IMMUNODISREGULATION IN CHRONIC HEPATITIS C INFECTION: THE MECHANISMS OF AUTOANTIBODY PRODUCTION

Ph.D. THESIS

HODA HASSAN GOHAR, M.D., M.Sc.

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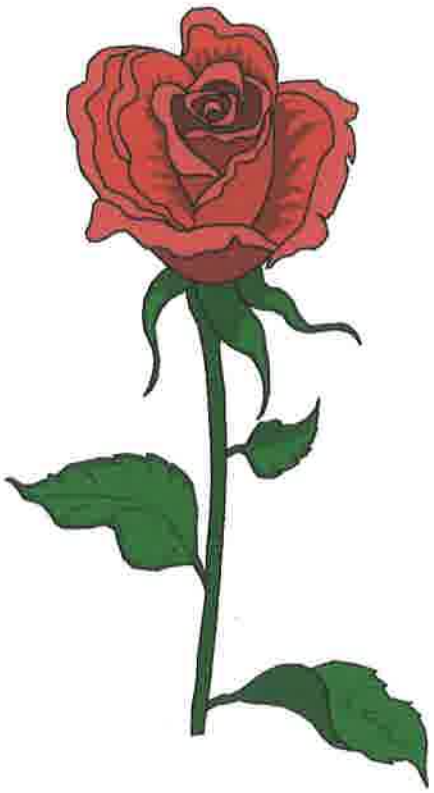

To My Family,
My Mother and
The memory of
My Father

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Summary

Chronic hepatitis C virus infection produces several alterations of immunological reactivity which may contribute to the disease progression and influence response to antiviral therapy and development of hepatocellular carcinoma. In this study, which includes 117 patients and 20 healthy volunteers, the presence of autoantibodies and their correlation with the degree of liver damage, host response to viral antigens, response to interferon-α (IFN-α) therapy and virus genotype, as well as T cell phenotype, NK cell activity and relevant cytokines production were analysed. The main findings include a direct correlation between the number of autoantibodies tested and Knodell's Score of liver damage and a direct correlation between HCV core IgM production and presence of antimitochondrial antibodies. There was no significant difference in overall frequency of autoantibodies in HCV-PCR positive and negative groups of patients. However, antimitochondrial autoantibodies as well as HCV-core IgM were associated with presence of HCV in the serum and non-responsiveness to IFN-α therapy. Geno (sero) type 4 was dominant in the analysed group of patients and, again, the presence of IgM to core antigens and antimitochondrial antibodies correlated with non-responsiveness to IFN-α in this subgroup. Further, non-responsiveness to IFN-α therapy correlated with higher interferon-γ (IFN-γ) and transforming growth factor-β (TGF-β) production, higher percentage of CD25+ cells and lower NK cell cytotoxic activity *in vitro*. Taken together the data obtained demonstrate that the viral genotype and immune response of the host define the outcome of hepatitis C virus infection and response to IFN-α therapy. They suggest for the first time that antimitochondrial autoantibodies may be surrogate parameter of virus persistence and support the notion that cytokine interplay, in particular, with TGF-β production and the activity of putative CD25+ regulatory cells may influence the natural history of HCV infection and response to antiviral therapy.

