, 2000, Azzari *et al.*, 2008) and HCV replication within PBMCs (Azzari *et al.*, 2000) were highly associated with HCV transmission to newborns. Viral load at delivery was shown to be a risk factor of vertical transmission of HCV to newborns (Ohto *et al.*, 1994) and had relatively higher titers of HCV when babies were infected in another study (Moriya *et al.*, 1995). Notwithstanding, there was no statistically significant difference in the viral load of HCV either in plasma of mothers who gave birth to infected children as opposed to mothers whose babies were not HCV infected after birth. Viral load was also similar in mothers whose PBMCs harboured the virus and mothers whose PBMCs were negative for HCV (Azzari *et al.*, 2008).

In addition, the finding of retention of HCV RNA despite clearance in blood of chronic infected patients in PBMCs (Cavalheiro *et al.*, 2007) and/or liver (Gil

et al., 1993) and PBMC in HCV infected patients in the hemodialysis setting (Thongsawat *et al.*, 2008) may suggest that mononuclear cell investigation for HCV RNA could be added to confirm sustained virological response. On the other hand, clearance of HCV from PBMCs when HCV RNA levels started to become low in plasma has been demonstrated (Meier *et al.*, 2001).

Of notice, patient 53 mixed infection of genotype 3a detected in plasma, CD19, CD14, CD45 cells, and genotype 4o in CD2 cells was highly unusual. Contamination is unlikely to account for, as apart from all preventive measures to avoid it (see chapter 2), this genotype is not seen usually at the VRD reference lab of the Health Protection Agency - UK. Another unusual case was detected in patient 63, where genotype 4d was detected only in CD14 cells of a patient that was under interferon plus ribavirin treatment for around 5 month at the time of examination. All the other PBMCs analysed were negative, so were saliva and plasma of this patient. Given that this later patient was under treatment, what could give an idea of a possible EVR, it is possible to infer that there was compartmentalization of the virus in macrophages of patient 63. It was confirmed only few years ago that HCV RNA can not only be detected in PBMCs of occult and chronic HCV infected patients, but so was its negative strand in both recipients, suggesting that these are site of HCV replication even in occult patients (Pham *et al.*, 2008). Both patients had unknown route of acquisition and low biochemical markers (AST, ALT, ALP and GGT).

There was one HIV-co-infected patient (28) in the present study that apart from having a high viral load in plasma (around 5 millions IU/mL) and moderate viral load in all PBMCs (below 100000 IU/mL) had HCV RNA levels in saliva below the level of quantification (<100 IU/mL). This patient was an injecting drug user (IDU) and has stopped injecting 6 months before examination. A further patient was known to be HIV seropositive (although HIV viraemia had not been assessed) (7). His HCV load in plasma was around 1000000 IU/mL and the load in PBMCs was around the same range as the previous patient, although it was negative for CD2 cells. HCV was genotyped in this latter patient's saliva, even though the viral load could not be quantified, the opposite of the earlier patient.

An eminent role of PBMCs in pathophysiology and viral persistence of HCV is highly likely if we take into account that higher HCV titres in CD19, CD14, CD2 and CD45 were observed in treated as opposed to untreated patients. Other findings such as HCV RNA detection in PBMCs in patients with SVR (Radkowski *et al.*, 2005), detection of HCV negative strand RNA in PBMCs (Kao *et al.* 1997; Mazur *et al.* 2001; Bouffard *et al.* 1992) and even a higher HCV titre detection after PBMC stimulation (Pham *et al.*, 2005) reinforce that possibility.

The present results indicate that the presence of HCV in saliva is influenced (or at least correlated with) the presence of HCV in plasma and PBMC. Despite the high frequency and load of HCV in blood compartments, HCV is rarely observed in high loads within whole saliva. Hence, it would suggest

that parenteral transmission of HCV is way more likely than transmission via oral fluid such as whole saliva.

Chapter 5

Genotypic distribution of HCV between oral and blood compartments

5.1. Introduction

As discussed in Chapter 1, there are six main HCV genotypes and molecular epidemiological studies indicate that there are marked geographic differences in the genotypic distribution across the globe. Genotypes 1, 2, and 3 are widely distributed throughout the USA, Europe, Australia and East Asia, while genotype 4 tends to be confined to the Middle East, Egypt and central Africa (Antaki et al, 2009; Kamal and Nasser, 2008). Genotype 5 is predominantly found in South Africa while genotype 6 in South East Asia (Lee et al, 2008; Antaki et al, 2009).

Studies of the genotype of HCV can provide insight into the spread of HCV within and between communities and countries (Magiorkinis et al, 2009) but additionally may have a clinical relevance. The genotype of HCV does not seem to greatly influence the clinical presentation of disease, although individuals with genotype 1b may be at greater risk factor hepatocellular carcinoma (HCC) (Lee et al, 2008) as may genotype 4 (Abdel Hamid et al, 2007) – which may also increase the risk of recurrence following liver transplantation (Zylberberg, Chaix and Brechot, 2000). With regards to oral disease there are few studies that have examined the impact of HCV genotype. Present evidence indicates that no specific HCV genotype correlates with the presence of possible HCV-associated oral lichen planus (Imhof et al, 1997; Lodi et al, 1997).

Certainly the genotype influences response to therapy. A sustained virological response (SVR) can be achieved in 81 to 84% of patients infected with genotypes 2 or 3 after 24 weeks of interferon alpha and ribavirin, however only 34 to 52% of those infected with genotype 1 will achieve SVR after 48 weeks of therapy. Hence genotype is the major contributing factor in treatment length and therapeutic response (Farnick et al, 2009; Webster et al, 2009).

HCV genotyping has been widely reported in studies involving individuals from South American countries. Most studies have been conducted in Brazil and Argentina although there has been a small number from Chile, Colombia, Peru and Venezuela (Table 5.1). To date, only about a third of the South American countries have published data on HCV genotype prevalence. Most of the studies undertaken in South American countries have used restriction fragment length polymorphism (RFLP) as the genotyping method (Table 5.1). This is a cheap and effective method for genotyping HCV but does not allow high resolution subtyping that HCV sequencing can and is discussed in more detail later in this chapter.

Genotype 1 is generally the most prevalent in South American countries (Table 5.1). There are some exceptions but these studies may not be representative of the population due to the cohorts of individuals being studied (Re *et al.*, 2003, Oubina *et al.*, 1995). Genotypes 4 and 5 are rarely detected in South American patients. Genotype 4 was seen in a number of studies but only in a few patients when detected in Argentina (Re *et al.*, 2007,

Oubina *et al.*, 1995), Brazil (Perone *et al.*, 2008, Oliveira *et al.*, 1999b, Mendes-Correa *et al.*, 2008, Campiotto *et al.*, 2005, Bassit *et al.*, 1999) and Chile (Barria *et al.*, 2008). Only a few cases of genotype 5 were detected in Argentina (Re *et al.*, 2003, Re *et al.*, 2008), Brazil (Campiotto *et al.*, 2005, Levi *et al.*, 2002) and Chile (Barria *et al.*, 2008), with the exception of one Argentinean study that detected 10 cases (over 30%) of genotype 5 HCV in their study group (Oubina *et al.*, 1995). Genotype 6 has not been detected in any genotyping study from South America (Table 5.1).

5.1.1. Genotype prevalence in Brazil

Genotype 1 is most prevalent in all Brazilian studies (Table 5.1). The first publication to enroll Brazilian patients for HCV genotyping was performed by researchers from Innogenetics in Belgium and used sera of 61 patients from Brazil (Stuyver *et al.*, 1993). Three years later, the first HCV genotyping study was carried out on Brazilian samples in Porto Alegre, Southern Brazil (Krug *et al.*, 1996).

5.1.2. Genotype distribution in Venezuela

Genotype 1b used to be the most prevalent in Venezuela (Pujol *et al.*, 1997), but it was replaced by genotype 2 over a 10-year period. There was no increase in frequency of genotype 3 and an insignificant increase in genotype 1a (Pujol *et al.*, 2007). Anti-HCV testing in blood banks since 1994 in Venezuela is thought to have contributed to this reduction in frequency of

HCV genotype 1b infection. As the prevalence of each genotype was not detailed in this study (Pujol *et al.*, 2007) it is not listed in Table 5.1. One year after the first study in Venezuela, the same group have published a theoretical genotype turnover in 6 patients under hemodialysis (Pujol *et al.*, 1998). This could mean reinfection with a different genotype, especially in a hemodialysis setting.

5.1.3. Genotype Distribution in Peru

There is only one study reporting genotypes associated with HCV infection in Peru, which was based in the capital city of Lima. Residents of the metropolitan area were enrolled if they were blood donors, patients undergoing hemodialysis, had haemophilia, had been admitted with acute or chronic hepatitis or were individuals undergoing drug abuse rehabilitation. From 2827 samples tested, 384 were anti-HCV positive in the referred risk groups tested (13.6 %). After randomly choosing 50 patients for genotyping from these 384, Genotype 1a was more prevalent, followed by genotypes 1b, 3 and 2a (Sanchez *et al.* 2000).

.

Table 5.1. Table summarising papers written on HCV genotyping in South American countries

Country of population studied (first author and year)	Number of patients	Clinical data	Methods	Genotypes in Plasma – N (%)	Genotypes in Saliva - N (%)	Genotypes in PBMC – N (%)
Brazil (Stuyver *et al.*, 1993)	61	23 hemodyalisis patients or blood donors with NANB liver disease, 24 positive or indeterminate for line immunoassay, 14 randomly chosen	Nested 5'-NCR PCR followed by RFLP and Reverse hybridization line probe assay - Inno-Lipa (Innogenetics)	1 – 20 (33); 1b – 23 (38); 2 – 1 (1.5); 3 – 15 (24.5); 1b/3 mix – 2 (3)	N/A	N/A

Argentina (Oubina *et al.*, 1995)	31	10 hemophiliac and 4 polytransfused children, 19 adults (3 polytransfused, 7 dialyzed and 9 sporadic cases)	RFLP analysis of nested 5'-NCR and if positive, core-based genotyping with PCR using specific primers	1 – 16 (48.4); 2 – 21 (63.6); 4 – 1 (3); 5 – 10 (30.3)	N/A	N/A
Brazil (Krug *et al.*, 1996)	100	HCV RNA positive patients in Southern Brazil	RFLP analysis of 5'-NCR nested PCR	1 – 55 (55); 2 – 8 (8); 3 – 37 (37)	N/A	N/A

Argentina (Picchio *et al.*, 1997)	48/59	HCV seropositive Hemophiliac patients with HIV (A=23/29) and without HIV (B=25/30)	RFLP analysis of nested 5'-NCR followed by Reverse hybridization line probe assay - Inno-Lipa (Innogenetics) for confirmation in 11 patients	A 1a – 13 (56.5); 1b – 5 (21.7); 1 – 1 (4.3); 2b – 1 (4.3); 3a – 1 (4.3); 3b – 1 (4.3); mixed – 1 (4.3); B 1a – 10 (40); 1b – 9 (36); 3a – 4 (16); 4/5 mix – 2 (8)	N/A	N/A
Venezuela	122	19 blood donors,	RFLP analysis of nested	1a – 28 (29);	N/A	N/A

(Pujol *et al.*, 1997)		43 chronic patients, 47 hemodialyzed, 13 hemophiliac patients	5’-NCR	1b – 45 (37); 2a – 18 (15); 2b – 7 (5); 3a – 3 (2.5); mixed – 12 (10)		
Venezuela (Pujol *et al.*, 1998)	14	Hemodialysis patients	RFLP and SSCP following nested 5’-NCR	1a – 3 (21.4); 1b – 5 (35.7); mixed – 6 (42.9)	N/A	N/A
Argentina (Quarleri *et al.*, 1998)	82	HCV chronically infected patients, 71 adults and 11 children (mean age 27.5 years-old, range 2 - 71 years)	RFLP analysis of nested 5’-NCR followed by direct sequencing in 50 cases	1a – 29 (35.3); 1b- 29 (35.3); 2a -16 (19.5); 2b – 2 (2.4); 3 – 6 (7.3)	N/A	N/A

Brazil (Bassit *et al.*, 1999)	348	34 blood donors, 23 hemophiliacs, 40 renal-transplant and 251 Chronic hepatitis C patients from São Paulo, Brazil	5'-NCR single round PCR followed by southern blot hybridization	1a – 65 (18.7); 1b – 109 (31.3); 1 – 45 (12.9); 2 – 15 (4.3); 3 – 109 (31.3); 4a – 1 (0.3)	N/A	N/A
Brazil (Oliveira *et al.*, 1999a)	43	39 chronic hepatitis C haemophiliacs (A) and 4 patients with other hematologic diseases (B) from Minas Gerais State – Brazil	RFLP analysis of nested 5'-NCR	A 1 – 33 (82.5); 3 – 6 (15); B 1 – 4 (100)	N/A	N/A
Brazil	150	Chronic hepatitis C	Nested 5'-NCR PCR	1 – 108 (72);	N/A	N/A

(Oliveira *et al.*, 1999b)		blood donors, haemophiliacs, cirrhotic patients, infectable drug users and under hemodialysis	followed by RFLP and Reverse hybridization line probe assay - Inno-Lipa (Innogenetics) for 60 samples	2 – 3 (2); 3 – 38 (25.3); 4 – 1 (0.7)		
Brazil (Martins *et al.*, 2000)	90	39 haemophiliacs (A) and 33 blood donors (B) from Goiânia City, Brazil	Nested 5'-NCR PCR followed by Reverse hybridization line probe assay - Inno-Lipa (Innogenetics)	A: 1a – 16 (41); 1b – 10 (25.6); 3a – 3 (7.7); 2/3a mix – 1 (2.6); B: 1a – 18 (54.6); 1b – 4 (12.1); 2b – 1 (3); 3a – 10 (30.3)	N/A	N/A

Brazil (Parana *et al.*, 2000)	232	HCV carriers candidates for antiviral therapy	Reverse hybridization line probe assay - Inno-Lipa (Innogenetics)	1a – 75 (32.3); 1b – 72 (31); 2a/b – 14 (6); 3a – 61 (26.3); mixed – 5 (2.2); ND – 5 (2.2)	N/A	N/A
Argentina (Quarleri *et al.*, 2000)	243	Three groups of viremic patients: with parenteral risk factors for viral infection, with nonparenteral risk and sporadic or community-acquired cases	RFLP analysis of nested 5'-NCR followed by direct sequencing for selected cases also for NS5b	1 – 130 (53.5); 2 – 56 (23); 3 – 21 (8.6); mixed – 36 (14.8)	N/A	N/A
Peru	50	Randomly	RFLP analysis of nested	1a – 37 (74);	N/A	N/A

(Sanchez *et al.*, 2000)		genotyped from 384 anti-HCV positives from several different risk groups (see text) from the metropolitan are of Lima, Peru	5’-NCR	1b – 6 (12); 2a – 1 (2); 3 – 5 (10); unresolved – 1 (2)		
Argentina (Oubina *et al.*, 2001)	18	Patients with histologically proven chonic hepatitis C and porhyiria cutanea tarda	RFLP analysis of nested 5’-NCR	1a – 2 (11.1) 1b – 9 (50); 2a/c– 1 (5.5); 3a – 1 (5.5); 1a/1b mix – 4 (22.2); 2b/c/3a mix – 1 (5.5)	N/A	N/A
Venezuela	16 HCV	Urban, rural and	RFLP analysis of nested	1a – 6 (37.5); 1	N/A	N/A

(Aguilar *et al.*, 2001)	RNA + / 2592	Amerindian populations from Venezuela	5’-NCR	b – 4 (25); 2b – 3 (18.8); 1a/1b mix– 1 (6.3); 1b/2a mix – 1 (6.3)		
Brazil (Levi *et al.*, 2002)	3	HCV carriers	Single round 5’-NCR PCR followed by direct sequencing	5 – 3 (100)	N/A	N/A
Colombia (Yepes *et al.*, 2002)	40	Anti-HCV positive patients from Medellín	RFLP analysis of nested 5’-NCR	1a, followed by 1b, then 2	N/A	N/A
Argentina (Re	96	Anti-HCV positive	RFLP of the 5´NCR, and	1 – 23 (38.3);	N/A	N/A

***et al.*, 2003)**		patients (60 patients were HCV RNA positive)	a nested PCR with type specific primers following RT-PCR - core region	2 – 33 (55); 3 – 3 (5); unresolved – 1 (1.7)		
Chile (Soza *et al.*, 2004)	147	HCV seropositive patients from Chile	RFLP – HCV region not shown	1a – 1/17 (6); 1b – 14/17 (82); 3a – 2 (12)	N/A	N/A
Brazil (Vogler *et al.*, 2004)	61	7 blood donors, 8 HCV chronic and 46 HIV-seropositive patients	Nested 5'-NCR PCR followed by direct sequencing	1 – 47 (77.1); 2 – 1 (1.6); 3 – 13 (21.3)	N/A	N/A
Brazil (Campiotto *et al.*, 2005)	1688	Viremic chronic hepatitis C patients	Nested 5'-NCR PCR followed by direct sequencing	1 – 1095 (64.9); 2 – 78 (4.6); 3 – 510 (30.2); 4 – 3 (0.2); 5 – 2 (0.1)	N/A	N/A

Argentina (Cusumano *et al.*, 2005)	7 HCV RNA+ / 16	Patients undergoing peritoneal dialysis	RFLP analysis of nested 5'-NCR, quantiplex HCV RNA branched DNA for viral load	1a – 3 (42.9); 1b – 4 (57.1)	N/A	N/A
Brazil (Lins *et al.*, 2005)	50	HCV seropositive patients	5'-NCR genotype specific nested PCR (detection of genotypes 1, 2, 3, and 4)	1 – 32 (64); 3 – 17 (34); 1/3 mix – 1 (2)	1 – 32 (64); 3 – 17 (34); 1/3 mix – 1 (2)	N/A
Brazil (Martins *et al.*, 2006)	165	Blood donors	Reverse hybridization line probe assay - Inno-Lipa (Innogenetics)	1 – 112 (67.9); 2 – 5 (3); 3 – 51 (29.1)	N/A	N/A
Argentina (Picchio *et al.*, 2006)	93 positives out of 1637	Seventy one percent of a rural town in Argentina enrolled to investigate HCV prevalence	RFLP analysis of nested 5'-NCR	1b – 92 (98.9); 1a/1b mix – 1 (1.1)	N/A	N/A

Brazil (Bezerra *et al.*, 2007)	119	HCV seropositive patients	5’-NCR nested PCR and RFLP	1 – 45/95 (47.3); 2 – 8/95 (8.4); 3 – 33/95 (34.7); ND – 9/95 (9.5)	N/A	N/A
Brazil (Cavalheiro *et al.*, 2007)	54	Chronic hepatitis C patients from the western Brazilian Amazon region (Acre) after combined therapy	5’-NCR single round PCR Amplicor (Roche)	1 – 31 (57); 2 – 2 (3.7); 3 – 11 (20); ND – 10 (19)	N/A	1 – 31 (57); 2 – 2 (3.7); 3 – 11 (20); ND – 10 (19)
Brazil (Parana *et al.*, 2007)	24	HCV antibody positives health care workers	Amplicor (Roche) with Inno-Lipa (Innogenetics) – HCV region not shown	1 – 16 (66.7); 2 – 1 (4.1); 3 – 6 (25); ND – 1 (4.1)	N/A	N/A

Argentina (Re *et al.*, 2007)	36	Chronic hepatitis (26), hemophiliacs (2), intravenous drug users (3), blood donors (5)	Nested core region PCR followed by direct sequencing	1a – 4 (11.1); 1b – 12 (33.3); 2c – 18 (50); 3a – 1 (2.7); 4a – 1 (2.7)	N/A	N/A
Chile (Barria *et al.*, 2008)	47	HCV seropositive patients for more than 6 months	Single round 5'-NCR and NS5b PCR followed by direct sequencing	1b – 39 (83); 2a – 1 (2.1); 3a – 5 (10.6); 4 – 1 (2.1); 5a – 1 (2.1)	N/A	1b – 39 (83); 2a – 1 (2.1); 3a – 5 (10.6); 4 – 1 (2.1); 5a – 1 (2.1)
Brazil (Mendes-Correa *et al.*, 2008)	100	HIV-HCV co-infected patients	5'-NCR nested PCR followed by direct sequencing	1 – 68 (68.7); 2 – 4 (4); 3 – 26 (26.3); 4 – 1 (1)	N/A	N/A
Brazil (Matos *et al.*, 2009)	5/878	Seropositive Afro-Brazilians from Goiás State	Reverse hybridization line probe assay - Inno-Lipa (Innogenetics)	1a – 3/3 (100)	N/A	N/A

Brazil (Perone *et al.*, 2008)	788	Chronic hepatitis C patients	Direct sequencing of 5'-NCR	1a – 291 (37.5); 1b – 314 (40.4); 1a/b – 4 (.5); 2a/c – 3 (0.4); 2b – 24 (3.1); 3a – 139 (17.9); 4 – 2 (0.2)	N/A	N/A
Argentina (Re *et al.*, 2008)	37	HIV infected patients from Cordoba, Argentina	RFLP analysis of nested 5'-NCR	1 – 27 (73); 2 – 1 (2.7); 3 – 5 (13.5)	N/A	N/A
Chile (Vera-Otarola *et al.*, 2009)	45	Chronic hepatitis C patients (HCV antibody positive and HCV RNA positive for more than 6 months)	Reverse hybridization line probe assay - Inno-Lipa (Innogenetics)	1 – 37 (82); 2 – 1 (2); 3 – 7 (15); 5 – 1 (2)	N/A	1 – 31 (94); 3 – 2 (6)

5.1.4. Genotype distribution in Colombia

The only published genotyping study from Colombia reports the prevalence of HCV genotypes in Medellín, the capital of Colombia. It did not specify the genotyping prevalence of HCV, although it is known that genotype 1a was the most prevalent and that 66.5% of these patients were younger than 44 years (Yepes *et al.*, 2002).

5.1.5. Genotype distribution in Argentina

Although genotype 1 is the most prevalent in Argentina (Picchio *et al.*, 2006, Quarleri *et al.*, 1998, Quarleri *et al.*, 2000, Re *et al.*, 2008), HCV genotype 2 was the most prevalent genotype in two studies with patients from Córdoba, Central Argentina. In the first study 55% of the patients were genotype 2 followed by genotype 1 (38.3%). Thirty nine samples could be sequenced (65% of the total samples), and genotype 2b was present in 16 (41%), 1b in 16 (41%), 1a in 5 (12.8), 3 in 1 (2.6%) and indeterminate in 1 (2.6%) of patients (Re *et al.* 2003). The same research group reported the following results: genotype 2c in 50%, 1b in 33.3%, 1a in 11.1%, 3a in 2.7% and 4a in 2.7% of patients (Re *et al.* 2007). These two studies are the only ones from outside East Argentina, and Córdoba is the second most populous city in the country, after Buenos Aires.

5.2. Aims

The first aim of this chapter was to determine if the genotype distribution of HCV of patients resident in the Recife area of Northern Brazil is consistent with other studies from South America. The second aim of this study was to determine if there were any genotype discordances between plasma, peripheral blood mononuclear cells and whole saliva taken from the same individuals. The effect of age on genotype carriage was also evaluated.

5.3. Patients and samples

5.3.1. Study population

Samples were collected from two different locations: a general public hospital and a private dialysis clinic in Recife, Northeast Brazil (details in Section 2.1). All participants were of Brazilian nationality. Clinical data on all patients were obtained from standard medical records and documented onto standardized forms, which were linked to patient samples by numerical code. These forms were used during anamnesis undertaken before clinical examination. Oral findings and route of HCV acquisition can be found in Table 2.1.

5.3.2. Sample collection

Matched oral and blood samples were obtained from all patients as described in Sections 2.2.1 and 2.2.2.

5.3.3. Sample processing

After separation of plasma from the blood, the CD19+, CD14+, CD2+ and CD45+ cell subsets were immunomagnetically fractionated as described in Sections 2.2.3. WMS was not subjected to centrifugation and it was stored at -80°C until required. RNA was extracted from blood cell subsets and oral samples as described in Section 2.3.2. The presence of DNA in randomly

selected extracts was verified by using the Microzone DNA OK kit as described in Section 2.3.3.

