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Occult Hepatitis C Virus Infection: Where are We Now?

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1. Introduction

Hepatitis C virus (HCV) is the major cause of chronic liver disease worldwide. Up to 30% of infected individuals spontaneously resolve their acute infection, while others develop chronic hepatitis C and replicate the virus seemingly indefinitely, but the features of the host and virus that are responsible for this difference are not yet clear. The persistence of the virus in the liver can lead to cirrhosis and hepatocellular carcinoma. End-stage liver disease due to a chronic HCV infection is currently the number one reason for liver transplantation in many parts of the world. No prophylactic vaccine is presently available and the current antiviral therapy successfully suppresses HCV replication in fewer than 50% of patients with a chronic infection. Until recently, patients who had eliminated HCV spontaneously or after treatment were considered to be definitively cured. But reports of low HCV RNA concentrations in the plasma, peripheral blood mononuclear cells and livers of patients who had cleared HCV has led to uncertainty in both patients and physicians. This new form of HCV infection is called occult HCV infection. This chapter summarises the data presently available on occult HCV infections and discusses its significance and reality.

1.1 HCV infection

1.1.1 HCV virus

Hepatitis C is a member of the *Flaviviridae* family. Its genome of approximately 9.5 kb is a positive RNA strand that encodes a large polyprotein of more than 3000 amino acid residues. The open reading frame is flanked by untranslated regions, the 5' UTR and 3' UTR. The 5'UTR region contains the internal ribosomal entry site (IRES). Processing and cleavage of the polyprotein yields structural and non-structural proteins (Figure 1). HCV has great genetic variability, with six genotypes and more than 70 subtypes (Simmonds et al., 2005).

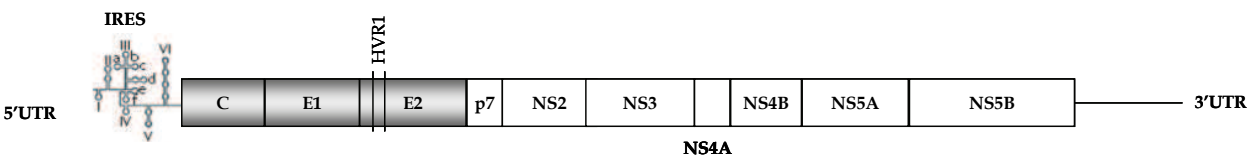


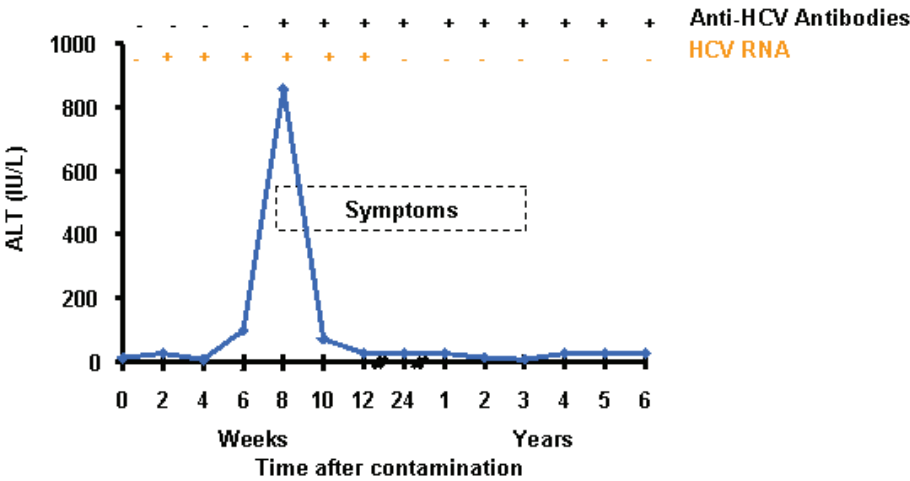
Fig. 1. Organisation of the HCV genome. The region in gray encodes structural proteins and the white one non-structural proteins.

1.1.2 Natural history of HCV infection

Patients infected with HCV virus first develop an acute disease that is usually asymptomatic. Diagnosis is confirmed by the detection of HCV genomic RNA in the plasma, which may also contain anti-HCV antibodies (Figure 2a). About 30% of infected patients spontaneously clear the virus, generally in the 3 months following clinical symptoms (Corey et al., 2006, Gerlach et al., 2003, Micallef et al., 2006)

Unfortunately, 70% of infected patients develop a chronic HCV infection (Figure 2b). The plasma of these patients contains anti-HCV antibodies and HCV RNA. These patients can be divided into two groups. One group has normal liver enzyme activities, while the other has abnormal liver enzyme activities. Thus, 7 - 53% of patients with a chronic HCV infection have normal alanine aminotransferase activities (Alter, 2005, Mathurin et al., 1998, Persico et al., 2000). Hepatic lesions are moderate, but liver biopsies have shown that 90% of patients suffering from chronic hepatitis have lesions (Marcellin et al., 1997b). Analysis of liver biopsies indicates that most patients with a chronic HCV infection have elevated liver enzymes and moderate to severe lesions. About 25% of them develop liver cirrhosis, and this results in hepatocellular carcinoma in 2% of them (Alberti et al., 1999). Liver transplantation is the only treatment available to these patients.

(a)



(b)

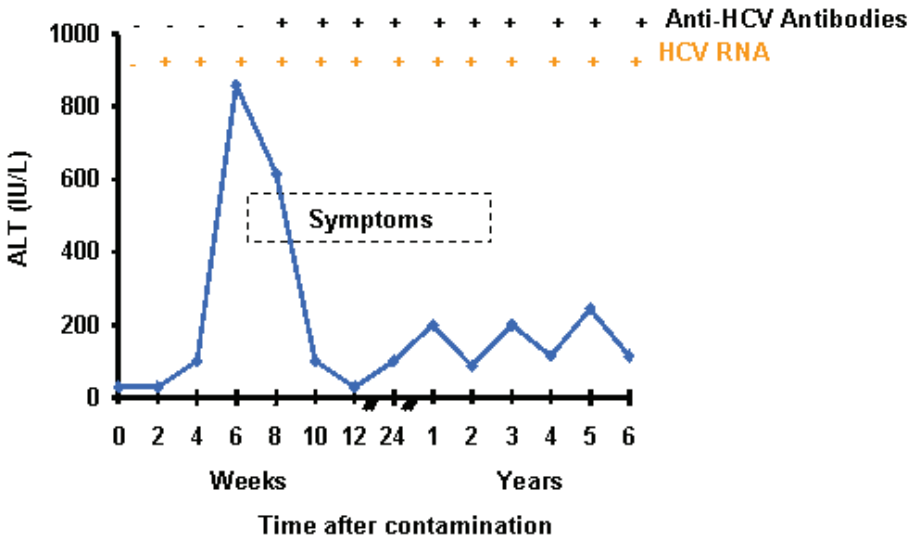


Fig. 2. Natural history of acute (a) and chronic (b) HCV infections.