CONTENTS

INTRODUCTION AND LITERATURE REVIEW

CHAPTER 1: INTRODUCTION & LITERATURE REVIEW

1.1 Structure and biology of hepatitis C virus (HCV)

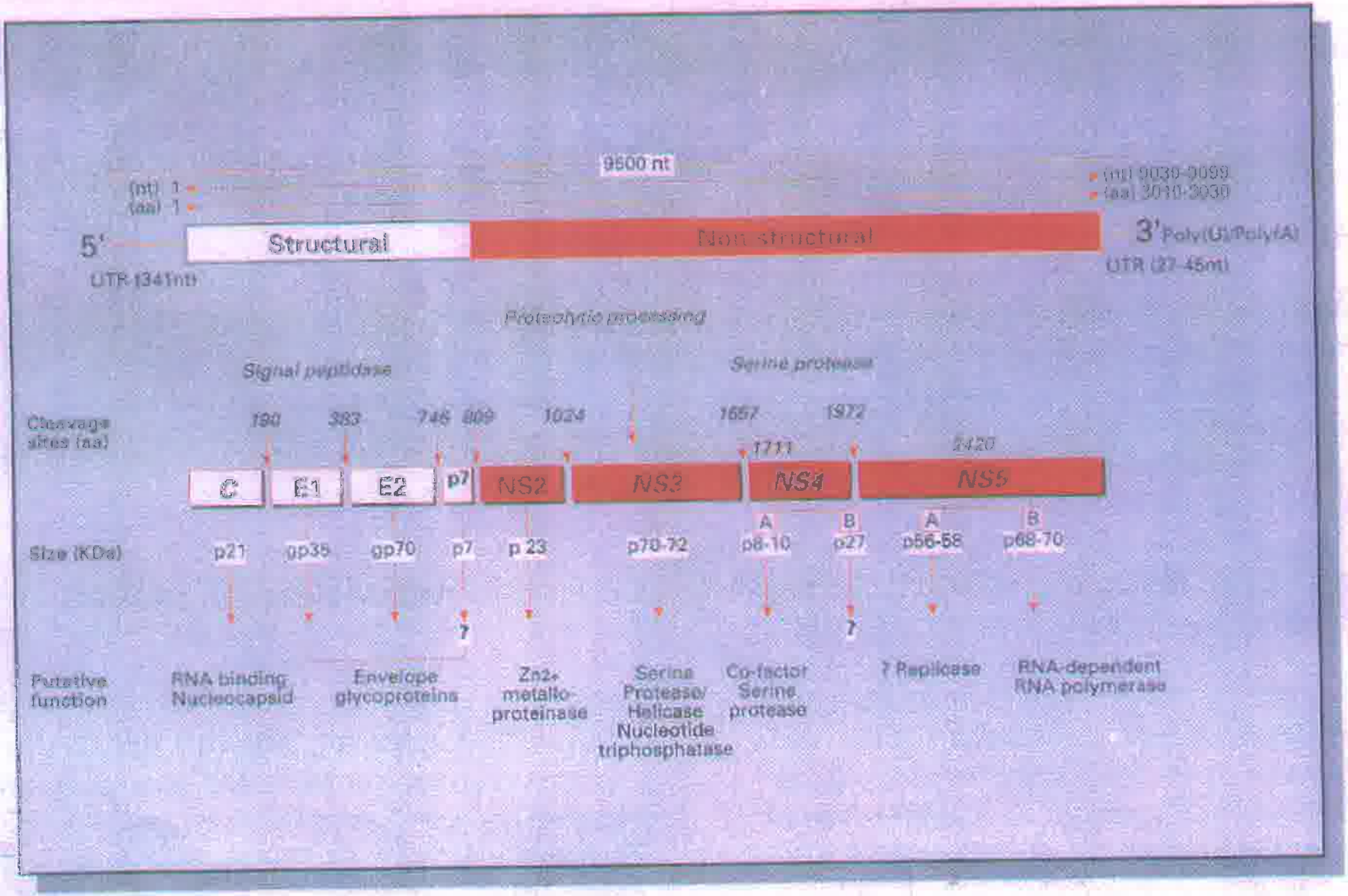
HCV has now been identified as the agent responsible for most cases of parentrally transmitted and many cases of sporadic non A, non B hepatitis (NANB). The discovery and characterization of the main causative agent of NANB hepatitis represent a major success in the application of cloning techniques in the identification of new infectious agents (Choo et al, 1989).

The structure and biology of HCV had been incompletely understood due to the absence of an efficient cell culture system permissive for HCV infection and replication, the lack of a small animal model of HCV infection, and the low viral titers commonly found in serum and liver of HCV-infected individuals. Only recently has the virus been visualized by electorn microscope (Shimizu et al, 1996).