5.3.4. Molecular detection of HCV

5.3.4.1. RNA extraction

RNA extraction was performed both manually (Section 2.3.2.1) for all samples except CD45 from patients 1 to 4 due to lack of blood for cell separation and automatically for whole saliva and plasma samples (Section 2.3.2.2).

5.3.4.2. Polymerase Chain Reaction (PCR)

Reverse transcription was carried out to generate cDNA for all samples (Section 2.3.4) followed by PCR of the 5'-noncoding region for plasma samples only (Section 2.3.5) and NS5b region for all samples (Section 2.3.6).

5.3.4.3. RFLP and direct sequencing

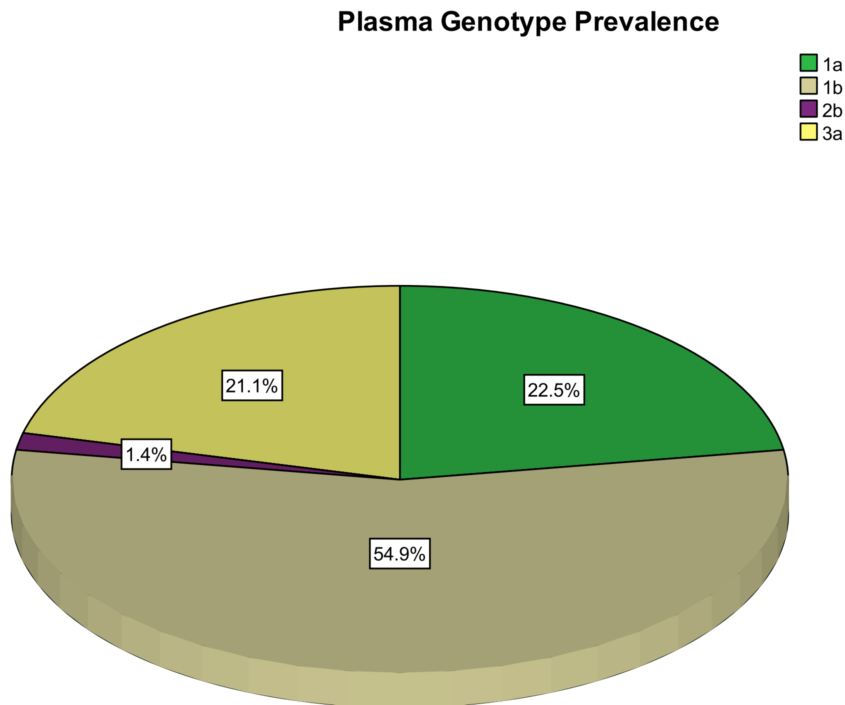
RFLP was performed for all plasma samples (Sections 2.3.8 and 2.3.9) and direct sequencing was performed for all samples (Section 2.3.12).

5.4. Genotyping results

5.4.1 Genotypes of HCV isolated from plasma

In the present study group, 71 patients were positive (83.5%) by HCV NS5b PCR in plasma and therefore could be genotyped. From these RNA positive patients, genotype 1a was detected in plasma of 16 patients (22.5%). Genotype 1b was detected in plasma of 39 patients (54.9%), whereas genotype 2b was only detected in one patient (1.4%) and genotype 3a in 15 patients (21.1%). The genotype distribution amongst plasma samples are shown in Figure 5.1. Genotyping of this compartment was also performed by RFLP. HCV RNA prevalence was again 83.5% and the genotyping results for the 71 patients were concordant with the NS5b sequencing, however there was a discordance at the subtype level in 3 patients which were genotyped as 1a by RFLP as opposed to 1b genotype via NS5b (patients 2, 34, N7), but the NCR region is told to be not accurate enough to predict differences between subtypes 1a and 1b (Verma *et al.*, 2008).

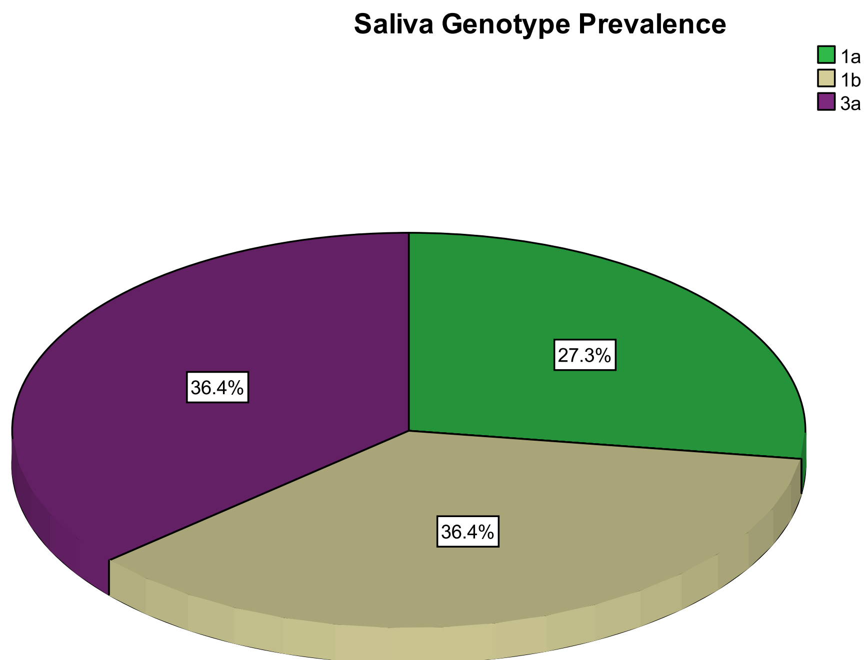
Figure 5.1. Genotype distribution of 71 HCV RNA positive plasma samples



5.4.2. Genotypes of HCV isolated from saliva

In the present study group, only 11 patients were positive (12.9%) for HCV NS5b PCR in saliva and therefore could be genotyped. From these RNA positive patients, genotype 1a was detected in plasma of 3 patients (22.5%), genotype 1b was detected in plasma of 4 patients (54.9%) and genotype 3a was detected in 4 patients (21.1%). The genotype distribution amongst saliva samples is shown in Figure 5.2.

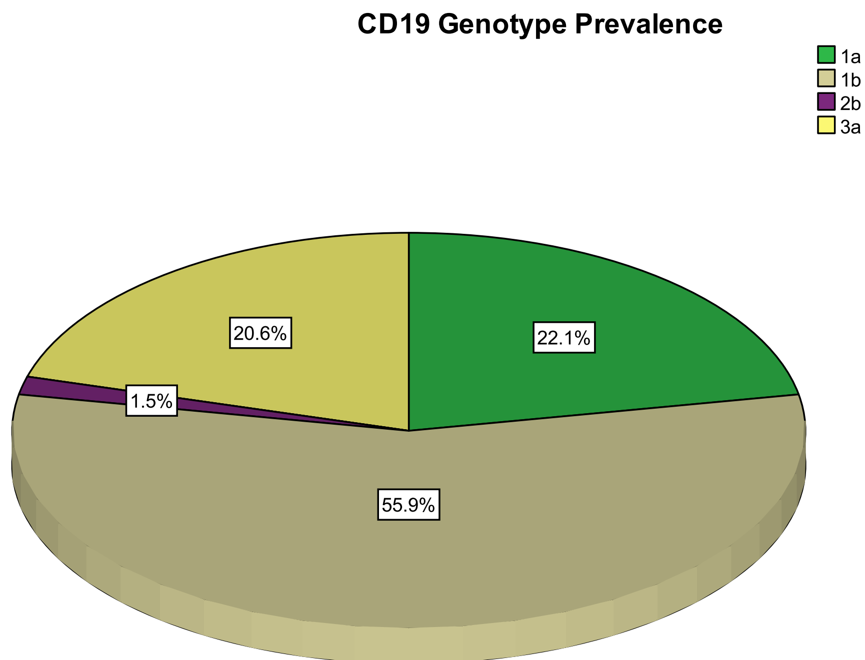
Figure 5.2. Genotype distribution of 11 HCV RNA positive saliva samples



5.4.3. Genotypes of HCV isolated from CD19

In the present study group, 68 patients were positive (80%) for HCV NS5b PCR in CD19 cells and therefore could be genotyped. From these RNA positive patients, genotype 1a was detected in CD19 cells of 15 patients (22.1%), genotype 1b was detected in CD19 cells of 38 patients (55.9%), genotype 2b (1.5) was detected only in one patient whereas genotype 3a in 14 patients (21.1%). Genotype distribution amongst CD19 samples is shown in a pie chart enclosed in Figure 5.3.

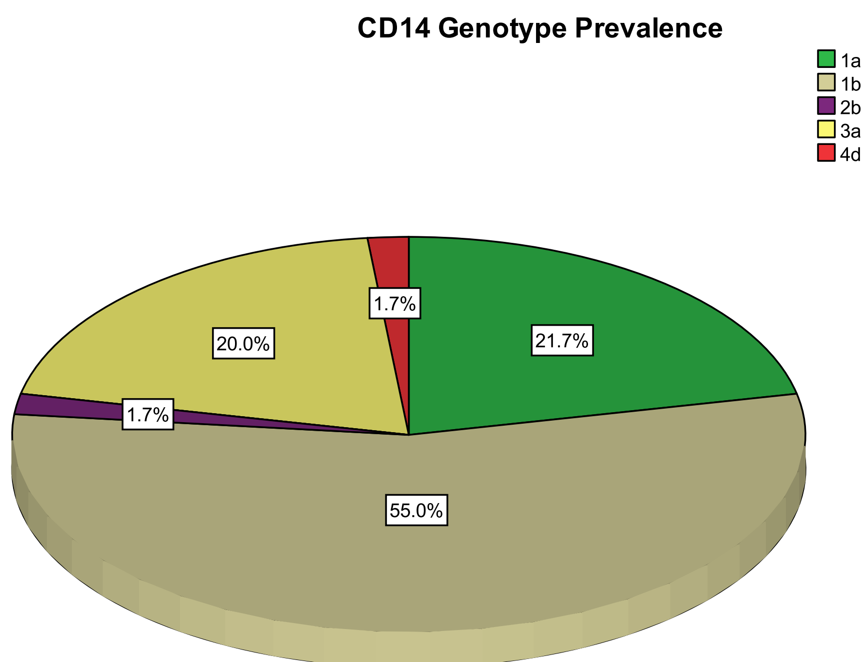
Figure 5.3. Genotype distribution of 68 HCV RNA positive CD19 samples



5.4.4. Genotypes of HCV isolated from CD14 cells

In the present study group, 60 patients were positive (70.6%) for HCV NS5b PCR in CD14 cells and therefore could be genotyped. From these RNA positive patients, genotype 1a was detected in CD14 cells of 15 patients (22.1%). Genotype 1b was detected in CD14 cells of 38 patients (55.9%), genotype 2b (1.7%) was detected only in one patient whereas genotype 3a in 14 patients (21.1%) and genotype 4d in only one patient (1.7%). Genotype distribution amongst CD14 samples are shown in a pie chart enclosed in Figure 5.4.

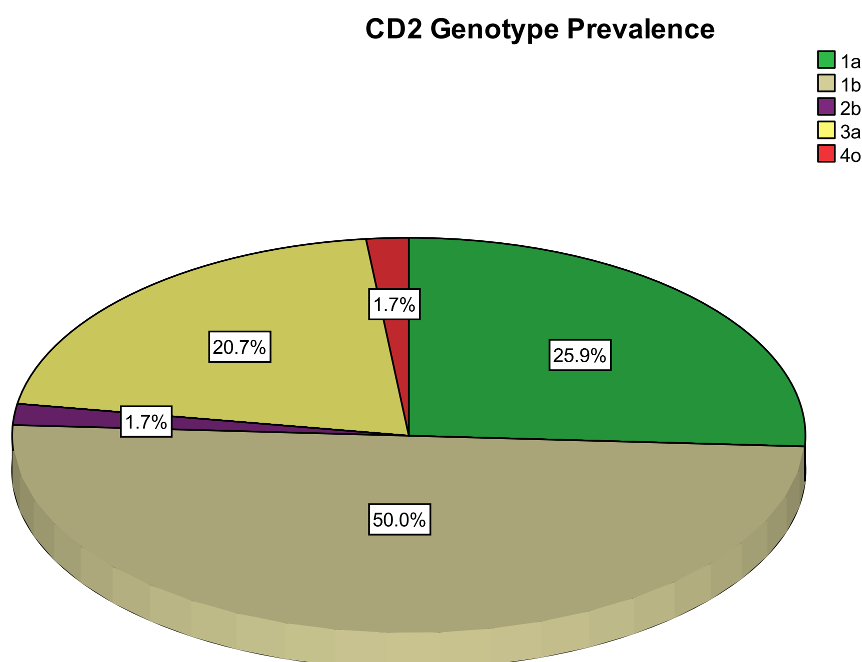
Figure 5.4. Genotype distribution of 60 HCV RNA positive CD14 cells



5.4.5. Genotypes of HCV isolated from CD2 cells

In the present study group, 58 patients were positive (68.2%) for HCV NS5b PCR in CD2 cells and therefore could be genotyped. From these RNA positive patients, genotype 1a was detected in CD2 cells of 15 patients (25.9%). Genotype 1b was detected in CD2 cells of 29 patients (50%), genotype 2b (1.7) was detected only in one patient whereas genotype 3a in 12 patients (20.7%) and genotype 4o in only one patient (1.7). Genotype distribution amongst CD2 samples are shown in a pie chart enclosed in Figure 5.5.

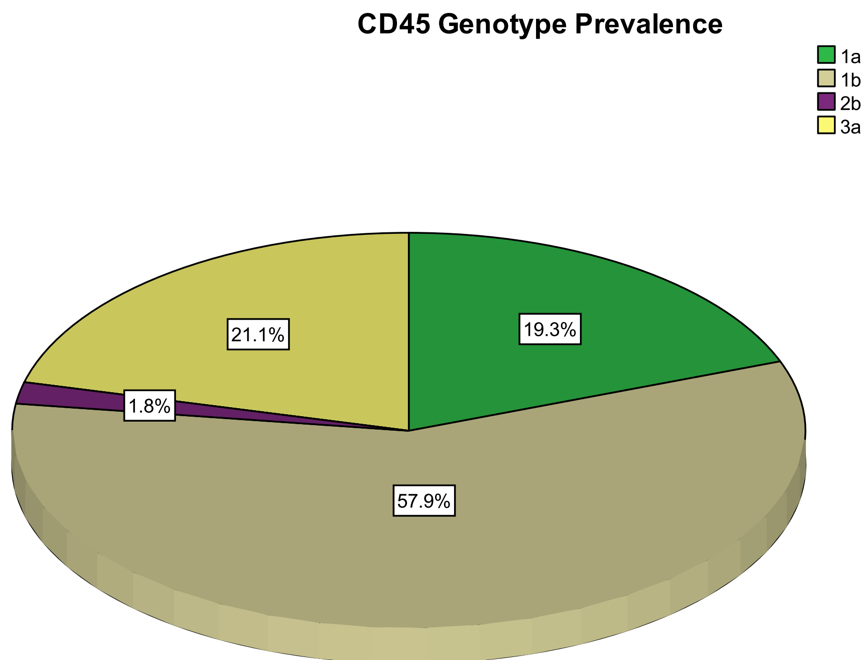
Figure 5.5. Genotype distribution of 58 HCV RNA positive CD2 cells



5.4.6. Genotypes of HCV isolated from CD45 cells

In the present study group, 57 patients were positive (67.1%) for HCV NS5b PCR in CD45 cells and therefore could be genotyped (samples 1, 2, 3 and 4 could not be genotyped at CD45 because they were unavailable). From these RNA positive patients, genotype 1a was detected in CD45 cells of 11 patients (19.3%). Genotype 1b was detected in CD45 cells of 33 patients (57.9%), genotype 2b (1.8) was detected only in one patient whereas genotype 3a in 13 patients (21.1%). Genotype distribution amongst CD45 samples are shown in a pie chart enclosed in Figure 5.6.

Figure 5.6. Genotype distribution of 57 HCV RNA positive CD45 cells



5.4.7. Distribution of HCV genotypes between compartments

The genotypes within plasma and each cell type was always identical (data not shown), with the exception of patients 53 where genotype 4o was identified in CD2 cells and patients 63 where genotype 4d in was identified in CD14 cells, in both cases all other compartments in these patients were genotype 3a.

5.5. Statistical correlations related to HCV genotypes

5.5.1. Correlation between HCV genotype in saliva and plasma

There was complete concordance between the genotype of HCV in plasma and whole saliva (data not shown).

5.5.2. Genotypes within different routes of acquisition

Fisher's exact test revealed no statistically significant difference in the frequency of the different genotypes in plasma and the route of acquisition ($p=0.391$).

5.5.3. Distribution of patient age versus HCV genotype

One way analysis of variance (ANOVA) revealed that there was a significant difference in the distribution of the three main genotypes (1a, 1b and 3a) Table 5.3. An additional comparison (post hoc) using the Bonferroni method revealed that there was a significant difference in the age distribution of genotypes 1a and 3a. Genotype 1a was more likely than 3a to be present in younger adults, while 3a was more likely than 1a to be present in older adults. One patient was removed from the analyses as they were the only individual with a genotype other than 1a, 1b or 3a (having genotype 2a).

Table 5.2. Plasma HCV genotypes according to route of acquisition

Route		Genotype				
		Negatives	1a	1b	3a	Total
Not available	Count	8	3	19	7	37
	% within routeofacquistion	21.6%	8.1%	51.4%	18.9%	100.0%
	% within Plasma_Gen	53.3%	18.8%	48.7%	46.7%	43.5%
Sexual	Count	0	1	1	0	2
	% within routeofacquistion	.0%	50.0%	50.0%	.0%	100.0%
	% within Plasma_Gen	.0%	6.3%	2.6%	.0%	2.4%
Transfusion	Count	5	7	15	5	32
	% within routeofacquistion	15.6%	21.9%	46.9%	15.6%	100.0%
	% within Plasma_Gen	33.3%	43.8%	38.5%	33.3%	37.6%
IV drug use	Count	1	0	1	0	2
	% within routeofacquistion	50.0%	.0%	50.0%	.0%	100.0%
	% within Plasma_Gen	6.7%	.0%	2.6%	.0%	2.4%
Dyalisis	Count	1	4	3	3	11
	% within routeofacquistion	9.1%	36.4%	27.3%	27.3%	100.0%
	% within Plasma_Gen	6.7%	25.0%	7.7%	20.0%	12.9%

Other	Count	0	1	0	0	1
	% within routeofacquistion	.0%	100.0%	.0%	.0%	100.0%
	% within Plasma_Gen	.0%	6.3%	.0%	.0%	1.2%
Total	Count	15	16	39	15	85
	% within routeofacquistion	17.6%	18.8%	45.9%	17.6%	100.0%
	% within Plasma_Gen	100.0%	100.0%	100.0%	100.0%	100.0%

Table 5.3. Distribution of patient age versus HCV genotype

Genotype	Mean	N	Std. Deviation	Minimum	Maximum	Median
1a	40.56	16	12.801	26	73	40.00
1b	50.92	39	15.660	20	74	53.00
3a	54.73	15	10.990	37	79	53.00
Total	49.37	70	14.859	20	79	50.00

Table 5.4. One way analysis of variance of distribution of HCV genotypes across patient age

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1766.703	2	883.351	4.395	.016
Within Groups	13467.640	67	201.010		
Total	15234.343	69			

Table 5.5. Post-hoc comparison of genotypes 1a, 1b and 3a using the Bonferroni method

Genotype (I)	Genotype (J)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
1a	1b	-10.361*	4.209	.049	-20.70	-.02
	3a	-14.171*	5.095	.021	-26.68	-1.66
1b	1a	10.361*	4.209	.049	.02	20.70
	3a	-3.810	4.308	1.000	-14.39	6.77
3a	1a	14.171*	5.095	.021	1.66	26.68
	1b	3.810	4.308	1.000	-6.77	14.39

*. The mean difference is significant at the 0.05 level (p value).

5.6. Discussion

The present study has examined the greatest number of patients from the north of Brazil than any other previous report. In addition it is the only investigation of genotype distribution across plasma, peripheral blood mononuclear cells (PBMC) and whole saliva. Many of the studies discussed in Table 5.1 employed gel-based assays which is applicable for clinical management of patients it does not provide the high level of resolution of sequencing/cloning which is considered the gold standard technique to assess viral diversity (Fan *et al.*, 2009), and was the chosen technique at this study for this analysis. In view of the latter the present results must be considered to be highly robust and thus present a true picture of HCV genodiversity in the present group of patients.

The results of the present study reveal that the genotype distribution is as might be expected for Brazil, namely that there is a dominance of genotype 1 in plasma and PBMC. Only one previous study of Brazil residents has performed genotyping in oral fluids and plasma, but not for any PBMC (Lins *et al.*, 2005) while three studies, one from Brazil (Cavalheiro *et al.*, 2007) and two from Chile (Vera-Otarola *et al.*, 2009, Barria *et al.*, 2008) have conducted a genotyping investigation on plasma and PBMCs, although not oral fluids, of residents of South America.

The consistency of the present results with those of other relevant studies indicates that the geographic locale is a principal factor that influences the

genotype of HCV infection. The geographic distribution of HCV genotypes in turn reflects the impact of changes in health care provision (e.g. increased use of blood and blood products, increased frequency of potential iatrogenic transmission and increased injecting drug use across the globe (Magiorkinis *et al.*, 2009). The role of genetic factors in the geographic distribution of HCV genotypes remains unclear (Jonsson *et al.*, 2008, Laouenan *et al.*, 2009, Zuniga *et al.*, 2009) but in the future may provide additional insight into this problem.

In the present study there were no significant differences in the distribution of genotypes across the different groups according to the potential route of acquisition. This is in accordance with the results of some (Maieron *et al.*, 2010) but not all similar studies. In particular Subtypes 1a, 3a and 4 have been reported to be associated with injecting drug use (IDU) in some countries (Esteban *et al.*, 2008) and 1b and 2 with blood transfusions – although this can depend upon the country in which the study patients are resident – as in China genotypes 3 and 6 may be associated with acquisition via IDU (Zhou *et al.*, 2009). The present group of Brazilian patients did contain a substantial number of individuals who may have acquired HCV via blood transfusions or IDU, hence in a country known to have a dominance of genotype 1 it is perhaps unsurprising to find that genotype 1 is common in the present cohort.

Interestingly, no patient in the present study had identifiable genotype 4 HCV in plasma or saliva, although, one previously untreated patient (53) which

was genotype 4o in CD2 cells, and genotype 3a in all other compartments, excluding saliva (which was negative). Another patient (63), who was under treatment at the time of examination, had no detectable HCV RNA in plasma, saliva or any PBMCs except for CD14 cells, which was genotyped as 4d via NS5b PCR. However, real time PCR could detect HCV RNA in all PBMCs of this patient, the viral load was low in all of the cell types and genotyping could not be performed. As the sequences derived from these compartments were unlike any typed in the laboratory previously, cross contamination from the laboratory setting can be excluded.

In the present study genotypes 1a and 1b were the dominant genotype in patients who had received haemodialysis. There was no notable genotypic difference detected between this group of patients from the hospital and the one from the private dialysis clinic (data not shown). In Brazil genotype 1 is the most prevalent isolate in haemodialysis recipients (Amorim *et al.*, 2010). A recently published study of samples obtained in 2002 from 51 haemodialysis recipients from the Federal state of Brazil found that 1a was the most frequent subtype and of note, as this differed from the frequency in the local blood donor community it suggests that nosocomial acquisition of HCV may be occurring during dialysis (Amorim *et al.*, 2010). It is of course well known that haemodialysis is itself a potential risk factor for HCV acquisition, although regular testing of all patients for HCV RNA, isolation of positive patients, use of dedicated dialysis machines and strict adherence to infection control policies are suggested as helpful measures to contain the HCV spread via dialysis units (Hussein *et al.*, 2010).