The natural history of an HCV infection can be modified by anti-HCV therapy. The current antiviral therapy for a chronic HCV infection is pegylated interferon plus ribavirin. It is recommended for patients with a liver fibrosis score of F2 or more. A sustained virological response, defined as undetectable HCV RNA in the serum 6 months after the end of treatment, is achieved in 40-70% of treated patients, depending on the HCV genotype. Patients infected with HCV genotype 1 have a lower sustained virological response (40%) than patients infected with HCV genotypes 2 or 3 (80%). New antiviral therapies targeting directly the HCV genome, anti-protease and anti-polymerase, will improve the sustained virological rate in HCV genotype 1 infected patients (Legrand-Abravanel et al., 2010, McHutchison et al., 2009). Recently, one genetic marker, interleukin 28B, was identified as a factor influencing the natural history of HCV infection and the treatment success of patients chronically infected: patients with a CC genotype clear the virus more easily (Ge et al., 2009, Thomas et al., 2009).

1.1.3 HCV infection in particular populations

The progression of HCV infections in patients with impaired immune systems differs from that in immunocompetent patients. We will focus on patients with end-stage renal disease and patients with an HIV coinfection.

The blood of patients undergoing regular dialysis is much more likely to contain anti-HCV antibodies (7 to 40%) than that of the general population (Rahnavardi et al., 2008), although the percentage has been lower in the past few years because of improved prevention measures, like using gloves, single-use material, and the isolation of HCV-infected people in dialysis units. But it still occurs in French hemodialysis units and requires appropriate management, even though the prevalence of HCV infection has decreased by 7.7% (Saune et al., 2010). It is difficult to evaluate the natural history of HCV in hemodialysis patients because the exact date of contamination is often unknown and the infection can be silent for several years. The liver enzyme activities cannot be used to predict the development of fibrosis in these patients; they can have hepatic lesions with normal liver enzyme activities (Furusyo et al., 2000, Martin et al., 2000). HCV infection is a significant cause of morbidity and mortality in patients with end-stage liver disease, and particularly in renal transplanted recipients. Death is generally due to liver dysfunction and loss of the renal graft. HCV infection has been linked to shorter allograft survival and more acute rejection episodes and virus-related glomerulonephritis (Pereira et al., 1998). Interferon-based treatment after renal transplantation is associated with graft loss and so is not recommended. It has been recommended that all kidney-transplant candidates be treated with alpha-interferon because of the relatively high rate of sustained virological response in HCV-positive dialysis patients given anti-HCV therapy (Izopet et al., 1997, Rostaing et al., 1998).

About 30% of patients infected with HIV are also infected with HCV. HCV infections have become the main cause of morbidity and mortality due to faster development of cirrhosis and hepatocellular carcinoma. These patients are less likely to spontaneously clear their HCV infection and their HCV virus load is often higher than average (Thomas et al., 2000). The virus load is especially increased in patients with low CD4+ T cells counts, probably due to loss of immune control. The rate of sustained virological response is lower in these patients than in those infected with HCV alone. Only 14-38% of patients infected with HCV genotypes 1 or 4 and HIV achieve a sustained virological response, while 44-73% of patients

infected with HIV and HCV genotype 2 or 3 do so (Carrat et al., 2004, Chung et al., 2004, Laguno et al., 2004, Torriani et al., 2004).

1.2 HCV tropism

HCV mainly replicates in the liver but there are other extra-hepatic replication sites.

1.2.1 The liver

The liver is the major replication site and contains high concentrations of HCV RNA (about 10^8 - 10^{11} copies per g tissue) (Sugano et al., 1995). HCV particles, free or associated with serum apolipoproteins, interact with multiple cell surface proteins on hepatocytes (Boonstra et al., 2009). The initial interaction involves glycosaminoglycans (GAGs) and lipoprotein receptors (LDLR), as well as scavenger receptor class B type I (SR-BI), followed by the recruitment of tetraspanin, CD81, and the later use of the tight junction proteins claudin-1 and occludin (OCLN).

In situ hybridization studies found HCV RNA in 5 to 50% of the liver cells of patients chronically infected with HCV genotype 1 (Pal et al., 2006). The proportion of hepatic cells with detectable negative strand HCV RNA (a marker of HCV replication) was closely correlated with the liver enzyme activities and histological changes. Histological damages observed in HCV infection are the results of productive infection of some liver cells.

1.2.2 Extra-hepatic sites

HCV RNA has been detected in the peripheral blood mononuclear cells of chronically infected patients (Blackard et al., 2005, Laskus et al., 2000, Lerat et al., 1996, Lerat et al., 1998, Muller et al., 1993, Navas et al., 1998), in the central nervous system (Forton et al., 2004, Morsica et al., 1997), and other tissues like pancreas, thyroid, spleen (Laskus et al., 1998), seminal fluid (Bourlet et al., 2002, Pasquier et al., 2000). Negative strand HCV RNA was also detected, suggesting HCV replication.

HCV compartmentalization has been described in lymphocytes B cells and dendritic cells (Ducoulombier et al., 2004). The HVR1-E2 sequences found in cells were different from those found in the plasma. Some of the 109 patients tested had very different strains in their peripheral blood mononuclear cells that was undetectable in their plasma (Roque-Afonso et al., 2005). The sequences found in the peripheral blood mononuclear cells of 9 patients were different enough to be classified as another genotype, distinct from the sequences found in the plasma. These strains could have been acquired during co-infection or super-infection. A significant proportion of patients with hepatitis C are infected with two or more HCV variants with distinct IRES sequences and distinct cellular tropism (Di Liberto et al., 2006, Durand et al., 2010). HCV compartmentalization was an independent predictor of treatment response in this study. They also found a correlation between the cellular tropism of HCV variants for liver or B cells *in vivo* and the translational efficiency of their IRES. The IRES of the B cell-specific strains all had a common activity profile and were consistently less efficient than paired plasma IRES in hepatocytes. These findings suggest that HCV replicates extra-hepatically in chronically infected patients. As HCV genotype 1 was more frequently detected than HCV genotypes 2 or 3, this genotype could be better adapted to mononuclear cells (Lerat et al., 1998). Some patients developed a cellular T cells response against a virus whose genotype

differed from that of the one detected in plasma (Sugimoto et al., 2005). Patients with HCV compartmentalization in mononuclear cells could develop a stronger immune response, so facilitating virus elimination.