HCV has been classified as a new genus in the flaviviridae family of viruses and important knowledge about HCV has initially been derived from these viruses (Francki et al, 1991). HCV is now believed to be an enveloped virus, approximately 50 nm in size and is known to possess a single stranded RNA genome of positive polarity and approximately 9,500 nucleotides length (Takamizawa et al, 1991). As in flavi- and pestiviruses, the viral genome is composed of a 5′ non-coding region (NCR), a long open reading frame encoding a polyprotein precursor of 3,010 to 3,033 amino acids and a 3′ NCR (Figure A). The 5′ NCR is highly conserved among different HCV genotypes and possesses an internal ribosomal entry site function which is essential for cap-independent translation of the viral RNA and thus holds a key position in the viral life cycle (Tsukiyama-Kohara et al, 1992). The polyprotein precursor is co- and post-translationally processed by both cellular and viral proteases to yield the mature structural and non-structural proteins (Grakoui et al, 1993). The first structural protein encoded by the HCV open reading frame is the core (nucleocapsid) protein

(P19) which is followed by two highly glycosylated envelope proteins, E1 and E2. The latter is thought to contain a region which is hypervariable and may be targeted by neutralizing antibodies. The structural proteins are released from the polyprotein precursor by the endoplasmic reticulum signal peptidase of the host cell (Santolini et al, 1994). The non-structural proteins NS2 through NS5 include two viral proteases essential for processing of the polyprotein precursor, an RNA helicase (Carboxyterminal region of NS3) and an RNA dependent RNA polymerase (NS5B). Cleavage of the polyprotein precursor at the NS2-NS3 junction is accomplished by a metalloprotease encoded by NS2 and NS3 (Hijikata et al, 1993), whereas the remaining non-structural proteins arise via proteolytic processing by a serine protease contained within the aminoterminal region of NS3 (Bartenschlager et al, 1993). NS4A functions as a cofactor for the NS3 protease. The functions of NS4B and NS5A remain to be established. As soon as the HCV genome was cloned, it became evident that viruses isolated from various geographic regions exhibited marked genetic heterogeneity (Moradpour et al, 1996).

Figure (A) Organization of Hepatitis C virus genome (Dusheiko, 1997)



1.2 Viral heterogeneity, genotype and nomenclature

The genetic variability of HCV has been demonstrated by comparative analysis of HCV isolates from distinct geographical areas and follow up studies of infected humans and chimpanzees. There is marked heterogeneity in the viral genome with regard to this variability : the 5′ NCR is highly conserved, in contrast to the envelope and NS5 encoding domains, which show significant changes. Sequence variability is found distributed throughout all genes of the viral genome, but the nucleotide and amino acid sequence of the nucleocapsid protein is highly conserved. High rates of sequence change have been observed in the part of the genome encoding the envelope glycoprotein 2 (E2) (Weiner et al, 1992). A hypervariable stretch of approximately 28 amino acids in the aminoterminal domain of E2 has been termed hypervariable region-1 (HVR1) (Weiner et al, 1991).

As the envelope proteins are likely to lie on the outside of the virus, they could be targets of the humoral immune response to HCV. It has been suggested that continuous genetic change in HVR1 allows HCV to 'escape' neutralisation by the host humoral immune system, and to act as a mechanism for the long term persistent carriage of the virus in the majority of those infected (Weiner et al, 1992).

The major antigenic difference, particularly in the envelope region, will be an important compounding factor in the development of vaccines for HCV. The capsid is more conserved among the different isolates. Thus, different types of HCV have been proposed based on analysis of the 5′ NCR, capsid and NS5 regions (Giannini et al, 1995). The known "genotypes" have been numbered 1 to 6 and the "subtypes" a, b and c in order of discovery. The current system of nomenclature includes 6 major genetic groups (Simmonds et al, 1994). The number of these varies according to the typing system used, but up to 80 different subtypes have been identified (Dusheiko, 1997).

One way to investigate the underlying relationships between the known varients of HCV is to perform phylogenetic analysis of nucleolide sequence of complete genome or subgenomic regions (Simmonds,1995). Nucleotide sequence comparisons were made in the NS5 region between HCV variants present in a worldwide panel of HCV-infected individuals and published sequences. Phylogenetic analysis revealed clustering of sequences into six major groups, some of which contained two or three distinct clusterings of more closely related variants (subgroups). Sequence similarities between members of the different groups ranged from 55% to 72% (mean 64.5%), whereas similarities of the more closely related variants in the subgroups ranged from 75% to 86% (mean 80%). Individual isolates within each of these clusters showed 88% sequence similarity (Simmonds et al, 1993 a).

Current patterns of HCV classification are based on genetic relatedness. Provisional classification of HCV have depended upon simple nucleotide sequence comparisons of complete genomes, or subgenomic fragments between variants found in different individuals. The variability of HCV is structured in a way that has suggested a two tiered classification. This nomenclature of "types" corresponding to the major branches of a phylogenetic tree of sequences from genomic or subgenomic regions of the genome, and "subtypes" corresponding to the more closely related sequences within some of the major groups has been widely adopted (Simmonds et al, 1994).