None of the patients who had received haemodialysis had more than one HCV subtype. This contrasts with the observation of mixed HCV genotypes in hemodialysis patients from Venezuela (Pujol *et al.*, 1998). However this latter study examined only 14 patients at different time points hence it is possible the patients were multiply exposed to HCV during this time and have acquired new HCV subtypes.

The present study found an age-related trend in which subtype 1a was more likely to be in younger persons while subtype 3a more likely in older individuals. Perhaps the most obvious reason for this trend would be an association with route of acquisition, but as there was no significant difference in the ages of groups according to route of acquisition this is unproven. Previous studies however have found that older individuals may acquire infection via iatrogenic routes (e.g haemodialysis or blood products) while younger persons become infected as a consequence of IDU (Esteban *et al.*, 2008, Maieron *et al.*, 2010) and as a consequence the infecting genotype is a reflection of the route of transmission rather than age alone (Di Tommaso *et al.*, 2003).

The present study found that there was good concordance of HCV subtypes between the different examined compartments. This concordance of subtypes between plasma and PBMC indicates that there is no compartmentalisation within blood of distinct subtypes and suggests that there may be some interplay (by unknown mechanisms) of HCV between

plasma and PBMC. The only exception was previously mentioned presence of genotype 4o in T lymphocytes (CD2) of patient 53, as opposed to genotype 3a present at his plasma and all other cellular compartments (saliva was negative for HCV), which could suggest compartmentalisation or maybe a case of mixed infections in a single patient. What draws even more attention towards genotype 4 detection in this study, which was also detected as previously mentioned in monocytes (CD14) of patient 63 (HCV negative in all other studied compartments), is the fact that genotype 4 is extremely rare in Brazil. There is a single case report for the whole state of Bahia, Northeast Brazil (Zarife *et al.*, 2006) and isolated cases in the largest genotyping study in Brazil found in São Paulo (Campiotto *et al.*, 2005), which highlight how unusual this genotype is in the country. The present study represents only the second study to report genotype 4 detection in Northeast Brazil.

The concordance of HCV subtypes between saliva and plasma (all be it with small numbers of saliva samples) suggests that the mouth and/or salivary glands are not sites of likely independent HCV acquisition and/or replication. Indeed the results of the present chapter and those of chapter 3, in which the frequency of salivary carriage of HCV is low, increasingly point towards the mouth as being an unlikely site of HCV replication.

Of relevance to the present chapter genotypic analysis has been employed to examine possible intrafamilial transmission of HCV. Such transmission has been suggested in some (Plancoulaine *et al.*, 2008) but not all (Ndong-Atome *et al.*, 2009) studies. However a recent study found some concordance of

HCV subtypes within Brazilian families and suggested that transmission could have occurred via non-sexual routes – including the sharing of toothbrushes (Cavalheiro *et al.*, 2009). Nevertheless, the cumulative results of chapters 3 to 5 of the present work would suggest that it is unlikely that such transmission is common as HCV is rarely present in saliva. The virus is generally present in low levels and there is no evidence of compartmentalisation demonstrating oral replication of the virus. It is important to consider, though, that salivary components may be causing the polymerase not to work by inhibiting its activity.

Chapter 6

Nucleotide changes and aminoacids variability between different compartments

6.1. Introduction

6.1.1. Quasispecies variability and sequencing analysis

Hepatitis C Virus (HCV) displays a high degree of genetic diversity. This arises from transcription errors (i.e nucleotide substitutions) due to a lack of proof reading by the HCV RNA polymerase and the absence of 5' to 3' exonuclease activity. The mean *in-vivo* frequency of nucleotide mutations varies between 1.4 X 10^3 to 1.9 X 10^3 substitutions per nucleotide per year (Le Guillou-Guillemette *et al.*, 2007) however *in-vitro* a mutation rate of 3.5 to 4.8 X 10^3 has been observed (Kato *et al.*, 2009). Some of the mutations are silent (i.e. synonymous) having no effect upon the amino acid sequence of the related protein but may affect the secondary structure of the viral RNA. Non-synonymous mutations do lead to changes in amino acids of the viral protein and can lead to the emergence of viral variants. Some of the non-synonymous mutants may of course be lethal to the virus, but the concern is whether such mutants have an impact upon the infectivity, pathogenicity of, and therapeutic response by HCV.

The non-coding 5' region is the most conserved region of the HCV genome. The most variable region of the HCV genome encodes the envelope proteins E1 and E2, such that the sequences of the hypervariable regions (HVR1 and HVR2) of E2 in strains from different individuals may vary by more than 50%, with the mutation rates of non-structural regions essential for RNA replication being lower than that of structural regions (Kato *et al.*, 2009).

A viral quasispecies is a group of viruses related by a similar mutation or group of mutations that compete within a mutagenic environment (e.g. a human host). Quasispecies can be considered to be “clouds” of related elements that behave almost (“quasi”) like a single virus (“species”). The precise influence of quasispecies upon HCV disease is not entirely evident. It would seem that the complexity of quasispecies influence the response to interferon alone or interferon plus ribavirin (Jain *et al.*, 2009). Anti-HCV therapy may itself drive the emergence of quasispecies (Jardim *et al.*, 2009). The presence and diversity of quasispecies prior to therapy may, in some but not all instances, predict response to therapy (Moreau *et al.*, 2008). Indeed this is no better than HCV viral load in predicting response to therapy (Salmeron *et al.*, 2006). Similarly there are conflicting reports of the association of the diversity of quasispecies with disease progression (Kumagai *et al.*, 2007) and histopathological hepatic change (Vallet *et al.*, 2007) although it may be that mutations influence progress to hepatocellular carcinoma (HCC) (De Mitri *et al.*, 2007) and that there is some compartmentalisation of quasispecies within tumoural hepatocytes when compared to hepatocytes from non-neoplastic tissue (Sobesky *et al.*, 2007). Quasispecies may allow HCV to escape from host cytotoxic T lymphocyte (CTL) and humoral immune responses (Domingo *et al.*, 2007, Guglietta *et al.*, 2009) for example mutations within NS3/4A protease allow escape from CD8 T cells (Salloum *et al.*, 2010).

Quasispecies analysis can aid epidemiological studies as well as investigation of possible nosocomial transmission of HCV (Almroth *et al.*, 2010, Gutelius *et al.*, 2010) and monitor potential resistance to therapy (Kuntzen *et al.*, 2008).

6.2. Quasispecies analysis within different compartments

An understanding of the quasispecies within saliva compared to plasma and PBMC may provide insight into the source of HCV within the mouth and potentially indicate if there is any preferential compartmentalisation of HCV quasispecies in the mouth or salivary glands. Hence the aim of this chapter was to determine whether there are differences in the distribution of quasipecies in plasma, saliva and peripheral blood mononuclear cells.

Compartmentalisation of HCV quasispecies in peripheral blood mononuclear cells (PBMC), serum, plasma and liver has been demonstrated (Vera-Otarola *et al.*, 2009). The sequences of HVR1 of HCV isolates from plasma can differ from those of isolates from CD19+ cells and monocytes, although the variants within the cells have a low rate of non-synonymous mutations (Ducoulombier *et al.*, 2004). Quasispecies within a specific cellular compartment may be statistically more genetically alike than quasispecies from different cellular compartments (Roque Afonso *et al.*, 1999). The responses of quasispecies within plasma and PBMC to interferon therapy may differ (Fornai *et al.*, 2001) and the complexity of quasispecies in liver may differ from those in plasma (Cabot *et al.*, 2000). However the majority of

the data concerning compartmentalisation of HCV quasispecies is based upon studies of small groups of patients. This study includes a higher number of patients and also attempts to analyse viral evolution between different compartments, comparing amplicons in different sites instead of the general quasispecies analysis within a single compartment, usually plasma. The HCV infection is composed of a pool of genetically distinct but closely related variants which are called quasispecies (Martell *et al.*, 1992). They arise from a combination of high error rate of viral RNA polymerase, the rapid replication rate of HCV and the large viral population size (Pawlotsky, 2006a) as previously seen in chapter 1 (refer to Section 1.6). By applying sequencing methods such as direct sequencing following block based PCR (Section 2.3.9), we could detect nucleotide substitutions and classify them as synonymous (when the nucleotide change does not affect the aminoacid that it will code in its specific codon) or non-synonymous changes (when the nucleotide substitution will result in a codon that is translated into a different aminoacid). This kind of analysis is possible thanks to specific software such as SeqMan and MegAlign from the LASERGENE package (Section 2.3.12).

Baseline quasispecies complexity is reported to be higher in patients that fail to achieve SVR as opposed to those who achieve SVR and baseline quasispecies complexity was also reported to predict treatment efficacy and was negatively associated with viral clearance rate (Shire *et al.*, 2006).

In summary, quasispecies dynamics has an impact on the biology of HCV, where escape variants contribute to viral persistence, disease progression

and treatment ineffectiveness (Domingo *et al.*, 2007). These quasispecies can only be detected if the single copies of the viruses are isolated and sequenced accordingly.

6.3. Aim

To establish if there are different quasispecies of HCV within plasma, saliva and peripheral blood mononuclear cells by means of analysing the compartments nucleotide changes and aminoacid variabilities.

6.4. Patients and samples

6.4.1. Study population

Samples were collected from two different locations. A general public hospital and a private dialysis clinic (refer to Section 2.1). All participants were of Brazilian nationality. Clinical data on all patients were obtained from standard medical records and documented onto standardized forms, which were linked to patient samples by numerical code. These forms were used during anamnesis undertaken before clinical examination. Oral findings and route of HCV acquisition can be found in Table 2.1.

6.4.2. Sample collection

Matched oral and blood samples were obtained from all patients as described in Sections 2.2.1 and 2.2.2.

6.4.3. Sample processing

After separation of plasma from the blood, the CD19+, CD14+, CD2+ and CD45+ cell subsets were immunomagnetically fractionated as described in Sections 2.2.3. WMS was not subjected to centrifugation and it was stored at -80°C until required. RNA was extracted from blood cell subsets and oral samples as described in Section 2.3.2. The presence of DNA in randomly

selected extracts was verified by using the Microzone DNA OK kit as described in Section 2.3.3.

6.4.4. Molecular detection of HCV

6.4.4.1. RNA extraction

RNA extraction was performed both manually (Section 2.3.2.1) for all samples, but CD45 from patients 1 to 4 due to lack of blood for cell separation and automatically for whole saliva and plasma samples (Section 2.3.2.2).

6.4.4.2. Polymerase Chain Reaction (PCR) and direct sequencing

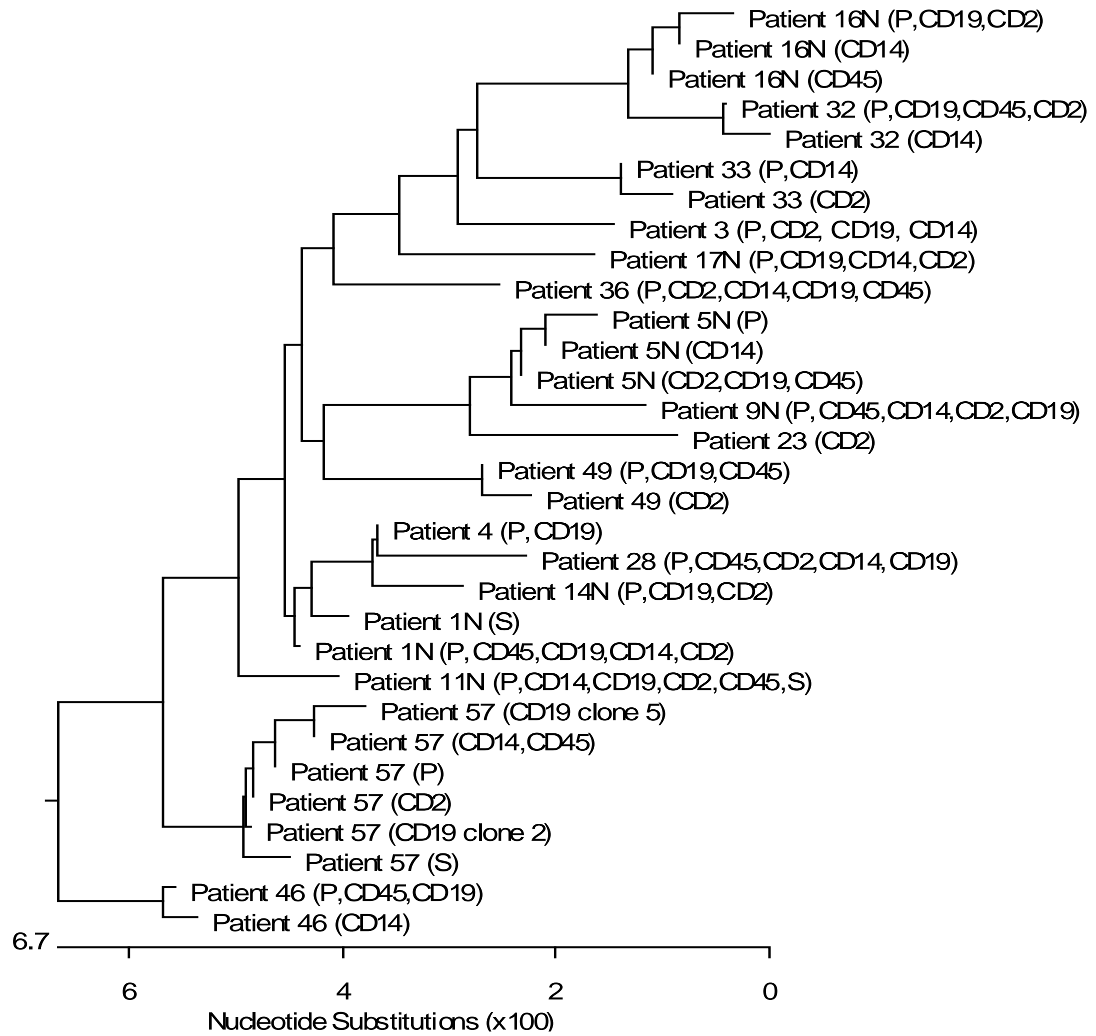
Reverse transcription was carried out to generate cDNA for all samples (Section 2.3.4) followed by PCR of the NS5b region for all samples (Section 2.3.6) and direct sequencing was performed for all compartments sampled from each patient, i.e. plasma, whole saliva, CD19, CD14, CD2 and CD45 (Section 2.3.12).

6.5. Phylogenetic analysis

Phylogenetic trees were created for genotypes 1a, 1b and 3a (Figures 6.1, 6.2 and 6.3 respectively). PBMC, saliva and plasma samples were either identical or closely related in each patient The adaptability of HCV via

quasispecies dynamics (refer to Chapter 1 for more details on quasispecies) could be clearly observed in our study as most of the patients had closely related sequences that usually would differ by variable number of nucleotides, resulting in silent or non-synonymous amino acid changes (more details later in this Chapter). It is possible to see that the majority of patients had closely related but not identical sequences, although some patients had identical sequences and one rare exception of a patient under dialysis (N12) where the sequence from the plasma was separate from the CD14 sequence.

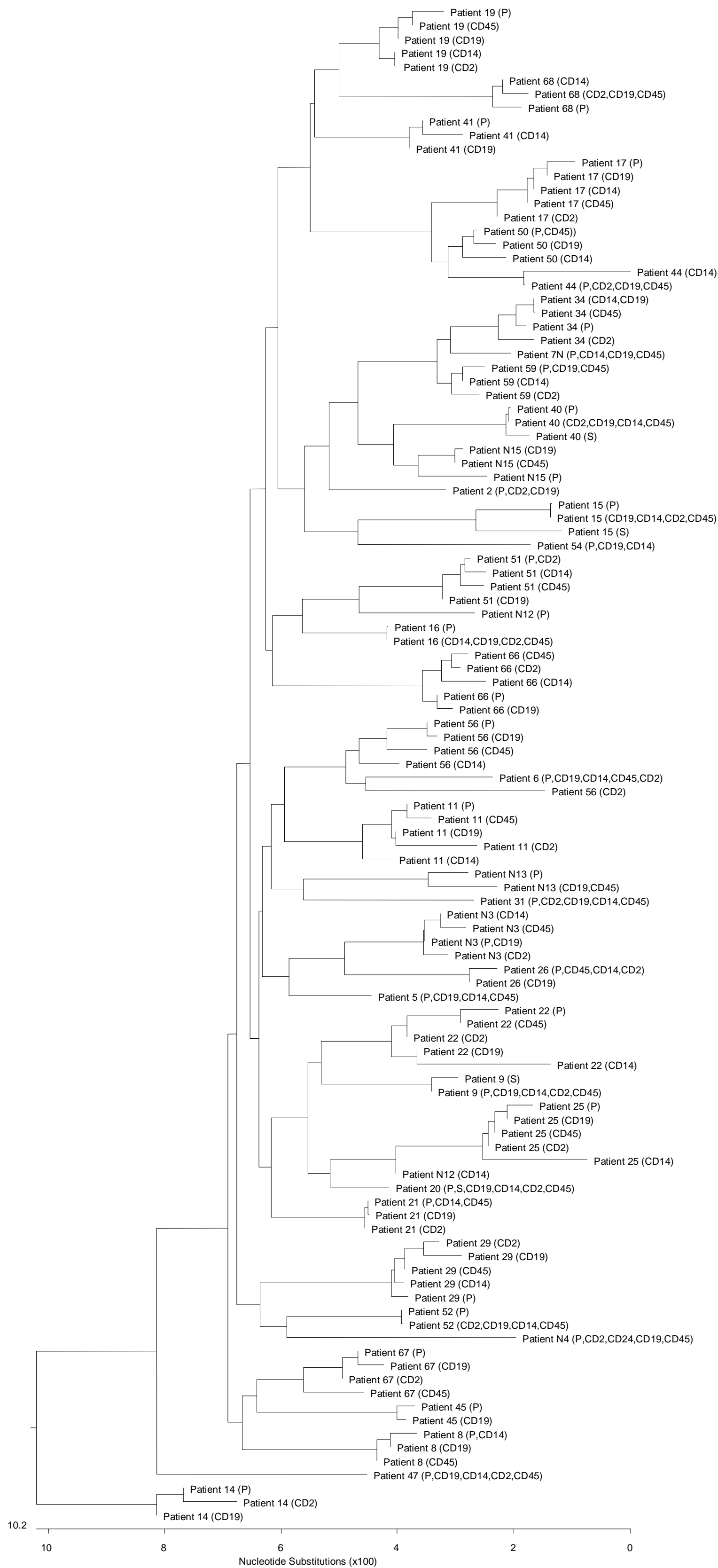
Figure 6.1. Phylogenetic tree of genotype 1a sequences



Identical sequences observed in multiple compartments are represented by a single sequence and the compartments in which that have been observed are detailed in brackets

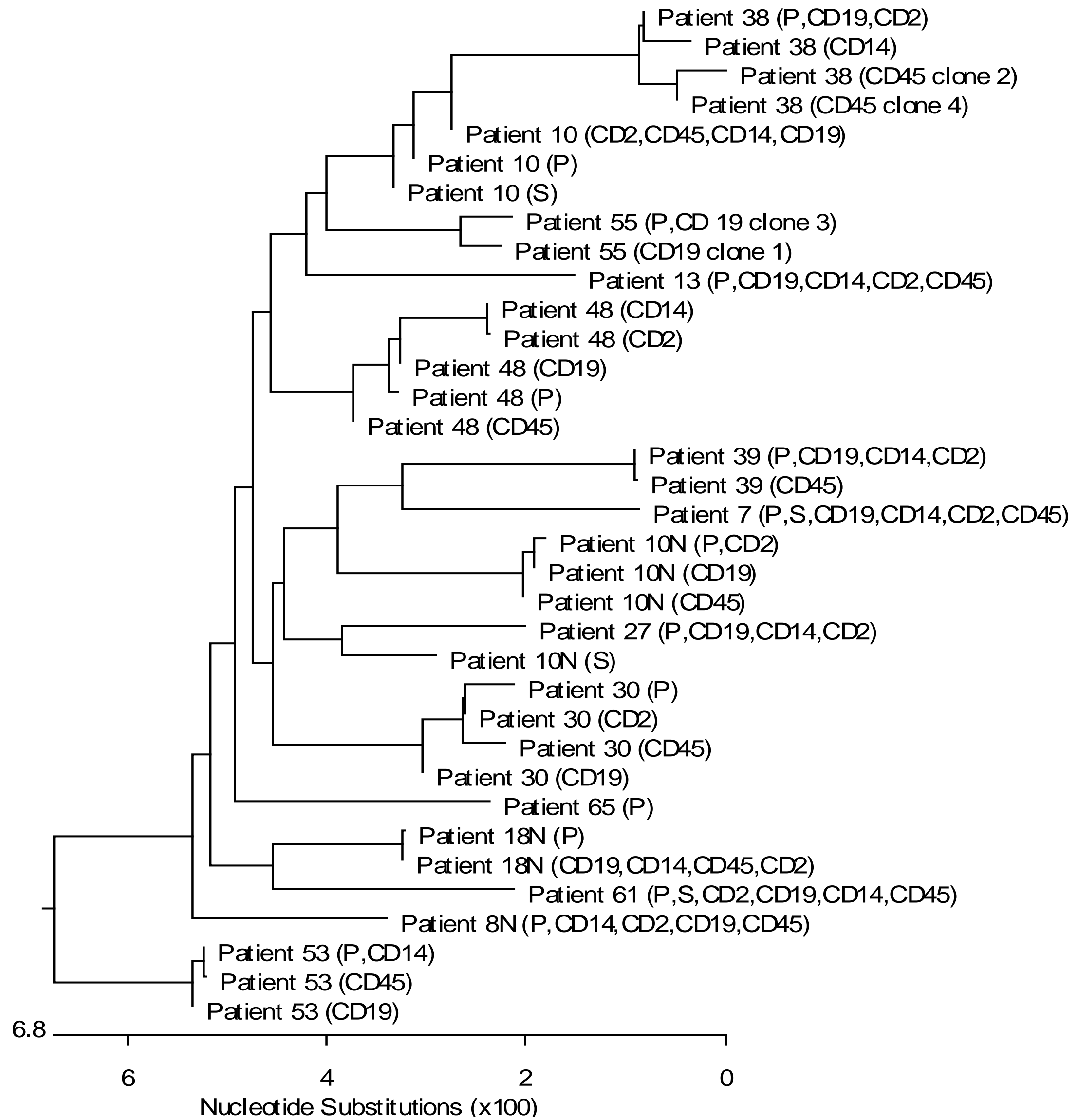
S = saliva, P = plasma

Figure 6.2. Phylogenetic tree of genotype 1b sequences



Identical sequences observed in multiple compartments are represented by a single sequence and the compartments in which that have been observed are detailed in brackets. S = saliva, P = plasma

Figure 6.3. Phylogenetic tree of genotype 3a samples



Identical sequences observed in multiple compartments are represented by a single sequence and the compartments in which that have been observed are detailed in brackets

S = saliva, P = plasma

6.6. Nucleotide changes and amino acids variability between compartments

For this study the direct sequence derived from the compartment and the associated predicted amino acid sequence were compared to that derived from the plasma. Compartments where the genotypes differed from the plasma were excluded from this analysis. This was a rare occurrence and was only observed in two patients; patient 53 in compartment CD2 which was genotyped as 4o and patient 63 in compartment CD14 which was genotyped as 4d.

To establish if there was any difference in the sequences from different compartments Fisher's exact test (refer to section 2.3.16) was employed to compare the expected amino acid sequences within the dominant quasispecies of individual HCV genotypes between the different compartments. This would thus detect the presence of non-synonymous mutations within the NS5b region of the HCV genome. In all analyses comparison was made to the plasma derived sequence.