2. Virological tools

The diagnosis of an HCV infection (acute and/or chronic) is based on the detection of serological and molecular markers in serum and plasma. They are also used to initiate treatment and to monitor treatment efficacy.

2.1 Serological assays

Anti-HCV antibodies in the blood plasma or serum are detected using third-generation enzyme immunoassays, which are specific for antibodies against various HCV epitopes. Recombinant antigens are used to capture circulating anti-HCV antibodies. Third-generation enzyme immunoassays are better than 99% specific for anti-HCV antibodies. Their sensitivity is more difficult to determine, given the lack of a reference (gold standard) method, but it is excellent in HCV-infected immunocompetent patients. Detection systems for serum HCV antibodies are insensitive in the acute phase because of long serological window. Immunoassays combining detection of anti-HCV antibodies and HCV antigen reduce the serological window of over 25 days (Laperche et al., 2005). Recently, quantitative immunoassays detecting HCV core antigen were developed. The HCV Ag level correlates the HCV RNA concentration. It can reduce the serological window of over 35 days and is highly specific to detect acute HCV infection in haemodialysis patients (Miedouge et al., 2010).

2.2 HCV RNA detection and quantification

The presence of HCV RNA in serum or plasma is first used to diagnose an HCV infection. The presence of HCV RNA alone, with no anti-HCV antibodies, strongly indicates an acute HCV infection, which must then be confirmed by seroconversion (the appearance of anti-HCV antibodies) a few days or weeks later. Acutely infected patients can also have both HCV RNA and anti-HCV antibodies at the time of diagnosis. It is difficult in this case to distinguish acute hepatitis C from an acute exacerbation of chronic hepatitis C. A patient with clinical or biological signs of chronic liver disease will have chronic hepatitis C if both the anti-HCV antibodies and the HCV RNA are present for at least 6 months. Description of HCV replication in the absence of anti-HCV antibodies is rare with the current enzyme immunoassays, but occurs in profoundly immunodepressed patients, hemodialysis patients or agammaglobulinemic subjects.

The presence of HCV RNA is checked regularly during anti-HCV treatment and treatment is stopped or continues depending on the result. The end-of-treatment and sustained virological responses should be assessed with a sensitive HCV RNA assay, with a lower detection limit of 50 IU/mL or less, according to Consensus Conference recommendations (Anonymous, 2002). The detection of HCV RNA at the end of therapy is highly predictive of a post-treatment relapse, whereas the absence of HCV RNA at the end of treatment indicates a virological response. Patients showing a virological response must be retested for HCV RNA with a sensitive method 24 weeks later to identify a sustained virological response, the endpoint of therapy.

Both qualitative and quantitative assays can be used to detect HCV RNA. Qualitative assays use the principle of target amplification with a “classic” polymerase chain reaction

(PCR), “real-time” PCR or “transcription-mediated amplification” (TMA). Quantitative assays are based on target amplification techniques (competitive PCR or real-time PCR) or signal amplification techniques (branched DNA (bDNA) assays). The commercial assays for HCV RNA are summarized in Table 1. Their ranges of quantification vary considerably.

	Supplier	Detection limit IU/mL	Quantification range IU/mL		
			Lower limit	Upper limit	
Qualitative assays					
TMA-based assay Versant	Siemens	5-10	NA	NA	
Cobas Amplicor v2.0	Roche	50	NA	NA	
APTIMA® HCV RNA	Genprobe	5.3	NA	NA	
Quantitative assays					
LCx®HCV RNA Quantitative Assay	Abbott	10	12	100 000 000	
Cobas Ampliprep/Cobas TaqMan	Roche	10	43	69 000 000	
Cobas Amplicor Monitor v2.0	Roche	600	600	500 000	
ARN HCV versant 3.0	Siemens	615	615	7 700 000	

Table 1. Detection limits and quantification ranges of commercial techniques for detecting HCV RNA. NA = not applicable

2.3 HCV genotyping

The HCV genotype is routinely determined before treating because the treatment duration, ribavirin dose and virological monitoring procedures depend on the HCV genotype. The reference method for HCV genotyping is direct sequencing of the NS5B or E1 regions of the HCV genome by means of “in-house” techniques, followed by sequence alignment with prototype sequences and phylogenetic analysis (Sandres-Saune et al., 2003, Simmonds et al., 2005). In clinical practice, HCV genotype can be determined by various commercial kits that use direct sequence analysis of the 5’UTR region (Halfon et al., 2001) or reverse hybridization analysis with genotype-specific probes located in the 5’UTR region (Verbeeck et al., 2008). Unfortunately, analysis of this region tends to misclassify a significant number of HCV subtypes. For example, 20-30% of subtype 1a are not correctly identified (Chen and Weck, 2002). Real-time PCR methods and DNA biochip methods are now available (Gryadunov et al., 2010, Mao et al., 2010, Martro et al., 2008, Nakatani et al., 2010, Park et al., 2009, Verbeeck et al., 2008). Methods based on analysis of the NS5B region are better for discriminating between HCV subtypes (Table 2) (Gryadunov et al., 2010, Sandres-Saune et al., 2003).

Analysis of HCV heterogeneity can be used to determine the source of contamination in cases of nosocomial transmission. The tools should be based on regions of the HCV genome that are variable enough to discriminate between HCV subtypes and even different clusters within a subtype. The best are the NS5B and hypervariable 1 (HVR1)-E2 regions (Figure 1). These are different enough to clearly indicate whether the viruses circulating in different people have a common source (Bracho et al., 2005, Izopet et al., 1999). These regions can also be analysed to determine whether a patient who suffers a late relapse does so because of reactivation of the former virus or because of a new infection with a virus belonging to the same subtype.

Assay	HCV region	Genotype identification	Subtype identification
Line Probe Assay	5'NC (I)	1-6 (mis-typing of G6)	Mis-subtyping
Sequencing	5'NC+core (II)	1-6 (better idenfication of G6)	Subtype 1a/1b
Real time PCR	NS5B or core/E1	1-6	Reference method
	5'NC	1-6 (false idenfication of G6)	Mis-subtyping
DNA biochip	5'NC or NS5B	1-6	Only subtypes 1a, 1b, 2a, 2b, 2c
	5'NC	1-6	Only subtypes 1a, 1b
	NS5B	1-6	More than 30 subtypes

Table 2. Performance of HCV genotyping methods.