Several classifications have been proposed, two of them being largely used in the literature (Table A). The first, proposed by Simmonds and co-workers is based on phylogenetic analysis of the E1 and NS5 regions (Simmonds et al, 1993 a) and distinguishes six major genotypes: 1 to 6 (labelled in Arabic numbers), four of these (types 1 to 4) containing several subtypes (a, b and c). The second classification, from Okamoto and co-workers, is based on analysis of the complete genome sequence of seven isolates (Okamoto et al, 1993) and identifies five HCV genotypes: I to V (labelled in Roman numbers). It is now recognized that these isolates described as types I, II, III, IV, and V correspond to 1a, 1b, 2a, 2b and 3a respectively (Simmonds et al, 1994)

Table A: Comparison of different HCV genotype classifications (modified from Simmonds et al, 1994)

Genotype	Chiron	Simmonds/ Chan	Okamoto/ Mori	Enomoto
1a	I	1a	I	K-PT
1b	II	1b	II	K-1
2a	III	2a	III	K-2a
2b	III	2b	IV	K-2b
3a	IV	3	V	n.c.
3b	IV	n.c.	n.c.	n.c.
4	n.c.	4	n.c	n.c.
5	V	n.c.	n.c.	n.c.
6	n.c.	n.c.	n.c.	n.c.

n.c.: not classified.

One potential problem of nomenclature based purely on genotypic classification is the possibility of hybrid viruses arising by recombination. It would be difficult to classify a variant that contained type 1a sequences at one end of the genome and type 2a sequences at the other. There is currently little evidence for the existence of such hybrids, as shown by the existing complete genome sequences and parallel analysis of samples in several regions of the genome, although recombination does occur in RNA viruses, and more work is needed to show whether or not it occurs in HCV (Simmonds, 1995).

Severe and progressive liver disease has been documented on infection with each of the well-characterized genotypes (type 1a, 1b, 2a, 2b, 3a and 4a). Possible variation in the rate of disease progression, differences between genotypes in routes and frequency of person to person transmission, or in the probability of achieving a sustained response to antiviral treatment would indicate the potential utility for the identification of the infecting genotype in certain clinical situations.

For example, if there were consistent differences between genotypes in response to interferon-alpha (IFN-α) treatment, genotyping might play an important role in patient selection and in calculating the most effective duration and dose of IFN treatment to achieve a long term response. Some reports from Japan and Europe have shown an association between infection with HCV 1b (II) and poor response to IFN-α, while infection with HCV 2a (III) seems to be associated with low HCV viraemia levels and good response to IFN-α (Takada et al, 1992; Yoshioka et al, 1992 and Nousbaum et al, 1993).

HCV genotypes may correlate to severity of liver disease, cirrhosis in particular (Brechot and Kremsdorf, 1993). Advanced fibrosis / cirrhosis were significantly correlated with genotype1b infection in patients with chronic hepatitis C with lower serum albumin levels and elevated serum α-fetoprotein levels (Chu et al, 2001).

In some patients, disease severity may be related to the duration of disease, but this is frequently difficult to establish in patients who may have acquired the disease by transmission in childhood. Other factors, including viral load, mode of acquisition, reinfections, host immunity, genetic factors, age, co-existent viral and parasitic infections and alcohol may conceivably determine the outcome of infection (Dusheiko et al, 1994).