6.6.1. Comparison of plasma and saliva sequences

In a comparison between matched saliva and plasma samples there were no amino acid changes in genotype 1a quasispecies while 50% of the genotype 1b and 25% of genotype 3a quasispecies differed in saliva when compared to plasma respectively (Table 6.1). There were no statistically significant

differences in the frequency of amino acid sequence changes between these two compartments (Fisher's exact test; P=0.709). No conclusions can be drawn from this analysis as there were very few patients who had detectable HCV in saliva.

Table 6.1. Amino acid changes within each genotype in matched saliva and plasma samples

Genotype	No changes	1 or more changes
1a	3	0
1b	2	2
3a	3	1

6.6.2. Comparison of plasma and CD19 sequences

In a comparison between matched CD19 cells and plasma only one (6.6%) of 15 patients with genotype 1a had amino acid changes. In contrast nine of 38 (23.1%) patients of genotype 1b had one or more amino acid difference between the two compartments. One of 14 patients with genotype 3a had a different amino acid sequence between the two compartments (Table 6.2). Fisher's exact test did not reveal any statistically significant difference in the frequency of amino acid changes between these two compartments (P = 0.255), although there appears to be a trend for changes to be more frequent with genotype 1b than 1a or 3a quasispecies.

Table 6.2. Amino acid changes within each genotype in isolates of matched CD19 cells and plasma samples

Genotype	No changes	1 or more changes
1a	14	1
1b	29	9
3a	13	1

6.6.3. Comparison of plasma and CD14 sequences

In a comparison between matched CD14 and plasma samples none of 13 patients with genotype 1a had amino acid changes between compartments. In contrast 12 of 33 (36.3%) of genotype 1b quasipecies had a change in one or more amino acid between the two compartments. There were no instances of amino acid change in 3a quasispecies (Table 6.3). Fisher's exact revealed that the difference in the frequency of amino acid changes between plasma and CD14 cells in the matched patients was significant (P=0.003), this reflects the high number of changes in genotype 1b quasipecies. Although the numbers of samples analysed were low the differences in amino acid changes in genotype 1b were significant because there were no changes observed within genotypes 1a and 3a (table 6.3).

Table 6.3. Amino acid changes within each genotype in isolates from matched CD19 cells and plasma samples

Genotype	No changes	1 or more changes
1a	13	0
1b	21	12
3a	11	0

6.6.4. Comparison of plasma and CD2 sequences

In a comparison between matched CD2 cells and plasma samples one of 14 (7.1%) patients with genotype 1a had one or more amino acid change in the quasispecies (Table 6.4). In contrast, nine of 29 (31%) of genotype 1b quasispecies had a change in one or more amino acid between the two compartments. One of 12 matched genotype 3a samples had one or more amino acid change in quasispecies (Table 6.4). Fisher's exact revealed that the difference in the frequency of amino acid changes between plasma and CD2 cells in the matched patients was not significant (P=0.153). There was however a trend for there to be differences in the amino acid sequence of the genotype 1b quasipecies.

Table 6.4. Amino acid changes within each genotype in isolates from matched CD2 cells and plasma samples

Genotype	No changes	1 or more changes
1a	13	1
1b	20	9
3a	11	1

6.6.5. Comparison of plasma and CD45 sequences

In a comparison between matched CD45 cells and plasma samples none of 14 genotype 1a patients had any amino acid changes (Table 6.5). In contrast 12 of 33 (36.3%) of genotype 1b quasipecies had a change in one or more amino acid between the two compartments. Two of 12 (16.6%) matched genotype 3a samples had one or more amino acid change in quasispecies. Fisher's exact test revealed that the difference in the frequency of amino acid changes between plasma and CD2 cells in the matched patients was significant (P=0.032), this reflects the differences within genotype 1b.

Table 6.5. Amino acid changes within each genotype in isolates from matched CD45 cells and plasma samples

Genotype	No changes	1 or more changes
1a	11	0
1b	21	12
3a	10	2

6.6.6. Positioning of amino acid changes

An analysis of all quasispecies from all compartments indicates that synonymous and non-synonymous mutations are spread across the entire NS5B region (Figure 6.4). Non-synonymous changes were generally more frequent at the 5' end, although there was were some "hotspots" towards the 3' end. There was no single codon that was more prone to mutation, although no mutations of either type occurred within codon 18. The amino acid changes and the compartments in which these occurred are summarised in Table 6.6.

Figure 6.4. Nucleotide mutations described per codon and association with synonymous or non-synonymous changes

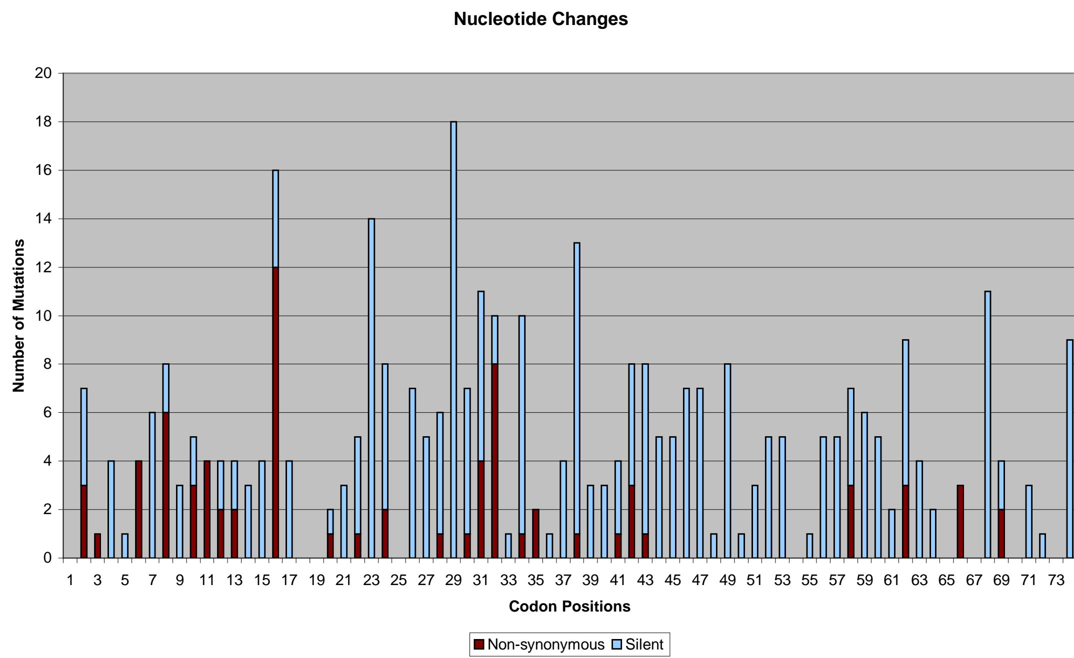


Table 6.6. Table of amino acid changes between plasma and the different compartments

S	1a	1b	3a	CD19	1a	1b	3a	CD14	1a	1b	3a	CD2	1a	1b	3a	CD45	1a	1b	3a
1				1				1				1				1			
2				2		I for Y (N15)		2				2				2		I for Y (N15)	Y for F (38)
3				3				3		Q for L (50)		3				3			
4				4				4				4				4			
5				5				5				5				5			
6				6		H for D (68)		6		H for D (68)		6		H for D (68)		6		H for D (68)	
7				7				7				7				7			
8		P for A (40)		8		P for A (40)		8		P for A (40); A for D (50)		8		P for A (40)		8		P for A (40)	
9				9				9				9				9			
10				10		E for G (29); K for E (41)		10				10	Q for H (33)			10			
11				11		P for A (16)		11		P for A (16)		11		P for A (16)		11		P for A (16)	
12				12				12				12		R for K (56)		12		R for K (56)	
13				13				13				13		Q for L (59)		13			
14				14				14				14				14			
15				15				15				15				15			
16		R for K (15)		16		K for R (22); R for K (50)		16		K for R (22, 56); R for W (44); R for K (66)		16		K for R (56); R for K (66)		16		K for R (56); R for K (66, 67)	
17				17				17				17				17			
18				18				18				18				18			
19				19				19				19				19			
20				20				20		E for K (N3)		20				20			
21				21				21				21				21			
22				22				22				22		L for P (34)		22			
23				23				23				23				23			
24				24		V for I (N13)		24				24				24		V for I (N13)	
25				25				25				25				25			
26				26				26				26				26			
27				27				27				27				27			
28		M for L (15)		28				28				28				28			
29				29				29				29				29			
30				30	N for S (57)			30				30				30			
31				31		P for S (68)		31		P for S (68)		31		P for S (68)		31		P for S (68)	
32				32		R for K (17)		32		R for K (17)		32		R for K (17)		32		R for K (17)	
33				33				33				33				33			
34				34				34		Q for H (44)		34				34			
35				35				35		N for S (N12), N for T (56)		35				35			
36				36				36				36				36			
37				37				37				37				37			
38				38				38		Y for N (44)		38				38			
39				39				39				39				39			
40				40				40				40				40			
41				41			C for R (53)	41				41				41			
42				42				42				42		R for P (29)		42		R for P (29)	
43				43			A for P (41)	43				43				43			
44				44				44				44				44			
45				45				45				45				45			
46				46				46				46				46			
47				47				47				47				47			
48				48				48				48				48			
49				49				49				49				49			
50				50				50				50				50			
51				51				51				51				51			
52				52				52				52				52			
53				53				53				53				53			
54				54				54				54				54			
55				55				55				55				55			
56				56				56				56				56			
57				57				57				57				57			
58				58			H for Y (30)	58				58			H for Y (30)	58			H for Y (30)
59				59				59				59				59			
60				60				60				60				60			
61				61				61				61				61			
62				62				62				62		T for S (56)		62		S for P (31); A for S (67)	
63				63				63				63				63			
64				64				64				64				64			
65				65				65				65				65			
66			R for K (10)	66		R for Q (50)		66		R for Q (41)		66				66			
67				67				67				67				67			
68				68				68				68				68			
69				69		R for K (N13)		69				69				69		R for K (N13)	
70				70				70				70				70			
71				71				71				71				71			
72				72				72				72				72			
73				73				73				73				73			
74				74				74				74				74			

6.7. Discussion

This chapter sought to establish if there are different quasispecies of HCV within plasma, saliva and peripheral blood mononuclear cells. Such knowledge would aid in establishing the source of HCV in oral fluids and indicate whether there is any notable compartmentalisation of a particular quasispecies in the mouth or salivary glands.

The biological implications of a quasispecies distribution are well established, at least for some RNA viruses. Variants of measles virus influence its virulence, and distribution within the host (Steinhauer *et al.*, 1987), while the rapid evolution of variants of Human Immunodeficiency Virus (HIV) leads to emergence of drug resistance (Rambaut *et al.*, 2004). Although this is mainly a feature of RNA viruses Hepatitis B virus (HBV), a DNA virus, can also exist as a quasispecies and can mutate under drug pressure, as it replicates through reverse transcription - which lacks proof reading - of an RNA intermediate (Chotiyaputta *et al.*, 2009). With regard to HCV, quasispecies can rapidly emerge in the early phases of infection such that quasispecies can differ by up to 7.8% (in the HVR1 region) (Herring *et al.*, 2005). Increased complexity of HVR1 sequences of quasispecies has been associated with progression from acute to chronic infection (Farci *et al.*, 2000). Compartmentalisation of HCV quasispecies in peripheral blood mononuclear cells (PBMC), serum, plasma and liver has previously been demonstrated (Vera-Otarola *et al.*, 2009). The sequences of HVR1 of HCV isolates from plasma can differ from those of isolates from CD19+ cells and monocytes, although the variants within the cells have a low rate of non-synonymous

mutation (Ducoulombier *et al.*, 2004). Quasispecies within a specific cellular compartment may be statistically more genetically alike than quasispecies from different cellular compartments (Roque Afonso *et al.*, 1999). The responses of quasispecies within plasma and PBMC to interferon therapy may differ (Fornai *et al.*, 2001) and the complexity of quasispecies in liver may differ from those in plasma (Cabot *et al.*, 2000). Iatrogenic immunosupression following liver transplantation and HIV-related immunodeficiency (Fishman *et al.*, 2009) may reduce quasispecies diversity suggesting that immune pressure may influence quasispecies diversification. However there is no consistent pattern of association between the diversity and complexity of e.g. HVR1 quasispecies with disease progression, risk of cirrhosis and hepatocellular carcinoma. Treatment responses may be influenced by the complexity and diversity of quasispecies (Fishman *et al.*, 2009). While there is increasing knowledge of HCV quasispecies there remain inconsistencies in the available data and at present their identification and characterisation are not part of the routine virological monitoring of this infection.

The present results indicate that there is no significant difference in HCV quasispecies of saliva when compared with plasma. This result is perhaps unexpected as so few of the patients had HCV in both saliva and plasma. Two of the 4 patients who had matched genotype 1b samples did have quasispecies that differed by more than one amino acid, thus mirroring the trend observed between plasma and all of the different PBMCs. However this does not provide any insight into the impact of the oral environment upon HCV mutation but would simply suggest that genotype 1b is more prone to mutation than the other genotypes, regardless of the site of host residence.

In the comparison of quasispecies between plasma and PBMC the most striking, and consistent observation was the predisposition for mutations, and hence differences, in genotype 1b quasispecies. This could suggest that within PBMC (particularly CD2, CD14 and CD 45) an environment is created in which there is some selective pressure that allows emergence of stable mutations. However this presupposes that there is active HCV replication within PBMC, which as discussed in chapter 5 remains debatable. Alternatively these different cell types may favour entry of certain quasispecies but as NS5B does not code for entry proteins this notion cannot presently be supported. Since higher 1b prevalence may only reflect infections that occurred several decades ago (Oubina *et al.*, 2001), the clear trend towards nucleotide changes more likely occurring in genotype 1b samples could be related to longer infections which would result in more quasispecies .

In the present study there was no consistent mutation hotspot within any of the demonstrable quasispecies, hence it is not possible to conclude that any particular mutation favours compartmentalisation within peripheral blood cells, plasma or saliva. This does not entirely exclude the possibility that HCV may compartmentalise in oral tissues as the present study only utilized the NS5B region. This region was chosen for the present study as it is a short sequence (273bp) that is applicable for molecular epidemiological studies but in hindsight may not be appropriate for tropism studies. Of note however NS5B is important in the RNA polymerase of HCV and mutations within this region may be of relevance to resistance to ribavirin and newer agents such as polymerase inhibitors (Chow *et al.*, 2010). Hipervariable region 1 (HVR-1)

would be an interesting HCV region to be studied with the compartmentalization approach of the current work, instead of NS5b as it has the potential to present more mutations than the region studied, which presents a higher rate of genetic stability (Blackard *et al.*, 2007, Briat *et al.*, 2005, Ducoulombier *et al.*, 2004, Harris *et al.*, 2001, Herring *et al.*, 2005, Kato *et al.*, 1995, Kumagai *et al.*, 2007, Moreau *et al.*, 2008, Nainan *et al.*, 2006, Roque Afonso *et al.*, 1999, Schramm *et al.*, 2008, Toyoda *et al.*, 1999, Zehender *et al.*, 2005).

Chapter 7

Concluding discussion

This thesis sought to determine the frequency and virological character of HCV within whole saliva in a group of patients from Northern Brazil. The present findings indicate that salivary carriage of HCV in this population is low and that such carriage is not influenced by local oral mucosal disease or HCV treatment status (chapter 3) but reflects the presence of HCV within peripheral blood mononuclear cells and plasma (chapter 4). Furthermore HCV isolates from whole saliva are genotypically identical to those of peripheral blood mononuclear cells and plasma (chapter 5) and there is no striking quasispecies phenomenon associated with whole saliva and hence the oral cavity (chapter 6).

The present findings thus suggest that saliva is an unlikely vehicle for the transmission of HCV (as reflected in the overall epidemiology of HCV disease) and that the mouth is not a frequent site of extra-hepatic replication of HCV. In particular the lack of any quasispecies unique to the mouth would indicate that, despite HCV negative strands being observed in oral epithelium, the oral mucosa and salivary glands are unlikely sites of local HCV replication and hence not tissues that will be easily spared from the effects of anti-HCV therapy.

However some caution must be placed upon entirely ruling out the mouth, or oral fluids, as being a possible source of transmission of HCV. As evidenced in chapter 1 and the introductions of chapters 3 and 4 there have been instances of possible intrafamilial HCV transmission and the HCV negative strand may be present within oral epithelial cells, hence salivary transmission of HCV must be possible, all be it very rare.

A striking feature of the present group of patients is the lack of evidence of a significant difference in hepatic function between the patients treated and not treated with appropriate HCV therapy. The treated patients did not reach a sustained virological response as evidenced by the presence of HCV in plasma. Of course the hepatic function and HCV load of the treated patients prior to therapy is not known, nevertheless this lack of response (possibly reflecting (unproven) variable compliance to therapy) does not detract from the overall conclusions as the trends observed in the treated patients were also seen in the untreated individuals.

The lack of evidence of quasispecies unique to the mouth might reflect a lack of samples (as a consequence of the low frequency of HCV in oral fluids) and perhaps the use of a larger panel of HCV-infected whole saliva samples might prove definitively that the oral epithelium and/or salivary glands do not promote quasispecies emergence. Nevertheless based upon the present evidence quasispecies of oral origin are unlikely to be of relevance to the clinical presentation, hepatic function or viral carriage of HCV.

It is presently concluded that, at least in a cohort of patients resident in Northern Brazil, oral carriage of HCV is uncommon and that the virological character of the virus in whole saliva is determined by that within plasma and peripheral blood mononuclear cells. While salivary antibodies to HCV may be a useful means of identifying infected individuals, detailed virological assessment for HCV within oral fluids will not reliably indicate the haematological and hepatic HCV status of patients with likely HCV disease.

There is no evidence for HCV compartmentalization as sequences from different compartments were closely related, although mutations were identified more frequently within genotype 1b in all compartments. This finding suggest a new trend towards hepatitis C evolution, as genotypes 1a and 1b do not usually differenciate in terms of treatment outcome, being both traditionally related to poor antiviral response.

References

Abe K, Inchauspe G (1991) Transmission of hepatitis C by saliva. *Lancet.,* **337,** 248.

Abe K, Kurata T, Shikata T, Sugitani M, Oda T (1987) Experimental transmission of non-A, non-B hepatitis by saliva. *J.Infect.Dis.,* **155,** 1078-1079.

Afdhal NH, McHutchison JG, Zeuzem S*, et al.* (2011) Hepatitis C pharmacogenetics: state of the art in 2010. *Hepatology,* **53,** 336-345.

Aguilar MS, Cosson C, Loureiro CL*, et al.* (2001) Prevalence of infection with hepatitis C virus in Venezuela, as assessed with an immuno-assay based on synthetic peptides. *Ann.Trop.Med.Parasitol.,* **95,** 187-195.

Aguilera GA, Romero YS, Regueiro BJ (2006) [Epidemiology and clinical manifestations of viral hepatitis]. *Enferm.Infecc.Microbiol.Clin.,* **24,** 264-276.

Ahmadian A, Ehn M, Hober S (2006) Pyrosequencing: history, biochemistry and future. *Clin.Chim.Acta.,* **363,** 83-94.

Akuta N, Suzuki F, Kawamura Y*, et al.* (2007) Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. *J.Hepatol.,* **46,** 403-410.

Almroth G, Ekermo B, Akerlind B, Mansson AS, Widell A (2010) Monitoring hepatitis C infection in a major Swedish nephrology unit and molecular resolution of a new case of nosocomial transmission. *J Med Virol,* **82,** 249-256.

Amorim RM, Raiol T, Trevizoli JE, Neves FA, Martins CR, Martins RM (2010) Hepatitis C virus genotypes in hemodialysis patients in the Federal District, Brazil. *Rev Inst Med Trop Sao Paulo,* **52,** 57-60.

Amorosa VK (2010) New frontiers of HCV therapy in HIV/HCV co-infection. *Curr HIV/AIDS Rep,* **7,** 117-126.

Antonishyn NA, Ast VM, McDonald RR, Chaudhary RK, Lin L, Andonov AP, Horsman GB (2005) Rapid genotyping of hepatitis C virus by primer-specific extension analysis. *J.Clin.Microbiol.,* **43,** 5158-5163.

Arrieta JJ, Rodriguez-Inigo E, Ortiz-Movilla N*, et al.* (2001) In situ detection of hepatitis C virus RNA in salivary glands. *Am.J.Pathol.,* **158,** 259-264.

Attstrom R, Egelberg J (1970) Emigration of blood neutrophils and monocytes into the gingival crevices. *J.Periodontal Res.,* **5,** 48-55.

Azzari C, Moriondo M, Indolfi G, Betti L, Gambineri E, de MM, Resti M (2008) Higher risk of hepatitis C virus perinatal transmission from drug user mothers is mediated by peripheral blood mononuclear cell infection. *J.Med.Virol.,* **80,** 65-71.

Azzari C, Resti M, Moriondo M, Ferrari R, Lionetti P, Vierucci A (2000) Vertical transmission of HCV is related to maternal peripheral blood mononuclear cell infection. *Blood,* **96,** 2045-2048.

Bagaglio S, Cinque P, Racca S, Pedale R, Grasso MA, Lazzarin A, Morsica G (2005) Hepatitis C virus populations in the plasma, peripheral blood mononuclear cells and cerebrospinal fluid of HIV/hepatitis C virus-co-infected patients. *AIDS,* **19 Suppl 3,** S151-S165.

Bakr I, Rekacewicz C, El HM, *et al.* (2006) Higher clearance of HCV infection in females compared to males. *Gut.,* **.**

Bare P, Massud I, Parodi C, *et al.* (2005) Continuous release of hepatitis C virus (HCV) by peripheral blood mononuclear cells and B-lymphoblastoid cell-line cultures derived from HCV-infected patients. *J.Gen.Virol.,* **86,** 1717-1727.

Barria MI, Vera-Otarola J, Leon U, *et al.* (2008) Influence of extrahepatic viral infection on the natural history of hepatitis C. *Ann.Hepatol.,* **7,** 136-143.

Bartolome J, Lopez-Alcorocho JM, Castillo I, Rodriguez-Inigo E, Quiroga JA, Palacios R, Carreno V (2007) Ultracentrifugation of serum samples allows detection of hepatitis C virus RNA in patients with occult hepatitis C. *J.Virol.,* **81,** 7710-7715.

Bartosch B, Vitelli A, Granier C, *et al.* (2003) Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. *J.Biol.Chem.,* **278,** 41624-41630.

Bassit L, Ribeiro-Dos-Santos G, Da Silva LC, *et al.* (1999) Genotype distributions of hepatitis C virus in Sao Paulo, Brazil: rare subtype found. *Hepatology,* **29,** 994-995.

Becheur H, Harzic M, Colardelle P, *et al.* (2000) [Hepatitis C virus contamination of endoscopes and biopsy forceps]. *Gastroenterol.Clin.Biol.,* **24,** 906-910.

Bedossa P, Poynard T (1996) An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology,* **24,** 289-293.

Belec L, Legoff J, Si-Mohamed A, Bloch F, Matta M, Mbopi-Keou FX, Payan C (2003a) Cell-associated, non-replicating strand(+) hepatitis C virus-RNA shedding in cervicovaginal secretions from chronically HCV-infected women. *J.Clin.Virol.,* **27,** 247-251.

Belec L, Legoff J, Si-Mohamed A, *et al.* (2003b) Mucosal humoral immune response to hepatitis C virus E1/E2 surface glycoproteins and HCV shedding in saliva and cervicovaginal fluids from chronically HCV-infected patients. *J.Hepatol.,* **38,** 833-842.

Berenguer M, Olaso V, Cordoba J, Gobernado M, Carrasco D, Berenguer J (1995) Genome detection in liver and peripheral blood mononuclear cells: predictor factors of sustained response in patients with chronic hepatitis C. *Eur J Gastroenterol Hepatol,* **7,** 899-903.

Bernardin F, Tobler L, Walsh I, Williams JD, Busch M, Delwart E (2008) Clearance of hepatitis C virus RNA from the peripheral blood mononuclear cells of blood donors who spontaneously or therapeutically control their plasma viremia. *Hepatology,* **47,** 1446-1452.