3. Definition of an occult HCV infection

The clinical resolution of a hepatitis C infection, either spontaneous or therapy-induced, has conventionally been deemed to reflect the complete eradication of HCV. However, the past 5 years have seen an emergence of several studies documenting the presence of HCV RNA in the liver or peripheral blood mononuclear cells of patients whose serum samples tested negative for HCV RNA by conventional PCR assays, with or without the presence of anti-HCV antibodies. This defined a new form of HCV infection, called occult HCV infection. Occult HCV infections were described using highly sensitive nucleic acid amplification assays with a sensitivity < 3 IU/mL. There are two forms of occult HCV infections: cryptogenic and secondary (Table 3).

Description	Cryptogenic occult HCV infection	Secondary occult HCV infection
<i>HCV RNA detection</i>		
Plasma (conventional PCR assay) *	-	-
Plasma (ultrasentive PCR assay) **	+/-	+/-
Peripheral blood mononuclear cells	+/-	+/-
Liver biospy	+	+/-
<i>Anti-HCV antibodies</i>	-	+
<i>Elevated liver enzymes</i>	yes	no

* Detection limit 50-600 UI/mL, ** detection limit ≤ 3 IU/mL

Table 3. Markers of occult HCV infection.

3.1 Cryptogenic occult HCV infection

Occult HCV infections are termed cryptogenic if the patient has (1) no anti-HCV antibodies and (2) elevated liver enzyme activities (Table 3). Castillo et al (Castillo et al., 2004) first described “occult HCV infections” in patients with hepatic disorders of unknown origin; they appeared to have no anti-HCV antibodies or HCV RNA by conventional techniques. All other known causes of liver disease were excluded (viruses, autoimmune hepatitis, alcoholism, drugs, metabolic and genetic liver disorders). They used very sensitive PCR methods to detect genomic HCV RNA in the livers of 57 of the 100 patients tested. This rate of positive sample was unexpectedly high. Negative strand HCV RNA was detected in 84% of these 57 patients by *in situ* hybridization. The peripheral blood mononuclear cells from 70% of these patients were also positive for HCV RNA.

Another study detected the HCV RNA genome in the hepatocytes of 27 of 31 patients, none of whom had markers of HCV infection or any abnormal liver function test (Idrees et al., 2010). Both positive and negative strand HCV RNA were found in the livers of 8 (25.8%) patients, suggesting ongoing virus replication in hepatocytes. The main studies describing cryptogenic occult HCV infections are shown in Table 4.

3.2 Secondary occult HCV infection

Occult HCV infections have also been described in patients (1) with anti-HCV antibodies and (2) with normal liver enzyme activities who had cleared their HCV infections, either spontaneously or after treatment. This defined cases of secondary occult HCV infection (Table 3).

The persistence of HCV after hepatitis C had been resolved spontaneously or by treatment was first described in 16 patients (Pham et al., 2004). A very sensitive method (detection limit: 10 copies/mL) revealed HCV RNA in the plasma of 88% of them and in the peripheral blood mononuclear cells of 81% of patients; it was also found in 86% of the monocytes tested. Similar results were obtained in 17 patients whose hepatitis C had been resolved by treatment: 24% had HCV RNA in their serum, 53% in peripheral blood mononuclear cells, 41% in lymphocytes and 65% in macrophages (Radkowski et al., 2005a). Castillo et al detected positive and negative-strand HCV RNA in liver biopsy specimens and cells of 20 sustained responders (Castillo et al., 2006). Positive-strand HCV RNA was detected in 95% of liver biopsy specimens and negative-strand HCV RNA (the replication intermediate) was found in 79% of liver biopsy samples that had positive-strand HCV RNA. Thirteen (65%) samples of peripheral blood mononuclear cells had positive-strand HCV RNA; and 12 of these (92%) also had negative-strand HCV RNA. This suggested that virus replication was taking place in the liver of these patients, which could explain the persistence of intrahepatic HCV years after successful antiviral therapy. In another study, HCV RNA was detected in the serum of 54% of patients who had spontaneously cleared their HCV infection; it was also found in the peripheral blood mononuclear cells of 50 to 64% of them (Carreno et al., 2006, Radkowski et al., 2005b). The main studies describing secondary occult HCV infections are shown in Table 4.

3.3 Diagnosis of occult HCV infections

As the first definition of an occult HCV infection was based on detecting HCV RNA in hepatocytes (Castillo et al., 2004), the presence of HCV RNA in the liver is the reference method. However, liver biopsies are not readily available and the newly available non-invasive methods for evaluating fibrosis will make biopsy-based methods less common. Carreño et al (Carreno et al., 2004) showed that HCV RNA can be detected in the peripheral blood mononuclear cells of 70% of patients with an occult HCV infection. So an alternative for diagnosing an occult HCV infection could be to look for HCV RNA in peripheral blood mononuclear cells. Ultrasensitive PCR assay can also detect HCV RNA in the plasma or serum, although it is undetectable by conventional PCR assay. Thus, HCV RNA concentrations of 60 - 160 copies/mL can be detected in the plasma of patients with an occult HCV infection using an ultrasensitive PCR assay (Bartolome et al., 2007)

All the groups that have described occult HCV infections used different methods to increase the chance of detecting low concentrations of HCV RNA (Pham et al., 2010).

- The first were highly sensitive molecular biological methods with a detection limit of about 3 IU/mL. They were based on nested PCR after reverse transcription targeting the 5'UTR region of the HCV genome. Amplification was often improved by nucleic acid hybridization (Southern blotting).

- The second method consisted of stimulating peripheral blood mononuclear cells *ex vivo* by culturing them with mitogens (interleukin-2 and phytohemagglutinin). This increased the detection of the HCV genome in cells apparently not infected with HCV (Pham et al., 2004, Pham et al., 2005). HCV RNA was detected in the peripheral blood mononuclear cells of about 30% of patients who had cleared HCV, but this percentage can increase up to 75% if the mononuclear cells are cultured with mitogens.
- The third method used an unconventional amount of plasma and number of cells, with plasma volumes of 1 - 4 mL and large numbers of cells (5x10⁵ - 4x10⁶ cells) (Bartolome et al., 2007, Castillo et al., 2009, Radkowski et al., 2005a). This improved the diagnosis of an occult HCV infection by 10-15%.
- Finally, it is also important to repeat tests on successive samples of plasma and peripheral blood mononuclear cells because HCV RNA detection is rarely permanent. Repeated testing leads to the detection of occult HCV infections in 100% of cases (Pham et al., 2004, Pham et al., 2008, Radkowski et al., 2005a).