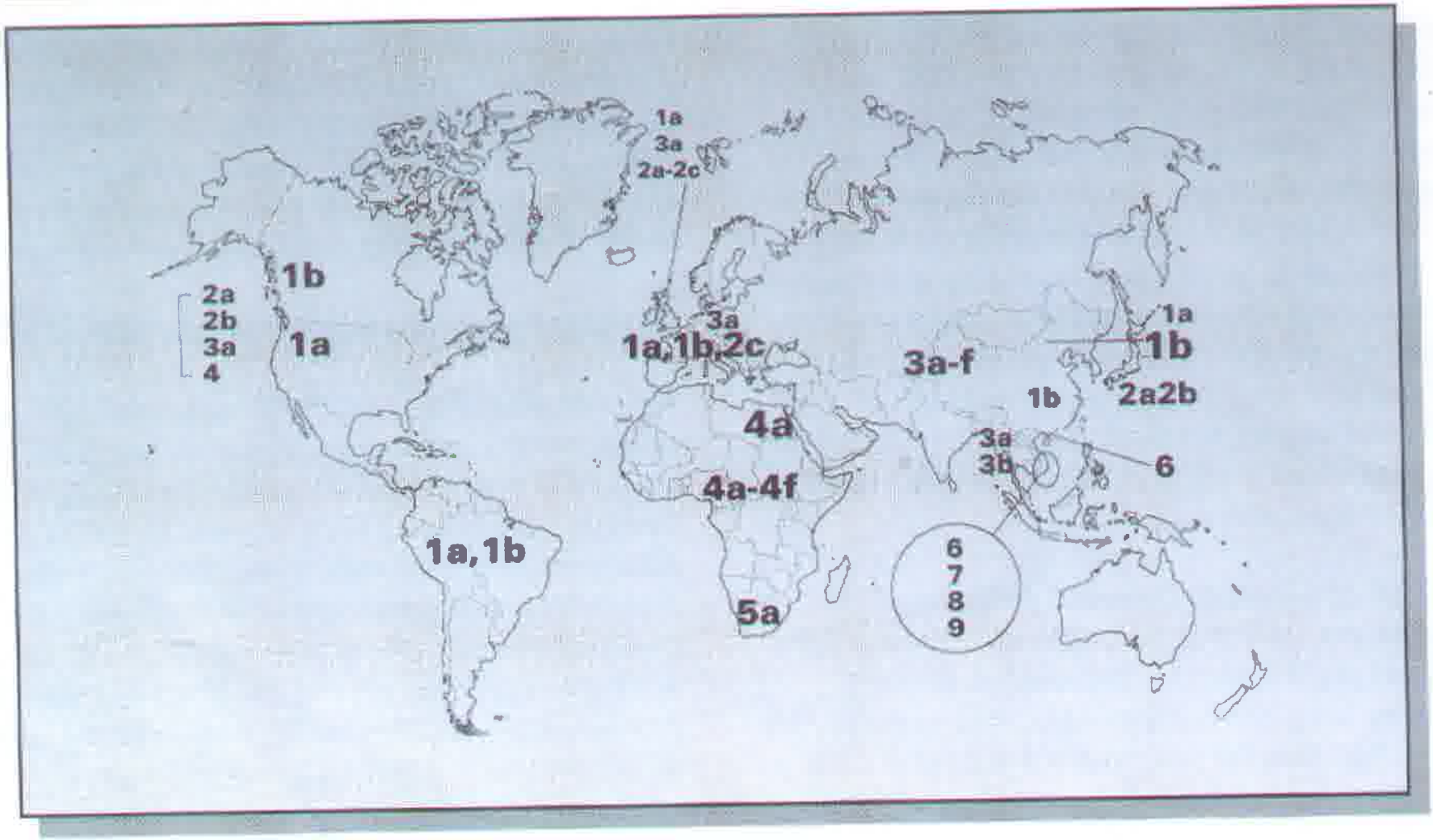
It has been assumed that defined HCV genotypes were confined to specific geographic regions (Figure B). With increasing sequence data it has become evident that multiple genotypes can co-exist in a given geographic region. Various HCV genotypes may even co-exist in a single patient (Tanaka et al, 1992). In both blood donors and patients with chronic hepatitis C in USA and Western Europe genotypes 1a, 1b, 2a, 2b and 3a are found. In Southern and Eastern Europe genotype 1b appears to be more frequent. In Japan, China and Taiwan genotypes 1b, 2a and 2b are found predominantly, there, genotype 1a is confined to individuals who have received blood products originating from the USA. Genotype 3 is prevalent in Singapore and Thailand. A striking geographic change in genotype distribution is apparent between Europe and the Middle East and parts

of North and Central Africa, where infection with genotype 4 is highly prevalent (Simmonds et al, 1993 a). This genotype actually comprises an array of subtypes, which may have resulted from long-term versus more recent introduction of HCV. In Egypt and elsewhere in the Middle East, at least 3 other subtypes of type 4 are identifiable which differ from those found in Central Africa. Genotype 5 is prevalent in South Africa and genotype 6 in Hong Kong (Bukh et al, 1995). Thirty to 50% of anti HCV positive blood donors are infected with type 5a in South Africa (Mc Omish et al, 1994).