Bezerra CS, Lima JMC, Vilar JL, Moreira JLB, Frota CC (2007), Vol. 38, pp. 656-661.

Biasi D, Colombari R, Achille A, Carletto A, Caramashi P, Corrocher R, Bambara LM (1995) HCV RNA detection in parotid gland biopsy in a patient with chronic hepatitis C virus liver disease. *Acta Gastroenterol.Belg.,* **58,** 465-469.

Blackard JT, Hiasa Y, Smeaton L, Jamieson DJ, Rodriguez I, Mayer KH, Chung RT (2007) Compartmentalization of hepatitis C virus (HCV) during HCV/HIV coinfection. *J.Infect.Dis.,* **195,** 1765-1773.

Blackard JT, Smeaton L, Hiasa Y*, et al.* (2005) Detection of hepatitis C virus (HCV) in serum and peripheral-blood mononuclear cells from HCV-monoinfected and HIV/HCV-coinfected persons. *J Infect Dis,* **192,** 258-265.

Boisvert J, He XS, Cheung R, Keeffe EB, Wright T, Greenberg HB (2001) Quantitative analysis of hepatitis C virus in peripheral blood and liver: replication detected only in liver. *J.Infect.Dis.,* **184,** 827-835.

Bouchardeau F, Cantaloube JF, Chevaliez S*, et al.* (2007) Improvement of hepatitis C virus (HCV) genotype determination with the new version of the INNO-LiPA HCV assay. *J.Clin.Microbiol.,* **45,** 1140-1145.

Bouffard P, Hayashi PH, Acevedo R, Levy N, Zeldis JB (1992) Hepatitis C virus is detected in a monocyte/macrophage subpopulation of peripheral blood mononuclear cells of infected patients. *J.Infect.Dis.,* **166,** 1276-1280.

Bren L (2001) Hepatitis C. An update. *FDA.Consum.,* **35,** 24-29.

Briat A, Dulioust E, Galimand J*, et al.* (2005) Hepatitis C virus in the semen of men coinfected with HIV-1: prevalence and origin. *AIDS,* **19,** 1827-1835.

Brok J, Gluud LL, Gluud C (2006) Ribavirin monotherapy for chronic hepatitis C infection: a Cochrane Hepato-Biliary Group systematic review and meta-analysis of randomized trials. *Am.J.Gastroenterol.,* **101,** 842-847.

Bronowicki JP, Loriot MA, Thiers V, Grignon Y, Zignego AL, Brechot C (1998) Hepatitis C virus persistence in human hematopoietic cells injected into SCID mice. *Hepatology.,* **28,** 211-218.

Buckton AJ, Ngui SL, Arnold C*, et al.* (2006) Multitypic hepatitis C virus infection identified by real-time nucleotide sequencing of minority genotypes. *J.Clin.Microbiol.,* **44,** 2779-2784.

Bull ME, Learn GH, Mullins JI, Frenkel LM (2007) Methods used to examine compartmentalization of viral populations between the genital tract and peripheral blood. *J.Infect.Dis.,* **196,** 493-494.

Cabot B, Martell M, Esteban JI*, et al.* (2000) Nucleotide and amino acid complexity of hepatitis C virus quasispecies in serum and liver. *J Virol,* **74,** 805-811.

Caldwell SH, Sue M, Bowden JH*, et al.* (1996) Hepatitis C virus in body fluids after liver transplantation. *Liver Transpl.Surg.,* **2,** 124-129.

Campiotto S, Pinho JR, Carrilho FJ*, et al.* (2005) Geographic distribution of hepatitis C virus genotypes in Brazil. *Braz.J.Med.Biol.Res.,* **38,** 41-49.

Carreno V, Pardo M, Lopez-Alcorocho JM, Rodriguez-Inigo E, Bartolome J, Castillo I (2006) Detection of hepatitis C virus (HCV) RNA in the liver of healthy, anti-HCV antibody-positive, serum HCV RNA-negative patients with normal alanine aminotransferase levels. *J Infect Dis,* **194,** 53-60.

Carrozzo M (2008) Oral diseases associated with hepatitis C virus infection. Part 1. sialadenitis and salivary glands lymphoma. *Oral Dis.,* **14,** 123-130.

Carrozzo M, Brancatello F, Dametto E*, et al.* (2005) Hepatitis C virus-associated oral lichen planus: is the geographical heterogeneity related to HLA-DR6? *J.Oral Pathol.Med.,* **34,** 204-208.

Carrozzo M, Gandolfo S (2003) Oral diseases possibly associated with hepatitis C virus. *Crit Rev.Oral Biol.Med.,* **14,** 115-127.

Carrozzo M, Gandolfo S, Carbone M, Colombatto P, Broccoletti R, Garzino-Demo P, Ghisetti V (1996) Hepatitis C virus infection in Italian patients

with oral lichen planus: a prospective case-control study. *J.Oral Pathol.Med.,* **25,** 527-533.
Carrozzo M, Quadri R, Latorre P*, et al.* (2002) Molecular evidence that the hepatitis C virus replicates in the oral mucosa. *J.Hepatol.,* **37,** 364-369.
Castillo I, Rodriguez-Inigo E, Bartolome J*, et al.* (2005) Hepatitis C virus replicates in peripheral blood mononuclear cells of patients with occult hepatitis C virus infection. *Gut.,* **54,** 682-685.
Cavalheiro NdP, De La Rosa A, Elagin S, Tengan FM, Araujo ES, Barone AA (2009) Hepatitis C: sexual or intrafamilial transmission? Epidemiological and phylogenetic analysis of hepatitis C virus in 24 infected couples. *Rev Soc Bras Med Trop,* **42,** 239-244.
Cavalheiro NdP, Filgueiras TC, Melo CE, Morimitsu SR, de Araujo ES, Tengan FM, Barone AA (2007) Detection of HCV by PCR in serum and PBMC of patients with hepatitis C after treatment. *Braz.J.Infect.Dis.,* **11,** 471-474.
Cha TA, Kolberg J, Irvine B*, et al.* (1991) Use of a signature nucleotide sequence of hepatitis C virus for detection of viral RNA in human serum and plasma. *J.Clin.Microbiol.,* **29,** 2528-2534.
Chambers TJ, Hahn CS, Galler R, Rice CM (1990) Flavivirus genome organization, expression, and replication. *Annu.Rev.Microbiol.,* **44:649-88.,** 649-688.
Champion JK, Taylor A, Hutchinson S, Cameron S, McMenamin J, Mitchell A, Goldberg D (2004) Incidence of hepatitis C virus infection and associated risk factors among Scottish prison inmates: a cohort study. *Am.J.Epidemiol.,* **159,** 514-519.
Chang TT, Young KC, Yang YJ, Lei HY, Wu HL (1996) Hepatitis C virus RNA in peripheral blood mononuclear cells: comparing acute and chronic hepatitis C virus infection. *Hepatology,* **23,** 977-981.
Chatzipantazi P, Roy KM, Cameron SO, Goldberg D, Welbury R, Bagg J (2004) The feasibility and acceptability of collecting oral fluid from healthy children for anti-HCV testing. *Arch.Dis.Child.,* **89,** 185-187.
Chen M, Yun ZB, Sallberg M, Schvarcz R, Bergquist I, Berglund HB, Sonnerborg A (1995) Detection of hepatitis C virus RNA in the cell fraction of saliva before and after oral surgery. *J.Med.Virol.,* **45,** 223-226.
Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989) Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science.,* **244,** 359-362.
Choo QL, Richman KH, Han JH*, et al.* (1991) Genetic organization and diversity of the hepatitis C virus. *Proc.Natl.Acad.Sci.U.S.A.,* **88,** 2451-2455.
Chotiyaputta W, Lok AS (2009) Hepatitis B virus variants. *Nat Rev Gastroenterol Hepatol,* **6,** 453-462.
Chow J, Liu Y, Comstock K*, et al.* (2010) Isolation and identification of ester impurities in RG7128, an HCV polymerase inhibitor. *J Pharm Biomed Anal,* **53,** 710-716.
Chung RT (2006) Assessment of efficacy of treatment in HCV: infection and disease. *J.Hepatol.,* **44,** S56-S59.
Cocquerel L, Voisset C, Dubuisson J (2006) Hepatitis C virus entry: potential receptors and their biological functions. *J.Gen.Virol.,* **87,** 1075-1084.

Couzigou P, Richard L, Dumas F, Schouler L, Fleury H (1993) Detection of HCV-RNA in saliva of patients with chronic hepatitis C. *Gut,* **34,** S59-S60.

Cribier B, Schmitt C, Bingen A, Kirn A, Keller F (1995) In vitro infection of peripheral blood mononuclear cells by hepatitis C virus. *J.Gen.Virol.,* **76,** 2485-2491.

Cummins AJ, Tedder RS (1992) Inadequate information on needlestick accidents. *Lancet,* **339,** 1178-1179.

Cunha KS, Manso AC, Cardoso AS, Paixao JB, Coelho HS, Torres SR (2005) Prevalence of oral lichen planus in Brazilian patients with HCV infection. *Oral Surg.Oral Med.Oral Pathol.Oral Radiol.Endod.,* **100,** 330-333.

Cusumano AM, Poratto F, del PN, Fernandez JL, Vilches A (2005) Identification of hepatitis C virus RNA in peritoneal dialysis fluid of patients with viremia. *Perit.Dial.Int.,* **25,** 478-482.

De Clercq E (2001) Antiviral drugs: current state of the art. *J.Clin.Virol.,* **22,** 73-89.

De Luca M, Ascione A, Vacca C, Zarone A (1992) Are health-care workers really at risk of HCV infection? *Lancet,* **339,** 1364-1365.

De Maddalena C, Zehender G, Bianchi BA, Monti G, Monteverde A, Invernizzi F, Galli M (1995) HCV-RNA detection using different PCR methods in sera, cryoglobulins and peripheral blood mononuclear cells of patients with mixed cryoglobulinemia. *Clin.Exp.Rheumatol.,* **13 Suppl 13,** S119-S122.

De Mitri MS, Cassini R, Bagaglio S, Morsica G, Andreone P, Marino N, Bernardi M (2007) Evolution of hepatitis C virus non-structural 5A gene in the progression of liver disease to hepatocellular carcinoma. *Liver Int,* **27,** 1126-1133.

De Sanjose S, Benavente Y, Vajdic CM*, et al.* (2008) Hepatitis C and non-Hodgkin lymphoma among 4784 cases and 6269 controls from the International Lymphoma Epidemiology Consortium. *Clin.Gastroenterol.Hepatol.,* **6,** 451-458.

Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ (1994) Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology,* **19,** 1513-1520.

Di Liberto G, Roque-Afonso AM, Kara R, Ducoulombier D, Fallot G, Samuel D, Feray C (2006) Clinical and therapeutic implications of hepatitis C virus compartmentalization. *Gastroenterology.,* **131,** 76-84.

Di Tommaso L, Macchia S, Morandi L, Leoncini S, Pession A, Dal Monte PR, Foschini MP (2003) Correlation between histologic staging, hepatitis C virus genotypes and clinical features in HCV chronic hepatitis: evidence of a new pattern. *Int.J.Surg.Pathol.,* **11,** 197-204.

Diz DP, Castro A, Rodriguez I, Reforma NG, Castro M, Eirea M, Hermida M (2005) HCV clearance patterns in saliva and serum of patients with chronic HCV infection under interferon plus ribavirin therapy. *J.Oral Pathol.Med.,* **34,** 308-311.

Domingo E, Gomez J (2007) Quasispecies and its impact on viral hepatitis. *Virus Res.,* **127,** 131-150.

Dougan S, Balogun MA, Elford J, Brant LJ, Sinka K, Evans BG, Ramsay ME (2007) Can current national surveillance systems in England and Wales

monitor sexual transmission of hepatitis C among HIV-infected men who have sex with men? *BMC.Public Health.,* **7:7.,** 7.

Dubuisson J, Hsu HH, Cheung RC, Greenberg HB, Russell DG, Rice CM (1994) Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. *J.Virol.,* **68,** 6147-6160.

Ducoulombier D, Roque-Afonso AM, Di LG*, et al.* (2004) Frequent compartmentalization of hepatitis C virus variants in circulating B cells and monocytes. *Hepatology.,* **39,** 817-825.

Dusheiko GM, Smith M, Scheuer PJ (1990) Hepatitis C virus transmitted by human bite. *Lancet.,* **336,** 503-504.

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

Eirea M, Dios PD, Hermida M, Rodriguez I, Castro A, Ocampo A (2005) Detection of HCV-RNA in saliva of HIV-HCV coinfected patients. *AIDS Res.Hum.Retroviruses,* **21,** 1011-1015.

el Kabir M, Scully C, Porter S, Porter K, Macnamara E (1993) Liver function in UK patients with oral lichen planus. *Clin.Exp.Dermatol.,* **18,** 12-16.

Elahi E, Pourmand N, Chaung R*, et al.* (2003) Determination of hepatitis C virus genotype by Pyrosequencing. *J.Virol.Methods.,* **109,** 171-176.

Esteban JI, Sauleda S, Quer J (2008) The changing epidemiology of hepatitis C virus infection in Europe. *J Hepatol,* **48,** 148-162.

Everhart JE, Di Bisceglie AM, Murray LM, Alter HJ, Melpolder JJ, Kuo G, Hoofnagle JH (1990) Risk for non-A, non-B (type C) hepatitis through sexual or household contact with chronic carriers. *Ann.Intern.Med.,* **112,** 544-545.

Fabris P, Infantolino D, Biasin MR*, et al.* (1999) High prevalence of HCV-RNA in the saliva cell fraction of patients with chronic hepatitis C but no evidence of HCV transmission among sexual partners. *Infection,* **27,** 86-91.

Fan X, Mao Q, Zhou D*, et al.* (2009) High diversity of hepatitis C viral quasispecies is associated with early virological response in patients undergoing antiviral therapy. *Hepatology,* **50,** 1765-1772.

Farajzadeh S, Shakibi MR, Moghaddam SD, Rahnama Z (2005) Behcet disease: clinical spectrum and association with hepatitis B and C viruses. *East Mediterr.Health J.,* **11,** 68-72.

Farci P, Shimoda A, Coiana A*, et al.* (2000) The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. *Science,* **288,** 339-344.

Feitelson MA (2006) *Hepatitis C Virus From Laboratory to Clinic.* Cambridge University Press.

Feld JJ, Hoofnagle JH (2005) Mechanism of action of interferon and ribavirin in treatment of hepatitis C. *Nature.,* **436,** 967-972.

Femiano F, Scully C (2005) Functions of the cytokines in relation oral lichen planus-hepatitis C. *Med.Oral Patol.Oral Cir.Bucal.,* **10 Suppl 1:E40-4.,** E40-E44.

Ferguson DB (1987) Current diagnostic uses of saliva. *J.Dent.Res.,* **66,** 420-424.

Fernandez A, Vazquez S, Rodriguez-Gonzalez L (2008) Tongue hyperpigmentation resulting from peginterferon alfa-2a and ribavirin treatment in a Caucasian patient with chronic hepatitis C. *J.Eur.Acad.Dermatol.Venereol.,* **.**

Fernandez JL, Valtuille R, Butera H, Fay F, Lef L, Rendo P (2004) Influence of hemodialysis procedure on HCV RNA detection in serum and peripheral blood mononuclear cells. *Ren Fail,* **26,** 369-373.

Ferrari C (2008) Therapeutic vaccination for hepatitis C: can protective T-cell responses be restored after prolonged antigen exposure? *Gastroenterology.,* **134,** 1601-1604.

Ferreiro MC, Dios PD, Scully C (2005) Transmission of hepatitis C virus by saliva? *Oral Dis.,* **11,** 230-235.

Figueiredo JF, Borges AS, Martinez R, Martinelli AL, Villanova MG, Covas DT, Passas AD (1994) Transmission of hepatitis C virus but not human immunodeficiency virus type 1 by a human bite. *Clin.Infect.Dis.,* **19,** 546-547.

Fink FM, Hocker-Schulz S, Mor W*, et al.* (1993) Association of hepatitis C virus infection with chronic liver disease in paediatric cancer patients. *Eur.J.Pediatr.,* **152,** 490-492.

Fishman SL, Branch AD (2009) The quasispecies nature and biological implications of the hepatitis C virus. *Infect Genet Evol,* **9,** 1158-1167.

Fleischmann M, Celerier P, Bernard P, Litoux P, Dreno B (1996) Long-term interferon-alpha therapy induces autoantibodies against epidermis. *Dermatology.,* **192,** 50-55.

Fornai C, Maggi F, Favilli F*, et al.* (2001) Rapid changes in hepatitis C virus quasispecies produced by a single dose of IFN-alpha in chronically infected patients. *J Interferon Cytokine Res,* **21,** 417-422.

Forns X, Bukh J (1999) The molecular biology of hepatitis C virus. Genotypes and quasispecies. *Clin.Liver Dis.,* **3,** 693-716, vii.

Freeman AJ, Marinos G, Ffrench RA, Lloyd AR (2001) Immunopathogenesis of hepatitis C virus infection. *Immunol.Cell Biol.,* **79,** 515-536.

Fried MW, Shindo M, Fong TL, Fox PC, Hoofnagle JH, Di Bisceglie AM (1992) Absence of hepatitis C viral RNA from saliva and semen of patients with chronic hepatitis C. *Gastroenterology.,* **102,** 1306-1308.

Furione M, Simoncini L, Gatti M, Baldanti F, Grazia RM, Gerna G (1999) HCV genotyping by three methods: analysis of discordant results based on sequencing. *J.Clin.Virol.,* **13,** 121-130.

Gabrielli A, Manzin A, Candela M, Caniglia ML, Paolucci S, Danieli MG, Clementi M (1994) Active hepatitis C virus infection in bone marrow and peripheral blood mononuclear cells from patients with mixed cryoglobulinaemia. *Clin.Exp.Immunol.,* **97,** 87-93.

Gaeta GB, Precone DF, Cozzi-Lepri A, Cicconi P, D'Arminio MA (2006) Multiple viral infections. *J.Hepatol.,* **44,** S108-S113.

Gallegos-Orozco JF, Rakela J, Rosati MJ, Vargas HE, Balan V (2008) Persistence of hepatitis C virus in peripheral blood mononuclear cells of sustained viral responders to pegylated interferon and ribavirin therapy. *Dig.Dis.Sci.,* **53,** 2564-2568.

Gameiro R, Venneno T, Prieto E, Castro R, Carnall V, Canas-Ferreira W, Exposto F (2001) Vaginal shedding of hepatitis C virus. *Int.J.STD AIDS,* **12,** 717-721.

Garcia F, Jr., Garcia F, Roldan C, *et al.* (2000) Detection of HCV and GBV-CHGV RNA in peripheral blood mononuclear cells of patients with chronic type C hepatitis. *Microbios,* **103,** 7-15.
Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K (2010) Development of monocytes, macrophages, and dendritic cells. *Science.,* **327,** 656-661.
Germani G, Hytiroglou P, Fotiadu A, Burroughs AK, Dhillon AP (2011) Assessment of fibrosis and cirrhosis in liver biopsies: an update. *Semin Liver Dis,* **31,** 82-90.
Gil B, Qian C, Riezu-Boj JI, Civeira MP, Prieto J (1993) Hepatic and extrahepatic HCV RNA strands in chronic hepatitis C: different patterns of response to interferon treatment. *Hepatology,* **18,** 1050-1054.
Goncalves PL, Cunha CB, Busek SC, Oliveira GC, Ribeiro-Rodrigues R, Pereira FE (2005) Detection of hepatitis C virus RNA in saliva samples from patients with seric anti-HCV antibodies. *Braz.J.Infect.Dis.,* **9,** 28-34.
Guglietta S, Garbuglia AR, Salichos L, *et al.* (2009) Impact of viral selected mutations on T cell mediated immunity in chronically evolving and self limiting acute HCV infection. *Virology,* **386,** 398-406.
Guijarro GB, Lopez Sanchez AF, Hernandez VG (2001) Presence of lichen planus during a course of interferon alpha-2a therapy for a viral chronic C hepatitis. *Med.Oral.,* **6,** 358-363.
Guillermo N, Hernandez Santana J, Borrego L, Jorge M (2009) [Lichenoid eruption in a patient with chronic hepatitis C virus infection treated with interferon and ribavirin]. *Gastroenterol Hepatol,* **32,** 130-131.
Gumber SC, Chopra S (1995) Hepatitis C: a multifaceted disease. Review of extrahepatic manifestations. *Ann.Intern.Med.,* **123,** 615-620.
Gurguta C, Kauer C, Bergholz U, Formann E, Steindl-Munda P, Ferenci P (2006) Tongue and Skin Hyperpigmentation During PEG-Interferon-alpha/Ribavirin Therapy in Dark-Skinned Non-Caucasian Patients with Chronic Hepatitis C. *Am.J.Gastroenterol.,* **101,** 197-198.
Gutelius B, Perz JF, Parker MM, *et al.* (2010) Multiple clusters of hepatitis virus infections associated with anesthesia for outpatient endoscopy procedures. *Gastroenterology,* **139,** 163-170.
Haddad J, Deny P, Munz-Gotheil C, *et al.* (1992) Lymphocytic sialadenitis of Sjogren's syndrome associated with chronic hepatitis C virus liver disease. *Lancet.,* **339,** 321-323.
Harle JR, Swiader L, Disdier P, Gerolami V, Cartouzou G, Weiller J (1993) [Identifying hepatitis C virus by the gene amplification technique in the saliva of patients for whom the search is simultaneously negative in their serum: 9 cases]. *Rev.Med.Interne,* **14,** 1005.
Harris KA, Teo CG (2001) Diversity of hepatitis C virus quasispecies evaluated by denaturing gradient gel electrophoresis. *Clin.Diagn.Lab Immunol.,* **8,** 62-73.
Heathcote J, Main J (2005) Treatment of hepatitis C. *J.Viral Hepat.,* **12,** 223-235.
Helle F, Dubuisson J (2008) Hepatitis C virus entry into host cells. *Cell Mol.Life Sci.,* **65,** 100-112.
Hermida M, Ferreiro MC, Barral S, Laredo R, Castro A, Diz DP (2002) Detection of HCV RNA in saliva of patients with hepatitis C virus infection by using a highly sensitive test. *J.Virol.Methods,* **101,** 29-35.

Herring BL, Tsui R, Peddada L, Busch M, Delwart EL (2005) Wide range of quasispecies diversity during primary hepatitis C virus infection. *J Virol,* **79,** 4340-4346.

Hnatyszyn HJ (2005) Chronic hepatitis C and genotyping: the clinical significance of determining HCV genotypes. *Antivir.Ther.,* **10,** 1-11.

Hou CH, Chen WY, Kao JH*, et al.* (1995) Intrafamilial transmission of hepatitis C virus in hemodialysis patients. *J.Med.Virol.,* **45,** 381-385.

Houghton M (2009) The long and winding road leading to the identification of the hepatitis C virus. *J.Hepatol.,* **51,** 939-948.

Hrachovec J, Patel P (2003) Weight-based ribavirin with pegylated interferon alfa 2b: benefits, risks, and the origin of the evidence. *Gastroenterology.,* **124,** 2003-2004.

Hsu HH, Wright TL, Luba D, Martin M, Feinstone SM, Garcia G, Greenberg HB (1991) Failure to detect hepatitis C virus genome in human secretions with the polymerase chain reaction. *Hepatology,* **14,** 763-767.

Hughes CA, Shafran SD (2006) Chronic hepatitis C virus management: 2000-2005 update. *Ann.Pharmacother.,* **40,** 74-82.

Hugle T, Fehrmann F, Bieck E*, et al.* (2001) The hepatitis C virus nonstructural protein 4B is an integral endoplasmic reticulum membrane protein. *Virology.,* **284,** 70-81.

Hussein MM, Mooij JM (2010) Methods used to reduce the prevalence of hepatitis C in a dialysis unit. *Saudi J Kidney Dis Transpl,* **21,** 909-913.

Ibrahim HA, Baddour MM, Morsi MG, Abdelkader AA (1999) Should we routinely check for hepatitis B and C in patients with lichen planus or cutaneous vasculitis? *East Mediterr.Health J.,* **5,** 71-78.