Unfortunately, while these techniques seem to be necessary for detecting an occult HCV infection, they are not really suitable for clinical diagnosis.

Study	Patient number	Negative HCV RNA		HCV RNA detection (ultrasensitive PCR assay)			HCV genotype
		Anti HCV antibodies	Hepatic cytolysis	Liver biopsy	Peripheral blood mononuclear cells	Plasma	
(Castillo et al., 2004)*	100	-	Yes	57/100 (57%)	40/57 (70%)	NA	1b
(Bartolome et al., 2007)*	106	-	Yes	106/106 (100%)	69/106 (65%)	62/106 (58%)	1b
(Barril et al., 2008)*	109	-	Yes	NA	49/109 (45%)	NA	1b
(Idrees et al., 2010)	31	-	Yes	23/31 (74%)	NA	NA	3a, 3b, 1a
(Pham et al., 2004)*†	16	+	No	NA	13/16 (81%)	15/17 (88%)	1a, 1b, 2a
(Radkowski et al., 2005a)*†	17	+	No	3/11 (27%)	9/17 (53%)	4/17 (24%)	1a, 1b, 2a, 3a
(Radkowski et al., 2005b)*†	11	+	No	NA	7/11 (64%)	6/11 (54%)	1a, 1b
(Carreno et al., 2006)*	12	+	No	10/12 (83%)	6/12 (50%)	NA	1b
(Castillo et al., 2006)	20	+	No	19/20 (95%)	13/20 (65%)	NA	1b, 2, 3
(Gallegos-Orozco et al., 2008)†	25	+	No	NA	5/25 (20%)	0/25 (0%)	1, 2

* Southern blot detection, † stimulation of cells in culture with mitogens, NA= not applicable

Table 4. Detection of HCV genomic RNA in different compartments in studies on occult HCV infections.

4. Clinical significance of occult HCV infections

To date, only one study had investigated the clinical characteristics of an occult HCV infection (Pardo et al., 2007). Two groups of patients were compared. One group of 68 patients had a cryptogenic occult HCV infection while the second group of 69 patients had a chronic HCV infection. The groups were matched for age, gender, body mass index and the estimated duration of abnormal liver function tests. Patients with an occult HCV infection had significantly higher plasma cholesterol and triglycerides that patient with a chronic HCV infection. But the activities of their alanine aminotransferases and gamma glutamyl

transpeptidases were lower. Liver biopsies showed that patients with chronic HCV infections had higher necro-inflammatory activities (96%) and fibrosis scores (75%) than did patients with an occult HCV infection (31% and 15%). However, the two groups had similar proportions of patients with cirrhosis or hepatic steatosis. Patients with an occult HCV infection had fewer HCV-infected hepatocytes (5.3%) than patients with a chronic HCV infection (10.1%). Therefore, an occult HCV infection results in fewer hepatic lesions than a chronic HCV infection. This could be due to the slow replication of HCV RNA observed in occult HCV infections.

Another study evaluated the efficacy of anti-HCV treatment in occult HCV infections (Pardo et al., 2006). They treated 10 patients with a cryptogenic HCV infection with pegylated interferon and ribavirin for twenty-four weeks. These patients had HCV RNA in their livers and peripheral blood mononuclear cells, and all had elevated liver enzymes activities. At treatment withdrawal, eight patients had normal liver enzyme activities and the peripheral blood mononuclear cells of eight tested negative for HCV RNA. Twenty-four weeks after treatment withdrawal, six patients still had normal liver enzymes and seven had peripheral blood mononuclear cells that were negative for HCV RNA. Two biopsies were taken from five patients, one before and one after treatment. HCV RNA was found in the second liver biopsy of all five patients. However, the HCV RNA concentrations in the liver biopsy taken after treatment were lower than in the pre-treatment samples and the number of infected hepatocytes was also lower (2.2%) after treatment than before (3.5%). The necro-inflammatory activity and fibrosis scores had also decreased in three of the five patients. Thus treating patients with a cryptogenic occult HCV infection can improve their liver histology.

5. HCV infectivity in occult HCV infection

The crucial questions are, first, whether detecting HCV RNA fragments, even from the negative strand, should be interpreted as ongoing virus replication, or as molecular residues of a resolved HCV infection and, second, whether patients with low concentrations of HCV RNA can transmit an infectious virus. The presence of negative stranded HCV RNA in peripheral blood mononuclear cells and in the liver of more than 50% of patients with occult HCV infection indicated virus replication. The virus detected in the plasma of patients with an occult HCV infection was identified in particles with densities of 1.03-1.04 to 1.08-1.19 g/mL (Bartolome et al., 2007). These densities are similar to those of the HCV viruses found in chronically infected patients.

Cultures of lymphoid cells established by treating peripheral blood mononuclear cells from healthy individuals with a T cell inducing mitogen *ex vivo* are susceptible to wild-type HCV and capable of supporting its complete replication cycle (MacParland et al., 2006). This system was used to investigate the infectious capacity of low concentrations of HCV RNA, derive from the blood plasma or from the supernatant of peripheral blood mononuclear cells of patients with a sustained virological response. The residual virus in the plasma of patients with an occult HCV infection can be infectious *in vitro* (MacParland et al., 2009). The HCV carried by three of the nine individuals studied produced an infection *in vitro*. Thus, the HCV RNA detected in the plasma of patients with an occult HCV infection was found to be infectious, however the number of experiments was small. If these data are confirmed, an occult HCV infection could facilitate the clinical reactivation of an HCV infection, especially in patients with a damaged immune system. The public health impact and significance for blood and organ donation of such a situation could be very serious.

However, the infection of peripheral blood mononuclear cells is surprising because they do not have some of the membrane receptors that are essential for HCV entry into hepatocytes. They have no SR-BI, claudin-1 or occludin receptors. Moreover, cell-culture produce HCV (HCVcc) could not replicate in peripheral blood mononuclear cells, whatever the cells are (Marukian et al., 2008).