Ilina EN, Malakhova MV, Generozov EV, Nikolaev EN, Govorun VM (2005) Matrix-assisted laser desorption ionization-time of flight (mass spectrometry) for hepatitis C virus genotyping. *J.Clin.Microbiol.,* **43,** 2810-2815.

Inokuchi M, Ito T, Uchikoshi M*, et al.* (2009) Infection of B cells with hepatitis C virus for the development of lymphoproliferative disorders in patients with chronic hepatitis C. *J.Med.Virol.,* **81,** 619-627.

Isherwood BJ, Patel AH (2005) Analysis of the processing and transmembrane topology of the E2p7 protein of hepatitis C virus. *J.Gen.Virol.,* **86,** 667-676.

Isobe K, Imoto M, Fukuda Y, Koyama Y, Nakano I, Hayakawa T, Takamatsu J (1995) Hepatitis C virus infection and genotypes in Japanese hemophiliacs. *Liver.,* **15,** 131-134.

Jaber MA, Porter SR, Bain L, Scully C (2003) Lack of association between hepatitis C virus and oral epithelial dysplasia in British patients. *Int.J.Oral Maxillofac.Surg.,* **32,** 181-183.

Jain MK, Yuan HJ, Adams-Huet B*, et al.* (2009) Pegylated interferon and ribavirin promote early evolution of nonstructural 5A protein in individuals with hepatitis C who demonstrate a response to treatment. *J Infect Dis,* **200,** 866-876.

Jardim AC, Yamasaki LH, de Queiroz AT*, et al.* (2009) Quasispecies of hepatitis C virus genotype 1 and treatment outcome with peginterferon and ribavirin. *Infect Genet Evol,* **9,** 689-698.

Jirillo E, Amati L, Caradonna L*, et al.* (1998) Soluble (s) CD14 and plasmatic lipopolysaccharides (LPS) in patients with chronic hepatitis C before

and after treatment with interferon (IFN)-alpha. *Immunopharmacol.Immunotoxicol.,* **20,** 1-14.

Jochen AB (1992) Occupationally acquired hepatitis C virus infection. *Lancet,* **339,** 304.

Jonsson JR, Purdie DM, Clouston AD, Powell EE (2008) Recognition of genetic factors influencing the progression of hepatitis C : potential for personalized therapy. *Mol Diagn Ther,* **12,** 209-218.

Jorgensen C, Legouffe MC, Perney P*, et al.* (1996) Sicca syndrome associated with hepatitis C virus infection. *Arthritis Rheum.,* **39,** 1166-1171.

Kage M, Ogasawara S, Kosai K*, et al.* (1997) Hepatitis C virus RNA present in saliva but absent in breast-milk of the hepatitis C carrier mother. *J.Gastroenterol.Hepatol.,* **12,** 518-521.

Kao JH, Chen PJ, Lai MY, Wang TH, Chen DS (1997) Positive and negative strand of hepatitis C virus RNA sequences in peripheral blood mononuclear cells in patients with chronic hepatitis C: no correlation with viral genotypes 1b, 2a, and 2b. *J.Med.Virol.,* **52,** 270-274.

Kato N, Mori K, Abe K*, et al.* (2009) Efficient replication systems for hepatitis C virus using a new human hepatoma cell line. *Virus Res,* **146,** 41-50.

Kato N, Nakazawa T, Mizutani T, Shimotohno K (1995) Susceptibility of human T-lymphotropic virus type I infected cell line MT-2 to hepatitis C virus infection. *Biochem.Biophys.Res.Commun.,* **206,** 863-869.

Kelly D, Skidmore S (2002) Hepatitis C-Z: recent advances. *Arch Dis Child,* **86,** 339-343.

Kim AI, Saab S (2005) Treatment of hepatitis C. *Am.J.Med.,* **118,** 808-815.

Klade CS, Wedemeyer H, Berg T*, et al.* (2008) Therapeutic vaccination of chronic hepatitis C nonresponder patients with the peptide vaccine IC41. *Gastroenterology.,* **134,** 1385-1395.

Klein RS, Freeman K, Taylor PE, Stevens CE (1991) Occupational risk for hepatitis C virus infection among New York City dentists. *Lancet,* **338,** 1539-1542.

Knodell RG, Ishak KG, Black WC*, et al.* (1981) Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology,* **1,** 431-435.

Koike A, Rolfs A (1990) *In PCR:Clinical Diagnostic and Research.* 11
-11 pp.

Komiyama K, Kawamura F, Arakawa Y, Mastuo H, Hayashi N, Shikata T, Moro I (1995) Detection of hepatitis C virus (HCV)-RNA in saliva and gastric juice. *Adv.Exp.Med.Biol.,* **371B,** 995-997.

Koziel MJ, Dudley D, Afdhal N, Choo QL, Houghton M, Ralston R, Walker BD (1993) Hepatitis C virus (HCV)-specific cytotoxic T lymphocytes recognize epitopes in the core and envelope proteins of HCV. *J.Virol.,* **67,** 7522-7532.

Krug LP, Lunge VR, Ikuta N, Fonseca AS, Cheinquer H, Ozaki LS, Barros SG (1996) Hepatitis C virus genotypes in Southern Brazil. *Braz.J.Med.Biol.Res.,* **29,** 1629-1632.

Kumagai N, Kaneko F, Tsunematsu S, Tsuchimoto K, Tada S, Saito H, Hibi T (2007) Complexity of the HVR-1 quasispecies and disease activity in patients with hepatitis C. *Eur J Clin Invest,* **37,** 566-572.

Kuntzen T, Timm J, Berical A*, et al.* (2008) Naturally occurring dominant resistance mutations to hepatitis C virus protease and polymerase inhibitors in treatment-naive patients. *Hepatology,* **48,** 1769-1778.

Kurauchi O, Furui T, Itakura A*, et al.* (1993) Studies on transmission of hepatitis C virus from mother-to-child in the perinatal period. *Arch.Gynecol.Obstet.,* **253,** 121-126.

Lamster IB, Ahlo JK (2007) Analysis of gingival crevicular fluid as applied to the diagnosis of oral and systemic diseases. *Ann.N.Y.Acad.Sci.,* **1098,** 216-229.

Lanford RE, Chavez D, Chisari FV, Sureau C (1995) Lack of detection of negative-strand hepatitis C virus RNA in peripheral blood mononuclear cells and other extrahepatic tissues by the highly strand-specific rTth reverse transcriptase PCR. *J.Virol.,* **69,** 8079-8083.

Laouenan C, Plancoulaine S, Mohamed MK*, et al.* (2009) Evidence for a dominant major gene conferring predisposition to hepatitis C virus infection in endemic conditions. *Hum Genet,* **126,** 697-705.

Laskus T, Radkowski M, Bednarska A*, et al.* (2002) Detection and analysis of hepatitis C virus sequences in cerebrospinal fluid. *J.Virol.,* **76,** 10064-10068.

Laskus T, Radkowski M, Wang LF, Cianciara J, Vargas H, Rakela J (1997) Hepatitis C virus negative strand RNA is not detected in peripheral blood mononuclear cells and viral sequences are identical to those in serum: a case against extrahepatic replication. *J.Gen.Virol.,* **78 (Pt 11),** 2747-2750.

Laskus T, Radkowski M, Wang LF, Nowicki M, Rakela J (2000) Uneven distribution of hepatitis C virus quasispecies in tissues from subjects with end-stage liver disease: confounding effect of viral adsorption and mounting evidence for the presence of low-level extrahepatic replication. *J.Virol.,* **74,** 1014-1017.

Lauer GM, Walker BD (2001) Hepatitis C virus infection. *N.Engl.J.Med.,* **345,** 41-52.

Le Guillou-Guillemette H, Vallet S, Gaudy-Graffin C, Payan C, Pivert A, Goudeau A, Lunel-Fabiani F (2007) Genetic diversity of the hepatitis C virus: impact and issues in the antiviral therapy. *World J Gastroenterol,* **13,** 2416-2426.

Leao JC, Teo CG, Porter SR (2006) HCV infection: aspects of epidemiology and transmission relevant to oral health care workers. *Int.J.Oral Maxillofac.Surg.,* **35,** 295-300.

Leicester KL, Olynyk JK, Brunt EM, Britton RS, Bacon BR (2006) Differential findings for CD14-positive hepatic monocytes/macrophages in primary biliary cirrhosis, chronic hepatitis C and nonalcoholic steatohepatitis. *Liver Int.,* **26,** 559-565.

Lerat H, Berby F, Trabaud MA, Vidalin O, Major M, Trepo C, Inchauspe G (1996) Specific detection of hepatitis C virus minus strand RNA in hematopoietic cells. *J.Clin.Invest,* **97,** 845-851.

Lerat H, Rumin S, Habersetzer F, Berby F, Trabaud MA, Trepo C, Inchauspe G (1998) In vivo tropism of hepatitis C virus genomic sequences in hematopoietic cells: influence of viral load, viral genotype, and cell phenotype. *Blood,* **91,** 3841-3849.

Levi JE, Takaoka DT, Garrini RH*, et al.* (2002) Three cases of infection with hepatitis C virus genotype 5 among Brazilian hepatitis patients. *J.Clin.Microbiol.,* **40,** 2645-2647.

Lim JK, Wooten D, Siegel R, Cheung RC (2005) Amantadine in treatment of chronic hepatitis C virus infection? *J.Viral Hepat.,* **12,** 445-455.

Lin C, Lindenbach BD, Pragai BM, McCourt DW, Rice CM (1994) Processing in the hepatitis C virus E2-NS2 region: identification of p7 and two distinct E2-specific products with different C termini. *J.Virol.,* **68,** 5063-5073.

Lin HJ, Shi N, Mizokami M, Hollinger FB (1992) Polymerase chain reaction assay for hepatitis C virus RNA using a single tube for reverse transcription and serial rounds of amplification with nested primer pairs. *J Med Virol,* **38,** 220-225.

Lindenbach BD, Rice CM (2005) Unravelling hepatitis C virus replication from genome to function. *Nature.,* **436,** 933-938.

Lindstrom H, Lundin M, Haggstrom S, Persson MA (2006) Mutations of the Hepatitis C virus protein NS4B on either side of the ER membrane affect the efficiency of subgenomic replicons. *Virus Res.,* **121,** 169-178.

Lins L, Almeida H, Vitvisk L, Carmo T, Parana R, Reis MG (2005) Detection of hepatitis C virus RNA in saliva is not related to oral health status or viral load. *J.Med.Virol.,* **77,** 216-220.

Liou TC, Chang TT, Young KC, Lin XZ, Lin CY, Wu HL (1992) Detection of HCV RNA in saliva, urine, seminal fluid, and ascites. *J.Med.Virol.,* **37,** 197-202.

Lodi G, Olsen I, Piattelli A, D'Amico E, Artese L, Porter SR (1997) Antibodies to epithelial components in oral lichen planus (OLP) associated with hepatitis C virus (HCV) infection. *J.Oral Pathol.Med.,* **26,** 36-39.

Lodi G, Pellicano R, Carrozzo M (2010) Hepatitis C virus infection and lichen planus: a systematic review with meta-analysis. *Oral Dis.*

Lodi G, Porter SR, Scully C (1998) Hepatitis C virus infection: Review and implications for the dentist. *Oral Surg.Oral Med.Oral Pathol.Oral Radiol.Endod.,* **86,** 8-22.

Lucidarme D, Decoster A, Delamare C*, et al.* (2003) [An inter-laboratory study of anti-HCV antibody detection in saliva samples]. *Gastroenterol.Clin.Biol.,* **27,** 159-162.

Lundin M, Lindstrom H, Gronwall C, Persson MA (2006) Dual topology of the processed hepatitis C virus protein NS4B is influenced by the NS5A protein. *J.Gen.Virol.,* **87,** 3263-3272.

Macparland SA, Pham TN, Gujar SA, Michalak TI (2006) De novo infection and propagation of wild-type Hepatitis C virus in human T lymphocytes in vitro. *J.Gen.Virol.,* **87,** 3577-3586.

Magiorkinis G, Magiorkinis E, Paraskevis D*, et al.* (2009) The global spread of hepatitis C virus 1a and 1b: a phylodynamic and phylogeographic analysis. *PLoS Med,* **6,** e1000198.

Maieron A, Metz-Gercek S, Hackl F*, et al.* (2010) Chronic hepatitis C in Austria, 1992-2006: genotype distribution and demographic factors. *Euro Surveill,* **15,** 19492.

Mandel ID (1990) The diagnostic uses of saliva. *J.Oral Pathol.Med.,* **19,** 119-125.

Mariette X, Loiseau P, Morinet F (1995) Hepatitis C virus in saliva. *Ann.Intern.Med.,* **122,** 556.

Marincovich B, Castilla J, del Romero J, Garcia S, Hernando V, Raposo M, Rodriguez C (2003) Absence of hepatitis C virus transmission in a prospective cohort of heterosexual serodiscordant couples. *Sex Transm.Infect.,* **79,** 160-162.

Martell M, Esteban JI, Quer J*, et al.* (1992) Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. *J.Virol.,* **66,** 3225-3229.

Martins RM, Barbosa AP, Oliveira JM, Vanderborght B, Yoshida CF (2000) Genotype analysis of hepatitis C virus in Brazilian hemophiliacs and blood donors. *Vox Sang.,* **78,** 255.

Martins RM, Teles SA, Freitas NR*, et al.* (2006) Distribution of hepatitis C virus genotypes among blood donors from mid-west region of Brazil. *Rev.Inst.Med.Trop.Sao Paulo,* **48,** 53-55.

Marukian S, Jones CT, Andrus L*, et al.* (2008) Cell culture-produced hepatitis C virus does not infect peripheral blood mononuclear cells. *Hepatology.*

Mastromatteo AM, Rapaccini GL, Pompili M, Ursino S, Romano-Spica V, Gasbarrini G, Vanini G (2001) Hepatitis C virus infection: other biological fluids than blood may be responsible for intrafamilial spread. *Hepatogastroenterology,* **48,** 193-196.

Maticic M, Poljak M, Kramar B*, et al.* (2001) Detection of hepatitis C virus RNA from gingival crevicular fluid and its relation to virus presence in saliva. *J.Periodontol.,* **72,** 11-16.

Maticic M, Poljak M, Seme K, Skaleric U (2003) The IgG antibody profile to various antigen regions of hepatitis C virus differs in oral fluid and serum of patients with chronic hepatitis C. *Oral Microbiol.Immunol.,* **18,** 176-182.

Matos MA, Reis NR, Kozlowski AG*, et al.* (2009) Epidemiological study of hepatitis A, B and C in the largest Afro-Brazilian isolated community. *Trans.R.Soc.Trop.Med.Hyg.,* **103,** 899-905.

Maylin S, Martinot-Peignoux M, Moucari R*, et al.* (2008) Eradication of hepatitis C virus in patients successfully treated for chronic hepatitis C. *Gastroenterology.,* **135,** 821-829.

Maynard M, Pradat P, Bailly F*, et al.* (2005) Amantadine triple therapy for non-responder hepatitis C patients. Clues for controversies (ANRS HC 03 BITRI). *J.Hepatol.,* **%20;.**

Mazur W, Mazurek U, Jurzak M, Wilczok T, Bulanowski Z, Gonciarz Z (2001) Positive and negative strands of HCV-RNA in sera and peripheral blood mononuclear cells of chronically hemodialyzed patients. *Med.Sci.Monit.,* **7,** 108-115.

Mazurek U, Wilczok T, Mazur W*, et al.* (2002) Distribution of hepatitis C virus RNA in whole blood of patients with HCV infection and HBV-HCV coinfection. *Med Sci Monit,* **8,** CR125-130.

Meier V, Mihm S, Braun WP, Ramadori G (2001) HCV-RNA positivity in peripheral blood mononuclear cells of patients with chronic HCV infection: does it really mean viral replication? *World J.Gastroenterol.,* **7,** 228-234.

Mellor J, Holmes EC, Jarvis LM, Yap PL, Simmonds P (1995) Investigation of the pattern of hepatitis C virus sequence diversity in different geographical regions: implications for virus classification. The International HCV Collaborative Study Group. *J.Gen.Virol.,* **76 (Pt 10),** 2493-2507.

Mendes-Correa MC, Cavalheiro NP, Mello C, Barone AA, Gianini RJ (2008) Genotypic distribution of hepatitis C among hepatitis C and HIV co-infected patients in Brazil. *Int.J.STD AIDS,* **19,** 595-599.

Minosse C, Calcaterra S, Abbate I, Selleri M, Zaniratti MS, Capobianchi MR (2006) Possible compartmentalization of hepatitis C viral replication in the genital tract of HIV-1-coinfected women. *J.Infect.Dis.,* **194,** 1529-1536.

Mizushima H, Hijikata M, Asabe S, Hirota M, Kimura K, Shimotohno K (1994) Two hepatitis C virus glycoprotein E2 products with different C termini. *J.Virol.,* **68,** 6215-6222.

Modi AA, Liang TJ (2008) Hepatitis C: a clinical review. *Oral Dis,* **14,** 10-14.

Molina S, Castet V, Fournier-Wirth C*, et al.* (2007) The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. *J.Hepatol.,* **46,** 411-419.

Moreau I, O'Sullivan H, Murray C, Levis J, Crosbie O, Kenny-Walsh E, Fanning LJ (2008) Separation of Hepatitis C genotype 4a into IgG-depleted and IgG-enriched fractions reveals a unique quasispecies profile. *Virol J,* **5,** 103.

Moriya T, Sasaki F, Mizui M, Ohno N, Mohri H, Mishiro S, Yoshizawa H (1995) Transmission of hepatitis C virus from mothers to infants: its frequency and risk factors revisited. *Biomed.Pharmacother.,* **49,** 59-64.

Morsica G, Bernardi MT, Novati R, Uberti FC, Castagna A, Lazzarin A (1997) Detection of hepatitis C virus genomic sequences in the cerebrospinal fluid of HIV-infected patients. *J.Med.Virol.,* **53,** 252-254.

Morsica G, Tambussi G, Sitia G, Novati R, Lazzarin A, Lopalco L, Mukenge S (1999) Replication of hepatitis C virus in B lymphocytes (CD19+). *Blood,* **94,** 1138-1139.

Muller HM, Pfaff E, Goeser T, Kallinowski B, Solbach C, Theilmann L (1993) Peripheral blood leukocytes serve as a possible extrahepatic site for hepatitis C virus replication. *J.Gen.Virol.,* **74 (Pt 4),** 669-676.

Munir S, Saleem S, Idrees M*, et al.* (2010) Hepatitis C treatment: current and future perspectives. *Virol J,* **7,** 296.

Nagao Y, Sata M, Noguchi S, Seno'o T, Kinoshita M, Kameyama T, Ueno T (2000) Detection of hepatitis C virus RNA in oral lichen planus and oral cancer tissues. *J.Oral Pathol.Med.,* **29,** 259-266.

Nainan OV, Lu L, Gao FX, Meeks E, Robertson BH, Margolis HS (2006) Selective transmission of hepatitis C virus genotypes and quasispecies in humans and experimentally infected chimpanzees. *J Gen Virol,* **87,** 83-91.

Nakano I, Imoto M, Fukuda Y, Koyama Y, Urano F, Kanda N, Isobe K (1992) Hepatitis C virus RNA in urine, saliva, and sweat. *Am.J.Gastroenterol.,* **87,** 1522.

Nakano T, Lu L, Liu P, Pybus OG (2004) Viral gene sequences reveal the variable history of hepatitis C virus infection among countries. *J.Infect.Dis.,* **190,** 1098-1108.

Navas S, Martin J, Quiroga JA, Castillo I, Carreno V (1998) Genetic diversity and tissue compartmentalization of the hepatitis C virus genome in blood mononuclear cells, liver, and serum from chronic hepatitis C patients. *J.Virol.,* **72,** 1640-1646.

Ndong-Atome GR, Njouom R, Padilla C, Bisvigou U, Makuwa M, Kazanji M (2009) Absence of intrafamilial transmission of hepatitis C virus and low

risk for sexual transmission in rural central Africa indicate a cohort effect. *J Clin Virol,* **45,** 349-353.

Neal KR, Ramsay S, Thomson BJ, Irving WL (2007) Excess mortality rates in a cohort of patients infected with the hepatitis C virus: a prospective study. *Gut.,* **56,** 1098-1104.

Nguyen MH, Keeffe EB (2005) Chronic hepatitis C: genotypes 4 to 9. *Clin.Liver Dis.,* **9,** 411-426, vi.

Noppornpanth S, Sablon E, De Nys K*, et al.* (2006) Genotyping hepatitis C viruses from Southeast Asia by a novel line probe assay that simultaneously detects core and 5' untranslated regions. *J.Clin.Microbiol.,* **44,** 3969-3974.

Nowicki MJ, Laskus T, Nikolopoulou G*, et al.* (2005) Presence of hepatitis C virus (HCV) RNA in the genital tracts of HCV/HIV-1-coinfected women. *J.Infect.Dis.,* **192,** 1557-1565.

Numata N, Ohori H, Hayakawa Y, Saitoh Y, Tsunoda A, Kanno A (1993) Demonstration of hepatitis C virus genome in saliva and urine of patients with type C hepatitis: usefulness of the single round polymerase chain reaction method for detection of the HCV genome. *J.Med.Virol.,* **41,** 120-128.

Oesterreicher C, Hammer J, Koch U, Pfeffel F, Sunder-Plassmann G, Petermann D, Muller C (1995) HBV and HCV genome in peripheral blood mononuclear cells in patients undergoing chronic hemodialysis. *Kidney Int,* **48,** 1967-1971.

Ogasawara S, Kage M, Kosai K, Shimamatsu K, Kojiro M (1993) Hepatitis C virus RNA in saliva and breastmilk of hepatitis C carrier mothers. *Lancet.,* **341,** 561.

Ogata N, Alter HJ, Miller RH, Purcell RH (1991) Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. *Proc.Natl.Acad.Sci.U.S.A.,* **88,** 3392-3396.

Ohki T, Tateishi R, Sato T*, et al.* (2008) Obesity is an independent risk factor for hepatocellular carcinoma development in chronic hepatitis C patients. *Clin.Gastroenterol.Hepatol.,* **6,** 459-464.

Ohoka S, Tanaka Y, Amako Y*, et al.* (2003) Sialadenitis in patients with chronic hepatitis C is not directly related to hepatitis C virus. *Hepatol.Res.,* **27,** 23-29.

Ohto H, Terazawa S, Sasaki N*, et al.* (1994) Transmission of hepatitis C virus from mothers to infants. The Vertical Transmission of Hepatitis C Virus Collaborative Study Group. *N.Engl.J.Med.,* **330,** 744-750.

Oliveira GC, Carmo RA, Rocha MO, Silva MO, Lima AT, Guimaraes MD, Correa-Oliveira R (1999a) Hepatitis C virus genotypes in hemophiliacs in the state of Minas Gerais, Brazil. *Transfusion,* **39,** 1194-1199.

Oliveira ML, Bastos FI, Sabino RR, Paetzold U, Schreier E, Pauli G, Yoshida CF (1999b) Distribution of HCV genotypes among different exposure categories in Brazil. *Braz.J.Med.Biol.Res.,* **32,** 279-282.

Oubina JR, Quarleri JF, Rudzinski M, Parks C, Badia I, Gonzalez Cappa SM (1995) Genomic characterization of hepatitis C virus isolates from Argentina. *J.Med.Virol.,* **47,** 97-104.

Oubina JR, Quarleri JF, Sawicki MA*, et al.* (2001) Hepatitis C virus and GBV-C/hepatitis G virus in Argentine patients with porphyria cutanea tarda. *Intervirology,* **44,** 215-218.

Panasiuk A, Prokopowicz D, Zak J, Wysocka J (2003) Peripheral blood T, B, and NK cells in relation to histological hepatitis activity and fibrosis stage in chronic hepatitis C. *Hepatogastroenterology.,* **50,** 178-182.

Parana R, Paiva T, Leite MR*, et al.* (2007) Infection with hepatitis C virus among health care workers in the Brazilian Western Amazon region (Rio Branco, State of Acre). *Am.J.Trop.Med.Hyg.,* **76,** 165-169.

Parana R, Vitvitski L, Berby F*, et al.* (2000) HCV infection in northeastern Brazil: unexpected high prevalence of genotype 3a and absence of African genotypes. *Arq Gastroenterol.,* **37,** 213-216.

Pastore L, Fiore JR, Tateo M*, et al.* (2006) Detection of hepatitis C virus-RNA in saliva from chronically HCV-infected patients. *Int.J.Immunopathol.Pharmacol.,* **19,** 217-224.

Pawlotsky JM (2002) Use and interpretation of virological tests for hepatitis C. *Hepatology.,* **36,** S65-S73.

Pawlotsky JM (2006a) Hepatitis C virus population dynamics during infection. *Curr.Top.Microbiol.Immunol.,* **299:261-84.,** 261-284.

Pawlotsky JM (2006b) Virology of hepatitis B and C viruses and antiviral targets. *J.Hepatol.,* **44,** S10-S13.

Pekler VA, Robbins WA, Nyamathi A, Yashina TL, Leak B, Robins TA (2003) Use of versant TMA and bDNA 3.0 assays to detect and quantify hepatitis C virus in semen. *J.Clin.Lab Anal.,* **17,** 264-270.

Perone C, Del Castillo DM, Pereira GL, Carvalho NO, Januario JN, Teixeira R (2008) [High prevalence of genotype 1 in individuals with hepatitis C in Belo Horizonte, MG]. *Rev.Soc.Bras.Med.Trop.,* **41,** 238-242.

Pham TN, King D, Macparland SA, McGrath JS, Reddy SB, Bursey FR, Michalak TI (2008) Hepatitis C virus replicates in the same immune cell subsets in chronic hepatitis C and occult infection. *Gastroenterology,* **134,** 812-822.

Pham TN, Macparland SA, Coffin CS, Lee SS, Bursey FR, Michalak TI (2005) Mitogen-induced upregulation of hepatitis C virus expression in human lymphoid cells. *J.Gen.Virol.,* **86,** 657-666.

Picchio GR, Bare PC, Descalzi VI*, et al.* (2006) High prevalence of infection with a single hepatitis C virus genotype in a small rural community of Argentina. *Liver Int.,* **26,** 660-665.

Picchio GR, Nakatsuno M, Boggiano C*, et al.* (1997) Hepatitis C (HCV) genotype and viral titer distribution among Argentinean hemophilic patients in the presence or absence of human immunodeficiency virus (HIV) co-infection. *J.Med.Virol.,* **52,** 219-225.

Pilli M, Penna A, Zerbini A*, et al.* (2002) Oral lichen planus pathogenesis: A role for the HCV-specific cellular immune response. *Hepatology.,* **36,** 1446-1452.

Plancoulaine S, Mohamed MK, Arafa N*, et al.* (2008) Dissection of familial correlations in Hepatitis C Virus (HCV) seroprevalence suggests evidence for intrafamilial viral transmission and genetic predisposition to infection. *Gut.,* **.**

Polis CB, Shah SN, Johnson KE, Gupta A (2007) Impact of maternal HIV coinfection on the vertical transmission of hepatitis C virus: a meta-analysis. *Clin.Infect.Dis.,* **44,** 1123-1131.

Pozzato G, Mazzaro C, Crovatto M*, et al.* (1994) Low-grade malignant lymphoma, hepatitis C virus infection, and mixed cryoglobulinemia. *Blood.,* **84,** 3047-3053.

Preston FE, Jarvis LM, Makris M, Philp L, Underwood JC, Ludlam CA, Simmonds P (1995) Heterogeneity of hepatitis C virus genotypes in hemophilia: relationship with chronic liver disease. *Blood.,* **85,** 1259-1262.

Puchhammer-Stockl E, Mor W, Kundi M, Heinz FX, Hofmann H, Kunz C (1994) Prevalence of hepatitis-C virus RNA in serum and throat washings of children with chronic hepatitis. *J.Med.Virol.,* **43,** 143-147.

Pujol FH, Devesa M, Loureiro CL, Capriles F, Liprandi F (1998) Turnover of hepatitis C virus genotypes in hemodialysis patients. *Arch.Virol.,* **143,** 823-827.

Pujol FH, Loureiro CL (2007) Replacement of hepatitis C virus genotype 1b by genotype 2 over a 10-year period in Venezuela. *J.Clin.Gastroenterol.,* **41,** 518-520.

Pujol FH, Loureiro CL, Devesa M, Blitz L, Parra K, Beker S, Liprandi F (1997) Determination of genotypes of hepatitis C virus in Venezuela by restriction fragment length polymorphism. *J.Clin.Microbiol.,* **35,** 1870-1872.

Pungpapong S, Kim WR (2008) Mortality in patients with hepatitis C. *Gastroenterology.,* **134,** 635-637.

Qian KP, Natov SN, Pereira BJ, Lau JY (2000) Hepatitis C virus mixed genotype infection in patients on haemodialysis. *J.Viral Hepat.,* **7,** 153-160.

Qiu P, Soder GJ, Sanfiorenzo VJ, Wang L, Greene JR, Fritz MA, Cai XY (2003) Quantification of single nucleotide polymorphisms by automated DNA sequencing. *Biochem.Biophys.Res.Commun.,* **%19;309,** 331-338.

Quarleri JF, Robertson BH, Mathet V*, et al.* (1998) Genomic and phylogenetic analysis of hepatitis C virus strains from Argentina. *Medicina (B Aires),* **58,** 153-159.

Quarleri JF, Robertson BH, Mathet VL*, et al.* (2000) Genomic and phylogenetic analysis of hepatitis C virus isolates from argentine patients: a six-year retrospective study. *J.Clin.Microbiol.,* **38,** 4560-4568.

Radkowski M, Gallegos-Orozco JF, Jablonska J*, et al.* (2005) Persistence of hepatitis C virus in patients successfully treated for chronic hepatitis C. *Hepatology,* **41,** 106-114.

Rai R, Deval J (2011) New Opportunities in Anti-Hepatitis C Virus Drug Discovery: Targeting NS4B. *Antiviral Res*.

Rambaut A, Posada D, Crandall KA, Holmes EC (2004) The causes and consequences of HIV evolution. *Nat Rev Genet,* **5,** 52-61.

Re V, Contigiani M, Yoshida CF, Lampe E (2007) Identification of hepatitis C virus subtype 2c by sequencing analysis in patients from Cordoba, Argentina. *Mem.Inst.Oswaldo Cruz,* **102,** 995-998.

Re V, Gallego S, Farias A, Barbas G, Kremer L, Diaz MP, Contigiani M (2008) Hepatitis C and HIV coinfection in central region of Argentina: prevalence, genotype characterization and risk factors. *Enferm.Infecc.Microbiol.Clin.,* **26,** 423-425.

Re V, Lampe E, Yoshida CF*, et al.* (2003) Hepatitis C virus genotypes in Cordoba, Argentina. Unexpected high prevalence of genotype 2. *Medicina (B Aires),* **63,** 205-210.

Rebora A, Rongioletti F, Drago F, Parodi (1999) Lichen planus as a side effect of HBV vaccination. *Dermatology.,* **198,** 1-2.

Rey D, Fritsch S, Schmitt C, Meyer P, Lang JM, Stoll-Keller F (2001) Quantitation of hepatitis C virus RNA in saliva and serum of patients coinfected with HCV and human immunodeficiency virus. *J.Med.Virol.,* **63,** 117-119.

Richardson D, Fisher M, Sabin CA (2008) Sexual Transmission of Hepatitis C in MSM May Not Be Confined to Those with HIV Infection. *J.Infect.Dis.,* **197,** 1213-1214.

Rodriguez-Inigo E, Casqueiro M, Bartolome J*, et al.* (2000) Hepatitis C virus RNA in kidney biopsies from infected patients with renal diseases. *J.Viral Hepat.,* **7,** 23-29.

Ronaghi M, Karamohamed S, Pettersson B, Uhlen M, Nyren P (1996) Real-time DNA sequencing using detection of pyrophosphate release. *Anal.Biochem.,* **242,** 84-89.

Roque-Afonso AM, Ducoulombier D, Di LG*, et al.* (2005) Compartmentalization of hepatitis C virus genotypes between plasma and peripheral blood mononuclear cells. *J.Virol.,* **79,** 6349-6357.

Roque Afonso AM, Jiang J, Penin F*, et al.* (1999) Nonrandom distribution of hepatitis C virus quasispecies in plasma and peripheral blood mononuclear cell subsets. *J.Virol.,* **73,** 9213-9221.

Roy KM, Bagg J, Bird GL, Spence E, Follett EA, Mills PR, Lau JY (1995) Serological and salivary markers compared with biochemical markers for monitoring interferon treatment for hepatitis C virus infection. *J.Med.Virol.,* **47,** 429-434.

Roy KM, Bagg J, Follett EA, Brewer A, Lowe GD (1996) Hepatitis C virus in saliva of haemophiliac patients attending an oral surgery unit. *Br.J.Oral Maxillofac.Surg.,* **34,** 162-165.

Roy KM, Bagg J, McCarron B (1999) The effect of saliva specimen collection, handling and storage protocols on hepatitis C virus (HCV) RNA detection by PCR. *Oral Dis.,* **5,** 123-127.

Roy KM, Bagg J, McCarron B, Good T, Cameron S, Pithie A (1998) Predominance of HCV type 2a in saliva from intravenous drug users. *J.Med.Virol.,* **54,** 271-275.

Sabbatani S, Manfredi R, Marinacci G, Pavoni M, Cristoni L, Chiodo F (2006) Reactivation of severe, acute pulmonary tuberculosis during treatment with pegylated interferon-alpha and ribavirin for chronic HCV hepatitis. *Scand.J.Infect.Dis.,* **38,** 205-208.

Salloum S, Kluge SF, Kim AY, Roggendorf M, Timm J (2010) The resistance mutation R155K in the NS3/4A protease of hepatitis C virus also leads the virus to escape from HLA-A*68-restricted CD8 T cells. *Antiviral Res,* **87,** 272-275.

Salmeron J, De Rueda PM, Ruiz-Extremera A*, et al.* (2006) Quasispecies as predictive response factors for antiviral treatment in patients with chronic hepatitis C. *Dig Dis Sci,* **51,** 960-967.

Sanchez JL, Sjogren MH, Callahan JD*, et al.* (2000) Hepatitis C in Peru: risk factors for infection, potential iatrogenic transmission, and genotype distribution. *Am.J.Trop.Med.Hyg.,* **63,** 242-248.

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc.Natl.Acad.Sci.U.S.A.,* **74,** 5463-5467.

Savoldi E, Gargiulo F, Marinone MG, Favilli F, Callea F, Sapelli PL, Turano A (2001) HCV-RNA occurrence in saliva of odontoiatric HCV seropositive patients. *Minerva Stomatol.,* **50,** 71-74.

Schackman BR, Oneda K (2007) Preventing vertical hepatitis C virus transmission. *Clin.Infect.Dis.,* **45,** 802.

Schackman BR, Oneda K, Goldie SJ (2004) The cost-effectiveness of elective Cesarean delivery to prevent hepatitis C transmission in HIV-coinfected women. *AIDS.,* **18,** 1827-1834.

Schramm F, Soulier E, Royer C*, et al.* (2008) Frequent Compartmentalization of Hepatitis C Virus with Leukocyte-Related Amino Acids in the Setting of Liver Transplantation. *J.Infect.Dis.*

Sharma V, Aggarwal S (2009) Spider nevi and chronic liver disease. *Indian J Dermatol Venereol Leprol,* **75,** 403.

Shepard CW, Finelli L, Alter MJ (2005) Global epidemiology of hepatitis C virus infection. *Lancet Infect.Dis.,* **5,** 558-567.

Shire NJ, Horn PS, Rouster SD, Stanford S, Eyster ME, Sherman KE (2006) HCV kinetics, quasispecies, and clearance in treated HCV-infected and HCV/HIV-1-coinfected patients with hemophilia. *Hepatology,* **44,** 1146-1157.

Simmonds P (2001a) The origin and evolution of hepatitis viruses in humans. *J.Gen.Virol.,* **82,** 693-712.

Simmonds P (2001b) Reconstructing the origins of human hepatitis viruses. *Philos.Trans.R.Soc.Lond B Biol.Sci.,* **356,** 1013-1026.

Simmonds P (2004) Genetic diversity and evolution of hepatitis C virus--15 years on. *J.Gen.Virol.,* **85,** 3173-3188.

Simmonds P, Bukh J, Combet C*, et al.* (2005) Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology.,* **42,** 962-973.

Simmonds P, Holmes EC, Cha TA*, et al.* (1993) Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. *J.Gen.Virol.,* **74,** 2391-2399.

Simmonds P, Mellor J, Sakuldamrongpanich T, Nuchaprayoon C, Tanprasert S, Holmes EC, Smith DB (1996) Evolutionary analysis of variants of hepatitis C virus found in South-East Asia: comparison with classifications based upon sequence similarity. *J.Gen.Virol.,* **77,** 3013-3024.

Simmonds P, Smith DB (1997) Investigation of the pattern of diversity of hepatitis C virus in relation to times of transmission. *J.Viral Hepat.,* **4 Suppl 1:69-74.,** 69-74.

Smith DB, Pathirana S, Davidson F, Lawlor E, Power J, Yap PL, Simmonds P (1997) The origin of hepatitis C virus genotypes. *J.Gen.Virol.,* **78,** 321-328.

Sobesky R, Feray C, Rimlinger F*, et al.* (2007) Distinct hepatitis C virus core and F protein quasispecies in tumoral and nontumoral hepatocytes isolated via microdissection. *Hepatology,* **46,** 1704-1712.

Sookoian S, Neglia V, Castano G, Frider B, Kien MC, Chohuela E (1999) High prevalence of cutaneous reactions to interferon alfa plus ribavirin combination therapy in patients with chronic hepatitis C virus. *Arch Dermatol,* **135,** 1000-1001.

Soza A, Arrese M, Gonzalez R*, et al.* (2004) Clinical and epidemiological features of 147 Chilean patients with chronic hepatitis C. *Ann.Hepatol.,* **3,** 146-151.

Steinhauer DA, Holland JJ (1987) Rapid evolution of RNA viruses. *Annu Rev Microbiol,* **41,** 409-433.

Steinmann E, Penin F, Kallis S, Patel AH, Bartenschlager R, Pietschmann T (2007) Hepatitis C virus p7 protein is crucial for assembly and release of infectious virions. *PLoS.Pathog.,* **3,** e103.

Stuyver L, Rossau R, Wyseur A, Duhamel M, Vanderborght B, Van Heuverswyn H, Maertens G (1993) Typing of hepatitis C virus isolates and characterization of new subtypes using a line probe assay. *J.Gen.Virol.,* **74,** 1093-1102.

Stuyver L, Wyseur A, van Arnhem W, Hernandez F, Maertens G (1996) Second-generation line probe assay for hepatitis C virus genotyping. *J.Clin.Microbiol.,* **34,** 2259-2266.

Sugimura H, Yamamoto H, Watabiki H*, et al.* (1995) Correlation of detectability of hepatitis C virus genome in saliva of elderly Japanese symptomatic HCV carriers with their hepatic function. *Infection,* **23,** 258-262.

Sulkowski MS, Ray SC, Thomas DL (2002) Needlestick transmission of hepatitis C. *JAMA,* **287,** 2406-2413.

Sung VM, Shimodaira S, Doughty AL*, et al.* (2003) Establishment of B-cell lymphoma cell lines persistently infected with hepatitis C virus in vivo and in vitro: the apoptotic effects of virus infection. *J.Virol.,* **77,** 2134-2146.

Suzuki T, Omata K, Satoh T*, et al.* (2005) Quantitative detection of hepatitis C virus (HCV) RNA in saliva and gingival crevicular fluid of HCV-infected patients. *J.Clin.Microbiol.,* **43,** 4413-4417.

Suzuki T, Tai H, Yoshie H*, et al.* (1997) Characterization of HIV-related periodontitis in AIDS patients: HIV-infected macrophage exudate in gingival crevicular fluid as a hallmark of distinctive etiology. *Clin.Exp.Immunol.,* **108,** 254-259.

Szabo E, Lotz G, Paska C, Kiss A, Schaff Z (2003) Viral hepatitis: new data on hepatitis C infection. *Pathol.Oncol.Res.,* **9,** 215-221.

Takamatsu K, Koyanagi Y, Okita K, Yamamoto N (1990) Hepatitis C virus RNA in saliva. *Lancet,* **336,** 1515.

Takamatsu K, Okayasu I, Koyanagi Y, Yamamoto N (1992) Hepatitis C virus propagates in salivary glands. *J.Infect.Dis.,* **165,** 973-974.

Takehara T, Hayashi N, Mita E*, et al.* (1992) Detection of the minus strand of hepatitis C virus RNA by reverse transcription and polymerase chain reaction: implications for hepatitis C virus replication in infected tissue. *Hepatology,* **15,** 387-390.

Takyar ST, Li D, Wang Y, Trowbridge R, Gowans EJ (2000) Specific detection of minus-strand hepatitis C virus RNA by reverse-transcription polymerase chain reaction on PolyA(+)-purified RNA. *Hepatology,* **32,** 382-387.

Taliani G, Badolato C, Lecce R*, et al.* (1995) Hepatitis C virus RNA in peripheral blood mononuclear cells: relation with response to interferon treatment. *J Med Virol,* **47,** 16-22.

Taliani G, Celestino D, Badolato MC*, et al.* (1997) Hepatitis C virus infection of salivary gland epithelial cells. Lack of evidence. *J.Hepatol.,* **26,** 1200-1206.

Tedder RS, Gilson RJ, Briggs M*, et al.* (1991) Hepatitis C virus: evidence for sexual transmission. *BMJ,* **302,** 1299-1302.

Thongsawat S, Maneekarn N, Kuniholm MH*, et al.* (2008) Occult hepatitis C virus infection during an outbreak in a hemodialysis unit in Thailand. *J.Med.Virol.,* **80,** 808-815.
Torres B, Martin JL, Caballero A, Villalobos M, Olea N (2000) HCV in serum, peripheral blood mononuclear cells and lymphocyte subpopulations in C-hepatitis patients. *Hepatol.Res.,* **18,** 141-151.
Toussirot E, Le HG, Mougin C, Balblanc JC, Bettinger D, Wendling D (2002) Presence of hepatitis C virus RNA in the salivary glands of patients with Sjogren's syndrome and hepatitis C virus infection. *J.Rheumatol.,* **29,** 2382-2385.
Toyoda H, Fukuda Y, Hayakawa T, Kumada T, Nakano S, Takamatsu J, Saito H (1999) Presence of multiple genotype-specific antibodies in patients with persistent infection with hepatitis C virus (HCV) of a single genotype: evidence for transient or occult superinfection with HCV of different genotypes. *Am.J.Gastroenterol.,* **94,** 2230-2236.
Tuke PW, Grant PR, Waite J, Kitchen AD, Eglin RP, Tedder RS (2008) Hepatitis C virus window-phase infections: closing the window on hepatitis C virus. *Transfusion,* **48,** 594-600.
Ustundag Y, Hizel N, Boyacioglu S, Akalin E (1997) Detection of hepatitis GB virus-C and HCV genomes in the saliva of patients undergoing maintenance haemodialysis. *Nephrol.Dial.Transplant.,* **12,** 2807.
Vallet S, Gouriou S, Nkontchou G*, et al.* (2007) Is hepatitis C virus NS3 protease quasispecies heterogeneity predictive of progression from cirrhosis to hepatocellular carcinoma? *J Viral Hepat,* **14,** 96-106.
van de Laar TJ, van der Bij AK, Prins M*, et al.* (2007) Increase in HCV incidence among men who have sex with men in Amsterdam most likely caused by sexual transmission. *J.Infect.Dis.,* **196,** 230-238.
van Doornum GJ, Lodder A, Buimer M, van Ameijden EJ, Bruisten S (2001) Evaluation of hepatitis C antibody testing in saliva specimens collected by two different systems in comparison with HCV antibody and HCV RNA in serum. *J.Med.Virol.,* **64,** 13-20.
Vedamurthy M, Vedamurthy A (2008) Spider nevi: a presenting feature of chronic liver disease. *Indian J Dermatol Venereol Leprol,* **74,** 397-398.
Vento S, Nobili V, Cainelli F (2006) Clinical course of infection with hepatitis C. *BMJ,* **332,** 374-375.
Vera-Otarola J, Barria MI, Leon U, Marsac D, Carvallo P, Soza A, Lopez-Lastra M (2009) Hepatitis C virus quasispecies in plasma and peripheral blood mononuclear cells of treatment naive chronically infected patients. *J.Viral Hepat.*
Verma V, Chakravarti A (2008) Comparison of 5' noncoding-core with 5' noncoding regions of HCV by RT-PCR: importance and clinical implications. *Curr Microbiol,* **57,** 206-211.
Viazov S, Widell A, Nordenfelt E (2000) Mixed infection with two types of hepatitis C virus is probably a rare event. *Infection.,* **28,** 21-25.
Vogler IH, Nishiya A, Morimoto HK*, et al.* (2004) Serological, epidemiological and molecular aspects of hepatitis C virus infection in a population from Londrina, PR, Brazil, 2001-2002. *Rev.Inst.Med.Trop.Sao Paulo,* **46,** 303-308.
Wang CC, Morishima C, Chung M*, et al.* (2006) High serum hepatitis C virus (HCV) RNA load predicts the presence of HCV RNA in saliva from

individuals with chronic and acute HCV infection. *J.Infect.Dis.,* **193,** 672-676.

Wang JT, Sheu JC, Lin JT, Wang TH, Chen DS (1992a) Detection of replicative form of hepatitis C virus RNA in peripheral blood mononuclear cells. *J.Infect.Dis.,* **166,** 1167-1169.

Wang JT, Wang TH, Lin JT, Sheu JC, Lin SM, Chen DS (1991) Hepatitis C virus RNA in saliva of patients with post-transfusion hepatitis C infection. *Lancet,* **337,** 48.

Wang JT, Wang TH, Sheu JC, Lin JT, Chen DS (1992b) Hepatitis C virus RNA in saliva of patients with posttransfusion hepatitis and low efficiency of transmission among spouses. *J.Med.Virol.,* **36,** 28-31.

Who (2000) Hepatitis C--global prevalence (update). *Wkly.Epidemiol.Rec.,* **75,** 18-19.

Widell A, Mansson S, Persson NH, Thysell H, Hermodsson S, Blohme I (1995) Hepatitis C superinfection in hepatitis C virus (HCV)-infected patients transplanted with an HCV-infected kidney. *Transplantation,* **60,** 642-647.

Willems M, Peerlinck K, Moshage H, Deleu I, Van den Eynde C, Vermylen J, Yap SH (1994) Hepatitis C virus-RNAs in plasma and in peripheral blood mononuclear cells of hemophiliacs with chronic hepatitis C: evidence for viral replication in peripheral blood mononuclear cells. *J Med Virol,* **42,** 272-278.

Yaari A, Tovbin D, Zlotnick M*, et al.* (2006) Detection of HCV salivary antibodies by a simple and rapid test. *J.Virol.Methods.,* **133,** 1-5.

Ye J, Liu W, Guo J, Zeng L (2001) The clinic study on synchronous detection of HCV RNA in the plasma and PBMC of hepatitis C patients. *J Tongji Med Univ,* **21,** 110-111.

Yepes A, Alvarez C, Restrepo JC, Correa G, Zapata JC, Arango AE (2002) [Viral genotypes in patients with hepatitis C virus infection in Medellin]. *Gastroenterol.Hepatol.,* **25,** 334-335.

Young KC, Chang TT, Liou TC, Wu HL (1993) Detection of hepatitis C virus RNA in peripheral blood mononuclear cells and in saliva. *J.Med.Virol.,* **41,** 55-60.