6. Immunology in occult HCV infections

Adaptive cellular immune response mechanisms, especially T cell responses, are believed to play a key role in the recovery from an HCV infection as well as in chronic hepatitis C. Proliferative CD4⁺ and CD8⁺ T cell responses are more efficient in patients with an occult HCV infection than in patients with a chronic HCV infection.

6.1 Humoral immune response

Patients with a classical HCV infection develop specific anti-HCV antibodies against virus structural and non-structural proteins. The antibodies appear late after the HCV infection and can never be detected in some cases. HCV can easily evade control by the humoral immune system. The titer of anti-HCV antibodies decrease rapidly in recovered chronically HCV infected patients given interferon-based therapy (Maylin et al., 2009, Toyoda et al., 2005) and can sometimes completely disappear about two decades after HCV recovery (Takaki et al., 2000). Loss of anti-HCV antibodies can be observed in 6 to 20% of immunocompetent patients (Kondili et al., 2002, Lefrere et al., 2004) and in hemodialysis and immunocompromised patients. Patients with a cryptogenic occult HCV infection could be patients who have lost their anti-HCV antibodies.

An immunoenzymatic test targeting the core protein has been developed recently. It detects immunoglobulin G that targets an immunodominant epitope of the HCV capsid (amino acids 5 to 19) that is not detected by other tests. Anti-HCV core antibodies are detected in 98.6% of chronically infected individuals. This test was used to examine the plasma of 145 serological silent patients with a cryptogenic occult HCV infection; 45 of them tested positive for anti-HCV antibodies (Quiroga et al., 2009). This IgG anti-HCV core test identifies occult HCV infections in seronegative, non-viremic patients and may be useful for tracking infections in patients who test negative for anti-HCV antibodies.

6.2 Cellular immune response

Specific T-cell responses alone can control an HCV infection even without an efficient humoral immune response (Post et al., 2004). The cellular immune response can still be detected decades after recovery from a chronic or acute HCV infection (Semmo et al., 2005, Takaki et al., 2000). The authors found that HCV-specific T helper cells and cytotoxic T-cell responses with an interferon-gamma phenotype persisted. HCV specific T-CD8⁺ cells have been detected in people who were not infected with HCV but were in continuous contact with chronically infected HCV patients (Scognamiglio et al., 1999). This suggests that an immune response can be constructed in response to a subclinical HCV infection. The persistence of a T-specific immune response could be the result of low concentrations of HCV RNA that are undetectable by present techniques. One study compared the cellular immune responses of 50 patients with a cryptogenic occult HCV infection, 141 patients with a chronic HCV infection and 21 patients with cryptogenic liver pathology (Quiroga et al.,

2004). Fifty-two per cent of the patients with a cryptogenic occult HCV infection had a specific proliferative CD4⁺ T cell response to HCV. Significantly fewer patients with a chronic HCV infection (26%) had this CD4⁺ T cell response. The specific HCV T cells detected targeted non-structural proteins and produced gamma interferon. The specific proliferative CD8⁺ T anti-HCV response in patients with an occult HCV infection was also stronger than in chronically infected patients. The cellular immune response may be more efficient in patients with an occult HCV infection, but it is not strong enough to definitively eliminate the virus. The same team demonstrated that HCV-specific CD4⁺ and CD8⁺ proliferative responses were observed more frequently in patients who have spontaneously eliminated the virus than in those whose chronic infection was eliminated by treatment (Quiroga et al., 2006).

The peripheral blood mononuclear cells like dendritic cells, monocytes, CD4⁺ and CD8⁺ T cells, and B cells of chronically infected patients can contain HCV virus particles. Pham et al found that the same cells were infected in patients with an occult HCV infection (Pham et al., 2008). However, the cytokine profiles differed depending on the type of infection (Pham et al., 2009). The peripheral blood mononuclear cells of patients with occult HCV infection produced more alpha-interferon, gamma interferon and tumor necrosis factor alpha than did those of patients with a chronic HCV infection. But transcription of the interleukin 10 gene was lower in patients with an occult HCV infection. Clearly, the impact of an occult HCV infection on the immunological function of T cells seems to be different from that of a chronic HCV infection, and needs further investigation.

7. Populations at risk of an occult HCV infection: hemodialysis and immunocompromised patients

Genomic HCV RNA was very recently found in the peripheral mononuclear cells of 45% of a group of serum HCV antibody-negative/HCV RNA-negative hemodialysis patients with elevated liver enzyme activities (Barril et al., 2008). This could have a big impact on the management of hemodialysis patients in dialysis units. But these results should be interpreted with caution for several reasons (Kamar et al., 2009). First, they found that the liver enzyme activities of patients with an occult HCV infection were abnormal, whereas the liver enzyme activities of dialysis patients with a chronic active HCV infection were often within the normal range. Second, they reported a very high percentage of deaths (39%) during the short follow-up, but these deaths were not due to HCV liver disease. This suggests that there was another underlying disease, other than an HCV infection, that was responsible for the increased liver enzyme activities. Third, seven of these hemodialysis patients had received a kidney transplant, but their serum remained HCV RNA-negative after kidney transplantation. There is usually a significant increase in the serum HCV RNA concentration of HCV RNA-positive patients after transplantation because of the loss of immune control under immunosuppression (Gane et al., 1996, Pereira and Levey, 1997, Rostaing et al., 2000). Hence, it is surprising that no HCV RNA was detected in the serum of the seven kidney transplant patients who had an occult HCV infection before transplantation.

HCV reactivation should readily occur in immunocompromised patients if HCV really does persist, due to the loss of immune control caused by the regimen of immunosuppressive drugs. We monitored 26 kidney-transplant patients for 10.5 years (range 2–16) after they had

eliminated their HCV while on hemodialysis. We search for the presence of HCV RNA in liver biopsies, blood plasma and peripheral blood mononuclear cells (Nicot et al., 2010). The peripheral blood mononuclear cells were stimulated with a mitogen in culture to increase the chance of detecting low concentrations of HCV RNA. We repeated the tests using a very sensitive RT-PCR assay with Southern blotting detection (detection limit, 2 IU/ml). We found no residual HCV RNA in samples tested at the last follow-up. Half the patients were given rabbit antithymocyte globulins and anti-CD3 monoclonal antibodies during induction therapy and for biopsy-proven acute rejection, twenty patients were given mycophenolic acid. These drugs increase HCV viremia in HCV-infected patients (Nelson et al., 2001, Rostaing et al., 2000, Zekry et al., 2004), but none of the kidney-transplant patients tested had any detectable HCV RNA in their plasma. And none of them developed any HCV-related glomerulopathy or liver disease during this long follow-up. No patients who underwent a post-treatment liver biopsy showed any deterioration of their liver histology. The fact that formerly HCV-infected immunocompromised patients did not suffer a relapse suggests the complete eradication of HCV after its elimination while on dialysis.

8. Occult hepatitis C virus infection: the controversy

The data that are presently available on occult HCV infections are conflicting. Studies carried out by three different groups are in favor of occult HCV infection (Carreno et al., 2006, Castillo et al., 2004, Castillo et al., 2005, Castillo et al., 2006, Radkowski et al., 2005a, Radkowski et al., 2005b), while those of many others support the recovery of an HCV infection (Bernardin et al., 2008, George et al., 2009, Maylin et al., 2008, Maylin et al., 2009, Nicot et al., 2010, Swain et al., 2010, Wiegand et al., 2004). Several arguments are in favor of the absence of persistent HCV RNA. HCV is an RNA virus that has no latent stage in its replication cycle and its genome cannot persist as DNA, unlike viruses like HIV, HBV and herpes viruses. It is therefore unclear how low concentrations of HCV can persist.

8.1 Relapse rate is low in patients successfully treated

Relapse rate is extremely low. However, for patients who experienced late relapse, it is not clear whether they suffer a true relapse or are re-infected.

8.1.1 Results of long-term follow-up studies

Long-term follow-up studies have indicated that patients achieving a sustained virological response are at little risk of a late virologic relapse. One conducted on more than 1300 patients given peginterferon alfa-2a, alone or in combination with ribavirin assessed whether a sustained virological response was synonymous with HCV elimination (Swain et al., 2010). It included HCV infected patients alone or co-infected with HIV, with elevated or persistently normal liver enzyme activities. They found that 99.1% of the patients who achieved a sustained virological response after treatment still had undetectable HCV RNA in their serum after a mean follow-up of 3.9 years (range, 0.8 –7.1 years). Another large review of more than 4000 patients derived from studies conducted between 1994 and 2008 concluded that only 3% of sustained virological responders showed evidence of a late recurrence of HCV RNA (between 6 months and 7 years) (Welker and Zeuzem, 2009). Immunocompetent and immunocompromised patients (liver or renal transplanted patients) treated with interferon or pegylated interferon alone or associated with ribavirin were

included. Smaller studies reported similar findings: serum HCV RNA remained undetectable in 92% to 100% of patients who achieved a sustained virological response after 2 to 13 years of follow-up (Desmond et al., 2006, Formann et al., 2006, Marcellin et al., 1997a, Veldt et al., 2004).

The description of occult HCV infections was followed by several large cohort studies looking for trace amounts of HCV in the plasma, peripheral blood mononuclear cells and/or liver of various populations. HCV RNA was not detected in the plasma or peripheral blood mononuclear cells of 156 of 344 successfully treated immunocompetent patients followed-up for a median of 3.3 years. While none of these patients suffered a virologic relapse, HCV RNA was detected in 2 of the 114 liver biopsies tested (Maylin et al., 2008). A study on 69 aviremic blood donors found no detectable HCV RNA in their peripheral blood mononuclear cells (Bernardin et al., 2008). Another on aviremic and viremic patients with cryptogenic liver disease HCV-associated systemic vasculitis, or connective tissue disease detected HCV RNA only in the peripheral blood mononuclear cells of patients with viremic HCV RNA (Halfon et al., 2008). These results were obtained using appropriate sample processing and highly sensitive methods; they do not support the idea of HCV persistence.

8.1.2 Improvement of liver histology after successful anti-HCV treatment

The vast majority of patients achieving sustained virological response demonstrate histologic improvements on post-treatment liver biopsies relative to pretherapy (Pearlman and Traub, 2011). The fibrosis scores of 82-88% patients were improved and cirrhosis regressed in 64% of patients. Only a small percentage of liver specimens taken after an interferon-induced sustained virological response contained persistent HCV RNA. For example, only 7 (2%) of 400 sustained virologic responders had detectable HCV RNA in post-treatment liver biopsies (McHutchison et al., 2002). And two of them had a virological relapse 12 months following completion of treatment. Histological studies showed that reduced liver inflammation and improved fibrosis scores in patients with a chronic HCV infection and advanced fibrosis or cirrhosis often accompanies a histological improvement (Formann et al., 2006, George et al., 2009, Marcellin et al., 1997a). This supports the idea of HCV elimination.

8.1.3 Few virological relapse in immunocompromised patients

A few cases of HCV recurrence have been reported in immunocompromised patients, supporting the hypothesis of HCV persistence. One patient was reinfected by the same HCV genotype after chemotherapy for lymphoma (Thomopoulos et al., 2008). The re-emergence of HCV was described in a patient who had been given a short course of prednisolone seven months after the end of HCV therapy, and in another who underwent a kidney transplantation seven months after the end of HCV therapy (Lin et al., 2008). Studies on larger cohorts of patients with an impaired immune system due to HIV coinfection (Page et al., 2010) or immunosuppressive treatment following renal transplantation (Kamar et al., 2003, Nicot et al., 2010) found no HCV RNA persisting in either the serum or peripheral blood mononuclear cells. If HCV really does persist, we should find a greater percentage of relapse in HIV coinfecting patients and renal transplanted patients. These data point to the definitive elimination of HCV in patients with undetectable HCV RNA.

8.2 How explain disagreement between studies?

The disagreement between reports of the definitive elimination of HCV or its persistence may be linked to differences in the criteria used to defined plasma viremia in apparently recovered patients. The standard techniques used to classify patients as sustained virological responders in studies describing occult HCV infections ranged from 50-600 IU/mL (135 - 1000 copies/mL), whereas sensitive techniques with detection limits of < 50 IU/mL have been recommended since 2002. About 60% of patients with an occult HCV infection had detectable HCV RNA in the plasma and their mean virus load was 71 HCV RNA copies/mL (range 18-192) (Bartolome et al., 2007). Therefore, HCV genomic RNA should have been detected by conventional tests if sensitive commercial RT-PCR methods had been used to screen the patients. This is supported by the demonstration that serum samples from 6.1% of 184 sustained virological responders that were HCV RNA negative with a standard PCR assay (detection limit 100 IU/mL) tested positive with the TMA assay (detection limit: 5 IU/mL) (Morishima et al., 2008). It is therefore important to use sensitive methods to determine the virological response after anti-HCV treatment.