Zarife MA, de Oliveira EC, Romeu JM, dos Reis MG (2006) [Detection of genotype 4 of the hepatitis C virus in Salvador, BA]. *Rev Soc Bras Med Trop,* **39,** 567-569.

Zehender G, De Maddalena C, Bernini F, Ebranati E, Monti G, Pioltelli P, Galli M (2005) Compartmentalization of hepatitis C virus quasispecies in blood mononuclear cells of patients with mixed cryoglobulinemic syndrome. *J.Virol.,* **79,** 9145-9156.

Zehender G, Meroni L, De Maddalena C, Varchetta S, Monti G, Galli M (1997) Detection of hepatitis C virus RNA in CD19 peripheral blood mononuclear cells of chronically infected patients. *J.Infect.Dis.,* **176,** 1209-1214.

Zein NN (2000) Clinical significance of hepatitis C virus genotypes. *Clin.Microbiol.Rev.,* **13,** 223-235.

Zeisel MB, Baumert TF (2006) Production of infectious hepatitis C virus in tissue culture: A breakthrough for basic and applied research. *J.Hepatol.,* **44,** 436-439.

Zhang M, O'Brien TR, Kopp WC, Goedert JJ (2005) Lymphocyte subsets in hemophilic patients with hepatitis C virus infection with or without

human immunodeficiency virus co-infection: a nested cross-sectional study. *BMC.Blood Disord.,* **5,** 2.

Zhou Y, Wang X, Mao Q*, et al.* (2009) Changes in modes of hepatitis C infection acquisition and genotypes in southwest China. *J Clin Virol,* **46,** 230-233.

Zignego AL, De Carli M, Monti M*, et al.* (1995) Hepatitis C virus infection of mononuclear cells from peripheral blood and liver infiltrates in chronically infected patients. *J Med Virol,* **47,** 58-64.

Zignego AL, Ferri C, Pileri SA, Caini P, Bianchi FB (2007) Extrahepatic manifestations of Hepatitis C Virus infection: a general overview and guidelines for a clinical approach. *Dig.Liver Dis.,* **39,** 2-17.

Zignego AL, Macchia D, Monti M*, et al.* (1992) Infection of peripheral mononuclear blood cells by hepatitis C virus. *J.Hepatol.,* **15,** 382-386.

Zoulim F, Chevallier M, Maynard M, Trepo C (2003) Clinical consequences of hepatitis C virus infection. *Rev.Med.Virol.,* **13,** 57-68.

Zuniga J, Romero V, Azocar J*, et al.* (2009) Protective KIR-HLA interactions for HCV infection in intravenous drug users. *Mol Immunol,* **46,** 2723-2727.

Appendices

Appendix 1. Gender, age, biopsy results, biochemical markers of liver fibrosis (AST, ALT, ALP, GCT) and comments about the medical history of patients including co-morbidities, when present

	Gender	Age	Biopsy result	Comments about medical history of the patients / co-morbidities	AST	ALT	ALP	GGT
1	M	50			26	15	96	25
2	M	34	A1F0		23	26	76	32
3	M	47	Chronic gastritis		180	283	92	227
4	F	37	A2F1	Under treatment when she was examined	31	25	53	22
5	M	73		HCV RNA and genotyping requested	N/A	N/A	N/A	N/A
6	M	33		HCV RNA and genotyping requested	271	213	174	214
7	F	65	A3F4	Anti-HIV was positive, scheduled to repeat exam	44	46	62	111
8	F	56			331	215	233	125
9	F	22		Waiting renal transplantation	67	73	165	48
10	F	53			34	38	79	51
11	F	39			31	32	107	69
12	F	43			11	10	79	23
13	M	45			62	108	91	68
14	F	69		Stopped Ribavirin because of Obsessive Compulsive Disorder	23	18	74	19
15	M	55			23	49	136	58
16	M	67			35	30	82	28
17	M	50		Biopsy to be scheduled	40	53	80	46
18	M	49		Under treatment when he was examined	33	22	60	51
19	M	73			132	106	111	337
20	M	65			16	20	100	41
21	F	58			44	42	87	70
22	F	71			43	27	149	288
23	M	53			20	23	100	68
24	M	30		Under treatment when she was examined (32th week)	31	47	68	139
25	F	62			47	24	123	101
26	M	52			57	90	103	78
27	F	37			28	30	87	15
28	M	36		HIV coinfected, IDU (stopped 6 months before examination)	29	31	122	119
29	F	64			166	154	116	155
30	M	57			84	86	131	94
31	F	25			25	21	92	26
32	M	27			31	22	136	53
33	M	50			227	103	376	58
34	F	27			75	107	122	94
35	M	53		Under treatment when she was examined (6th week)	42	37	93	46
36	F	51		Biopsy performed at the day of the examination	76	90	203	341
37	F	48			62	50	269	194
38	F	60			33	33	367	300
39	F	62		Biopsy performed at the day of the examination	45	56	117	28
40	M	55		Biopsy performed at the day of the examination	52	82	99	183
41	M	20			33	45	64	265
42	M	47			23	19	100	19
43	M	44		Biopsy to be scheduled	21	19	126	35
44	F	30			24	28	238	362

	Gender	Age	Biopsy result	Comments about medical history of the patients / co-morbidities	AST	ALT	ALP	GGT
45	F	65	A2F3		41	45	213	65
46	M	53	A1F2	Treatment set to start in November 2006	64	100	60	301
47	F	49	A3F3	-	32	64	167	61
48	M	67			93	93	151	333
49	M	43			103	142	96	64
50	F	28			26	30	83	86
51	F	64			91	97	130	80
52	F	38			35	50	91	31
53	M	45			40	41	84	38
54	M	54			106	164	91	139
55	F	61		Treatment set to start in November 2006	77	136	100	117
56	F	53			170	169	182	147
57	M	45		HCV RNA and genotyping requested	68	116	131	520
58	M	47		Under treatment when he was examined	24	20	100	33
59	M	44		Fever and headaches because of IFN, no benefits	29	36	95	58
60	F	48			51	35	130	146
61	M	48			54	88	201	68
62	F	39			15	10	65	18
63	F	56		Under treatment when she was examined	24	15	86	40
64	F	51			93	135	69	25
65	F	46			18	25	62	26
66	M	57		Biopsy to be scheduled soon	141	274	170	669
67	M	43			21	40	65	94
68	M	51		Biopsy performed at the day of the examination	26	25	55	26
N1	M	44		Stopped treatment 2 months ago, without improvements	28	91	98	621
N2	M	40			10	<30	882	11
N3	M	52			20	15	153	39
N4	M	45			10	19	34	40
N5	F	34			21	21	919	125
N6	M	73			40	35	58	28
N7	M	74			11	28	38	93
N8	M	47			11	9	82	34
N9	M	26			54	113	141	67
N10	M	49			24	33	20	60
N11	F	27			20	10	115	185
N12	M	36			15	16	111	33
N13	M	63			25	<6	88	26
N14	M	27			19	13	1527	55
N15	M	70			24	33	143	165
N16	M	73			27	18	142	98
N17	M	29			3	14	103	24
N18	M	79			9	8	129	23

Biopsy results (METAVIR scoring system): The fibrosis is graded on a 5-point scale from 0 to 4. The activity, which is the amount of inflammation (specifically, the intensity of necro-inflammatory lesions), is graded on a 4-point scale from A0 to A3. It is a simplified and widely used scoring system for chronic liver disease grading (Bedossa *et al.*, 1996).

Activity score / Fibrosis score (F4 grades cirrhosis):

A0 = no activity / F0 = no fibrosis

A1 = mild activity / F1 = portal fibrosis without septa

A2 = moderate activity / F2 = portal fibrosis with few septa

A3 = severe activity / F3 = numerous septa without cirrhosis

Appendix 2. Individual patient data concerning age, estimated cell count (PDH), SVR, genotyping details, viral load and viral load per 1000 for specific compartments studied (plasma, saliva, CD19, CD14, CD2 and CD45)

	Treatment	PDH (CD19)	PDH (CD19)	PDH (CD19)	PDH (CD19)	SVR	Plasma (NS5b)	Saliva (NS5b)	CD19	CD14	CD2	CD45	Viral Load (P	Viral Load (S)	VL/1000 cells (S)	Viral Load (CD19)	Viral Load (CD14	Viral Load (CD2)	Viral Load (CD45)	VL/1000 cells (CD19)	VL/1000 cells (CD14)	VL/1000 cells (CD2)	VL/1000 cells (CD45)
1	Yes	9930	6670	14200	-	Yes	-	-	-	-	-	N/A	0	0	0,0	0	0	0	-	0,0	0,0	0,0	-
2	No	2990	18300	33200	-	N/A	1b	-	1b	-	1b	N/A	1.410.000	0	0,0	21200	3430	4580	-	7090,3	187,4	138,0	-
3	No	7740	6680	6930	-	N/A	1a	-	1a	1a	1a	N/A	294.000	0	0,0	23	5450	1680	-	3,0	815,9	242,4	-
4	Yes	5100	9360	11800	-	No	1a	-	1a	-	-	N/A	12	0	0,0	0	0	0	-	0,0	0,0	0,0	-
5	No	1090	9300	61600	363000	N/A	1b	-	1b	1b	-	1b	2.940.000	0	0,0	9420	96900	490	0	8642,2	10419,4	8,0	0,0
6	No	3150	2090	5470	36200	N/A	1b	-	1b	1b	1b	1b	3.510.000	13	0,1	199000	4410	3740	719000	63174,6	2110,0	683,7	19861,9
7	No	7380	2580	873	142000	N/A	3a	3a	3a	3a	3a	3a	1.180.000	0	0,0	16800	6660	0	5940	2276,4	2302,3	7628,0	0,0
8	Yes	640	787	8950	29100	No	1b	-	1b	1b	-	1b	712.000	0	0,0	6070	0	2	2250	9488,0	0,0	0,2	77,3
9	No	4230	3660	8470	15300	N/A	1b	1b	1b	1b	1b	1b	758.000	0	0,0	5190	617	35000	13100	1227,0	168,6	4132,2	856,2
10	Yes	1010	3210	3690	97500	No	3a	3a	3a	3a	3a	3a	2.060.000	37	1,8	3136	4588	521	98132	3105,0	1429,3	141,2	1006,5
11	No	12700	17700	3140	52800	N/A	1b	-	1b	1b	1b	1b	457.000	11	0,1	2573	1265	881	3858	202,6	71,5	280,6	73,1
12	No	2830	15200	50100	242000	N/A	-	-	-	-	-	-	0	0	0,0	0	0	0	0	0,0	0,0	0,0	0,0
13	No	10300	16400	20100	33700	N/A	3a	-	3a	3a	3a	3a	9.840.000	1	0,1	34057	2755	9460	5105	3306,5	168,0	470,6	151,5
14	Yes	10600	10200	34700	140000	No	1b	-	1b	-	1b	-	53.100	0	0,0	1887	0	118	17	178,0	0,0	3,4	0,1
15	No	5480	20400	12500	77500	N/A	1b	1b	1b	1b	1b	1b	19.600.000	3910	1275,1	103046	21249	42300	62750	18804,0	1041,6	3384,0	809,7
16	No	5570	6310	54200	91000	N/A	1b	-	1b	1b	1b	1b	6.500.000	9	0,8	13761	8531	2281	1402	2470,6	1352,0	42,1	15,4
17	No	9690	30000	22300	477000	N/A	1b	-	1b	1b	1b	1b	278.000	0	0,0	86593	1560	664	571	8936,3	52,0	29,8	1,2
18	Yes	6020	32400	27600	23000	?	-	-	-	-	-	-	0	0	0,0	0	0	0	0	0,0	0,0	0,0	0,0
19	No	32000	6730	41300	405000	N/A	1b	-	1b	1b	1b	1b	2.210.000	0	0,0	9219	365	98	404	288,1	54,2	2,4	1,0
20	Yes	9840	16800	16600	304000	No	1b	1b	1b	1b	1b	1b	30.800.000	26	173,5	233862	46044	7416	14834	23766,5	2740,7	446,7	48,8
21	No	10100	33900	85300	24000	N/A	1b	-	1b	1b	1b	1b	1.080.000	0	0,0	10014	715	237	585	991,5	21,1	2,8	24,4
22	No	19400	29200	41700	77200	N/A	1b	-	1b	1b	1b	1b	1.110.000	6	0,1	727	5	178	1657	37,5	0,2	4,3	21,5
23	No	13600	31000	24500	60800	N/A	-	-	-	-	1a	-	0	0	0,0	0	0	0	0	0,0	0,0	0,0	0,0
24	Yes	1210	8060	9140	25400	?	-	-	-	-	-	-	0	0	0,0	0	0	0	0	0,0	0,0	0,0	0,0
25	No	4540	11300	36600	40300	N/A	1b	-	1b	1b	1b	1b	5.910.000	0	0,0	7088	241	581	12390	1561,2	21,3	15,9	307,4
26	No	17900	34200	19600	19800	N/A	1b	-	1b	1b	1b	1b	9.730.000	0	0,0	42296	1354	775	1592	2362,9	39,6	39,5	80,4
27	No	23900	54700	30600	93200	N/A	3a	-	3a	3a	3a	-	2.520.000	88	17,0	20561	830	203	145	880,3	15,2	6,6	1,6
28	No	17200	10600	145000	8090	N/A	1a	-	1a	1a	1a	1a	5.160.000	74	1,1	84384	4772	1072	9675	4906,0	450,2	7,4	1195,9
29	No	18700	30600	195000	101000	N/A	1b	-	1b	1b	1b	1b	5.130.000	0	0,0	32114	555	51	230	1717,3	18,1	0,3	2,3
30	No	17500	27400	46100	61000	N/A	3a	-	3a	-	3a	3a	67.500	0	0,0	38	112	127	10	2,2	4,1	2,8	0,2
31	No	10600	49000	93400	91000	N/A	1b	-	1b	1b	1b	1b	1.350.000	0	0,0	6945	904	110	1057	655,2	18,4	1,2	11,6
32	No	7310	18500	37900	20800	N/A	1a	-	1a	1a	1a	1a	178.000	0	0,0	114	0	40	0	15,6	0,0	1,1	0,0
33	Yes	451	11000	41300	52200	No	1a	-	-	1a	1a	-	2.760	0	0,0	0	196	0	0	0,0	17,8	0,0	0,0
34	No	4000	8600	231000	199000	N/A	1b	-	1b	1b	1b	1b	507.000	78	0,7	1039	232	164	162	259,8	27,0	0,7	0,8
35	Yes	648	15100	25700	90100	?	-	-	-	-	-	-	0	0	0,0	0	0	0	0	0,0	0,0	0,0	0,0
36	No	6480	12600	17800	103000	N/A	1a	-	1a	1a	1a	1a	7.650.000	0	0,0	3350	1900	774	724	517,0	150,8	43,5	7,0
37	No	1590	180000	267000	470000	N/A	-	-	-	-	-	-	0	0	0,0	0	0	0	0	0,0	0,0	0,0	0,0
38	No	1530	12200	15400	29800	N/A	3a	-	3a	3a	3a	3a	173.000	0	0,0	0	13	2	0	0,0	1,1	0,1	0,0
39	No	3770	15100	154000	71200	N/A	3a	-	3a	3a	3a	3a	621.000	0	0,0	5205	4893	1316	9801	1380,6	324,0	8,5	137,7
40	No	3610	19900	86200	47200	N/A	1b	1b	1b	1b	1b	1b	3.810.000	63	0,9	21038	12826	3811	37034	5827,7	644,5	44,2	784,6
41	No	1640	30100	6800	53000	N/A	1b	-	1b	1b	-	-	562.000	0	1,0	420	768	148	34	256,1	25,5	21,8	0,6
42	No	6060	29000	14200	525000	N/A	-	-	-	-	-	-	0	0	0,0	0	0	0	0	0,0	0,0	0,0	0,0
43	Yes	25000	27100	44500	551000	Yes	-	-	-	-	-	-	0	0	0,0	0	0	44500	0	0,0	0,0	134,0	0,0
44	No	3100	48500	17200	122000	N/A	1b	-	1b	1b	1b	1b	4.390.000	3	0,4	94400	5520	160000	41900	30451,6	113,8	9302,3	343,4
45	Yes	2620	1690	13500	43900	No	1b	-	1b	-	-	-	270.000	0	0,0	67	0	0	0	25,6	0,0	0,0	0,0
46	No	3090	3240	5420	27500	N/A	1a	-	1a	1a	-	1a	372.000	0	0,0	3028	0	35	328	979,9	0,0	6,5	11,9
47	No	877	5760	8050	14200	N/A	1b	-	1b	1b	1b	1b	17.900.000	0	0,0	45300	2499	159	20120	51681,2	433,9	19,8	1416,9
48	No	83	4950	4680	42600	N/A	3a	-	3a	3a	3a	3a	1.470.000	0	0,0	23	0	13	294	278,3	0,0	2,8	6,9
49	No	3750	4790	16600	18000	N/A	1a	-	1a	-	1a	1a	72.200	0	0,0	169	0	0	0	45,1	0,0	0,0	0,0
50	No	3030	3690	8100	46700	N/A	1b	-	1b	1b	-	1b	6.890.000	43	4,3	1831	1098	393	3196	604,3	297,6	48,5	68,4
51	No	151	1520	7710	19100	N/A	1b	-	1b	1b	1b	1b	2230000	3	0,3	49	26	312	179	323,6	17,1	40,5	9,4
52	No	2760	10600	14700	56300	N/A	1b	-	1b	1b	1b	1b	433.000	0	0,0	633	4804	2086	1539	229,3	453,2	141,9	27,3
53	No	4590	5130	20400	73400	N/A	3a	-	3a	3a	4o	3a	1.580.000	0	0,0	1624	2309	52	121	353,8	450,1	2,5	1,6
54	Yes	8460	2710	13300	16400	No	1b	-	1b	1b	-	-	1.250.000	6	0,1	13576	869	6	2768	1604,7	320,7	0,5	168,8
55	No	1740	3520	37700	9620	N/A	3a	-	3a	-	-	-	278.000	22	0,5	1198	0	0	372	688,5	0,0	0,0	38,7
56	No	1340	6350	11400	7070	N/A	1b	-	1b	1b	1b	1b	1.760.000	0	0,0	5950	456	49	799	4440,3	78,1	4,3	0,0
57	No	1480	9500	97600	24600	N/A	1a	1a	1a	1a	1a	1a	8.160.000	31	0,3	265	594	35	1551	179,1	62,5	0,4	63,0
58	Yes	UNAVAILABLE	UNAVAILABLE	UNAVAILABLE	UNAVAILABLE	?	-	-	-	-	-	-	3.500	0	0,0	UNAVAILABLE	UNAVAILABLE	UNAVAILABLE	UNAVAILABLE	UNAVAILABLE	UNAVAILABLE	UNAVAILABLE	UNAVAILABLE
59	Yes	365	3960	6550	7280	No	1b	-	1b	1b	1b	1b	3.090.000	95	4,1	7271	4572	71	651	19939,9	1154,5	10,8	89,4
60	No	4050	35200	8720	26400	N/A	-	-	-	-	-	-	0	0	0,0	0	0	3.030	0	0,0	0,0	347,5	0,0
61	No	747	12700	11100	17200	N/A	3a	3a	3a	3a	3a	3a	2.390.000	26	0,2	2731	1957	5753	4700	3655,5	154,1	518,3	273,3
62	No	2260	13800	6490	81500	N/A	-	-	-	-	-	-	0	0	0,0	0	0	0	0	0,0	0,0	0,0	0,0
63	Yes	1110	29700	32800	17300	?	-	-	-	4d	-	-	0	0	0,0	0	0	0	0	0,0	0,0	0,0	0,0
64	No	4680	1560	17900	8240	N/A	2b	-	2b	2b	2b	2b	1.650.000	0	0,0	3437	0	2	127	734,4	0,0	0,1	15,4
65	No	3530	5160	13700	15500	N/A	3a	-	-	-	-	-	107.000	0	0,0	0	0	0	0	0,0	0,0	0,0	0,0
66	No	3230	2250	10500	11100	N/A	1b	-	1b	1b	1b	1b	119.000	0	0,0	582	0	2	281	180,2	0,0	0,2	25,3
67	Yes	19800	18200	49900	28800	No	1b	-	1b	-	1b	1b	134.000	0	0,0	11300	157	942	3490	570,7	8,6	18,9	121,2
68	No	6660	32500	26100	23600	N/A	1b	-	1b	1b	1b	1b	6.740.000	32	0,4	412000	14000	2580	114000	61861,9	430,8	98,9	4830,5
N1	Yes	278000	9450	27700	18100	No	1a	1a	1a	1a	1a	1a	17.700.000	0	0,0	1770000	215000	129000	747000	6366,9	22751,3	46570,4	41270,7
N2	No	2160	9930	86600	180000	N/A	-	-	-	-	-	-	0	0	0,0	0	0	0	0	0,0	0,0	0,0	0,0
N3	No	3240	20000	37000	203000	N/A	1b	-	1b	1b	1b	1b	209.000	0	0,0	7.820	611	3	193	2413,6	30,6	0,1	1,0
N4	No	4950	19600	41100	278000	N/A	1b	-	1b	1b	1b	1b	236.000	0	0,0	2140	13800	0	613	432,3	704,1	0,0	2,2
N5	No	12300	31800	120000	25800	N/A	1a	-	1a	1a	1a	1a	483.000	0	0,0	0	955	0	68	0,0	30,0	0,0	2,6
N6	No	937	15200	34200	83100	N/A	-	-	-	-	-	-	0	0	0,0	0	0	0	0	0,0	0,0	0,0	0,0
N7	No	5220	31000	21700	12200	N/A	1b	-	1b	1b	-	1b	22.900	0	0,0	11	0	0	174	2,1	0,0	0,0	14,3
N8	No	12500	37800	42900	54800	N/A	3a	-	3a	3a	3a	3a	79.000	0	0,0	1300	19	100	384	104,0	0,5	2,3	7,0
N9	No	18700	9460	28400	88800	N/A	1a	-	1a	1a	1a	1a	167.000	4	1,9	304	0	13	1270	16,3	0,0	0,5	14,3
N10	No	8310	19300	138000	589000	N/A	3a	3a	3a	3a	3a	3a	3.070.000	0	0,0	13200	30	0	28	1588,4	1,6	0,0	0,0
N11	Yes	2280	2390	18600	79400	No	1a	1a	1a	1a	1a	1a	4.970.000	0	0,0	5320	2230	703	1110	2333,3	933,1	37,8	14,0
N12	No	5430	15200	225000	495000	N/A	1b	-	-	1b	-	-	48.700	0	0,0	110	524	248	0	20,3	34,5	1,1	0,0
N13	No	15000	13600	43000	51400	N/A	1b	-	1b	-	-	1b	224.000	0	0,0	5420	50	0	0	361,3	3,7	0,0	0,0
N14	No	7610	12100	51100	71900	N/A	1a	-	1a	-	1a	-	1.450.000	0	0,0	1640	58	258	0	215,5	4,8	5,0	0,0
N15	No	7690	9670	21300	41700	N/A	1b	-	1b	-	-	1b	195.000	0	0,0	12800	20	0	2350	1664,5	2,1	0,0	56,4
N16	No	368	11300	30800	37200	N/A	1a	-	1a	1a	1a	1a	280.000	0	0,0	356000	523.000	452000	258000	967528,0	46283,2	14675,3	6935,5
N17	No	5170	29600	28800	67800	N/A	1a	-	1a	1a	1a	-	12.300	0	0,0	2370	2300	0	5	458,4	77,7	0,0	0,1
N18	No	5990	32900	27800	36600	N/A	3a	-	3a	3a	3a	3a	90.500	0	0,0	41700	5.750	5640	1650	6961,6	174,8	202,9	45,1

Colour coding in Appendix 2: cells highlighted in yellow represent negative results in a given assay, while positive results were obtained in the other assay. On the other hand, cells highlighted in blue represent the positive results in a given assay, while negative results were obtained in the other assay. Discrepancies have been approached in the discussion of Chapter 3.

Appendix 3. Two examples of RFLP gels photographed under UV light