New HCV infection cannot be excluded in studies showing persistent HCV infections because detailed viral molecular analysis was not used. The patients in these studies were often infected by intravenous drug use or unknown routes and so possibly became re-exposed to the same source of contamination. Most analyses were done on the conserved 5'UTR region, which do not discriminate well subtypes. No accurate phylogeny was done on the NS5B or HVR1-E2 region of the HCV genome. It is therefore difficult to be sure that it was exactly the same virus that reappeared or to exclude re-infection with a common source and a similar virus. This must be borne in mind in studies describing late relapses.

8.3 Kinetics of HCV RNA in cells under treatment

Analysis of the kinetics of HCV RNA in compartments other than the plasma may help us to understand HCV replication and identify clinically significant patterns of response to treatment. The declines in the concentrations of HCV RNA in the peripheral blood mononuclear cells and plasma of patients treated with pegylated interferon and ribavirin were comparable during the initial 12 weeks of therapy (Pugnale et al., 2008). The decrease in HCV RNA in the peripheral blood mononuclear cells started as early as in plasma in many patients, while the kinetics in the two compartments differed markedly for some of them, hinting at compartment-specific HCV replication and response to treatment. HCV RNA was undetectable in the peripheral blood mononuclear cells in 0% of patients on day 0, in 5% on day 1, in 15% on day 4, in 23.6% on day 8, in 48.6% on day 22, in 58% on day 43, in 73% on day 71, and in 81% on day 85. This progression reflects the overall decay of HCV RNA in these cells. The rapid loss of virus from peripheral blood mononuclear cells was associated with a sustained virological response. Another study explored the presence of HCV RNA in different cell subsets (CD4+, CD8+, NK and B cells) of 34 HIV-HCV coinfecting patients (23 sustained virological responders and 11 relapsers) at the end of antiviral therapy (de Felipe et al., 2009). HCV RNA was detected in cell subsets of 9 patients: two who achieved a sustained virological response and 7 who relapsed. There is thus a significant association between the presence of HCV RNA in cell subsets at the end of treatment and viral relapse. In the light of the high proportion of HCV-infected cells in cases of occult HCV infection, it is rather surprising that no more cases of HCV relapse were observed in this population.

8.4 Immune control of residual HCV RNA?

A recent study provides data that could resolve this controversy. It seems that low concentrations of HCV RNA reappear sporadically after successful therapy in a small proportion of patients and that this is associated with stimulation of the cellular immune response that controls HCV infection (Veerapu et al., 2011). The plasma and peripheral blood mononuclear cells of 117 patients who had recovered from an HCV infection (tested with Cobas Amplicor HCV test, limit of detection: 100 IU/mL or 270 copies/mL) were re-tested for HCV RNA with a more sensitive method (detection limit: < 40 copies/mL). The plasma of none of the 19 spontaneously recovered patients contained detectable HCV RNA. The cells of one of them tested positive for HCV RNA. The intensity of the PCR band decreased in cells collected later until complete clearance of HCV RNA from the cell compartment by week 93. This suggests that the persistence of a low concentration of HCV RNA is not a common feature of spontaneous HCV clearance. Plasma samples of 15 of 98 (15%) recovered treated patients tested positive for HCV RNA and the peripheral blood mononuclear cells of 3 of 76 patients tested were positive. All the samples obtained later tested negative. The time that had passed since the cessation of therapy differed between those patients with detectable HCV RNA and those without. HCV RNA was mostly detected in the first 8 years after the end of therapy. All later samples tested were HCV RNA negative. The HCV-specific T cell response was more vigorous at the HCV RNA-positive time points than at HCV RNA-negative ones. It triggered non-structural proteins, which are not part of the virus particle but are present only when the virus infects cells and virus RNA is translated into proteins. There could therefore be a correlation between an increased antiviral T-cell response and persistent low concentrations of virus, with the T-cell response stimulated by antigen from the newly translated HCV RNA. These data suggest that trace amounts of HCV RNA may persist for a limited but not indefinite time after successful therapy and may sporadically reappear in the circulation. The residual virus is perpetually kept in check by the immune response. This may be missed in standard clinical evaluations, which typically assess the presence of HCV RNA at a single time point 6 months after cessation of therapy. Moreover, this study shows that peripheral blood mononuclear cells are unlikely to be a long-lived reservoir of HCV in aviremic patients.

9. Conclusion

Most reports of HCV RNA sequences in cell or liver specimens despite HCV RNA being undetectable have come from a relatively few teams studying small cohorts of patients, but none of these patients experienced a real virological relapse. The clinical impact of low levels of HCV replication is not yet well defined. It is not clear whether these findings are replication-competent virus, have any clinical significance, or whether the situation predisposes to virus breakthrough. However, the majority of data from long-term follow-up studies on large cohorts of patients have failed to confirm occult HCV infection. Patients achieving a sustained virological response are very unlikely to suffer a relapse. Very few of them had detectable HCV RNA in their livers, the main replication site, and the liver histology of the majority of them improved. Further well-designed, multicenter, prospective trials are necessary for interferon-based therapies and also in the future for antiviral therapies based on specifically targeted antiviral therapy for HCV to finally resolved these conflicting data.

Waiting new results, we must therefore conclude that a sustained virological response should be considered to be a durable marker of virus eradication because there is limited evidence for occult HCV infection. The patients can be considered not infectious and cured from a virological standpoint.

10. References

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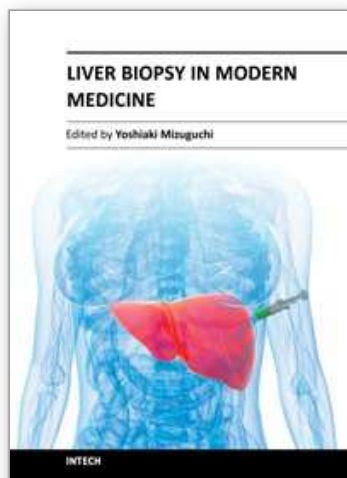
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Liver biopsy, first performed by Paul Ehrlich in 1883, remains an important diagnostic procedure for the management of hepatobiliary disorders and the candidate/donated organ for transplantation. The book "Liver biopsy in Modern Medicine" comprises 21 chapters covering the various aspects of the biopsy procedure in detail and provides an up-to-date insightful coverage to the recent advances in the management of the various disorders with liver biopsy. This book will keep up with cutting edge understanding of liver biopsy to many clinicians, physicians, scientists, pharmaceuticals, engineers and other experts in a wide variety of different disciplines.

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