Knowledge of the geographic distributions of HCV genotypes not only provides information on virus origin and transmission, but will also be important for antiviral treatment and vaccine development. A vaccine for HCV will probably have to be multivalent because of the high degree of sequence divergence in the envelope gene that makes cross-protection between genotypes unlikely. An active area of research into HCV is the investigation of possible differences in the course of disease associated with different genotypes, such as the rate of development of cirrhosis and hepatocellular carcinoma, and whether certain genotypes are more or less likely to respond to IFN-α treatment. Dusheiko et al (1994) examined the correlation of HCV genotypes and response to interferon or Ribavirin. These two agents have different antiviral actions against HCV. Their data support the possibility that patients with type I, who tend to have more advanced diseases, respond poorly to IFN-α. Japanese patients infected with HCV type II (1b) have lower response rates to IFN-α than do patients with HCV type III (2a) (Giannini et al, 1995).

Figure (B) geographical distribution of hepatitis C virus

(Dusheiko, 1997)



1.3 The clinical signs of hepatitis C infection

The possibility of chronic hepatitis C should be considered in patients presenting with biological signs and symptoms of chronic liver disease associated with a history of a "major" or "minor" risk factor for HCV infection. Major risk factors are blood transfusion prior to 1990 or a history of current or past intravenous drug use. Minor risk factors are related to less obvious parentral or percutaneous contacts with contaminated blood. The mean incubation period of hepatitis C is 6-12 weeks. However, with a large inoculum, such as in cases following administration of factor VIII, the incubation period is reduced to 4 weeks or less (Lee et al, 1991).

Hepatitis C virus causes both acute and chronic hepatitis. However, typical acute hepatitis C is rarely, if ever, seen in general practice since the disease is asymptomatic in 95% of patients. The acute course of HCV infection is clinically mild, and the peak serum ALT elevations are less than those encountered in acute hepatitis A or B. Only 25% of cases are icteric. Subclinical disease is common; such patients may first present decades later with sequelae such as cirrhosis or hepatocellular carcinoma. During the early clinical phase the serum ALT levels may fluctuate and may become normal or near normal, making the determination of true convalescence difficult (Dusheiko, 1997).

In chronic hepatitis C, 50% of patients are without symptoms and many only complain of unexplained fatigue. Since symptoms may be non-existent or too vague, patients may not see their doctors until more serious liver disease has developed. Physical examination may typically be unremarkable; enlarged liver is discerned in only 25% of patients. Typical acute hepatitis C presenting with jaundice and actually elevated liver enzymes (e.g. ALT) and elevated bilirubin, is seldom seen, and symptoms when present are indifferentiable from those associated with acute hepatitis A or B (Gérard and Delwaide, 1997).

Circulating immune complex associated manifestations (e.g. urticaria, purpura, skin rash, arthralgia) are infrequently seen and fulminant hepatitis or subacute

hepatic failure is rare during acute primary HCV infection. Chronic hepatitis C classically is defined as presence of persistent elevation of liver transaminases, particularly ALT, due to HCV for at least 6 months. Chronic hepatitis C is usually a chance finding discovered incidentally as a result of anti-HCV antibody screening of blood donors or in the context of a general health workup or other medical examinations calling for liver function testing. Symptoms when present may include one or more of the followings: Prolonged Flu, malaise, myalgia, anorexia, nausea, fever, muscle and or joint pain and pain over the liver (i.e. right upper quadrant pain) (Gérard and Delwaide, 1997).

1.4 Transmission and risk factors of hepatitis C infection

Although the precise mode of acquisition of hepatitis C is often uncertain, hepatitis C is known to be transmitted by parenteral, or inapparent parenteral contact routes. The virus circulates in relatively low titer in blood, but transmission by blood transfusion and blood products including factor VII, factor IX, fibrinogen and cryoglobulin has been unequivocally documented. Similarly, transmission among intravenous drug abusers through shared needles accounts for the high prevalence of infection in this group (Dusheiko, 1997).

Age, male gender, marriage, anti-schistosomiasis injection treatment, blood transfusion, invasive medical procedure (surgery, catheterization, endoscopy and / or dialysis), receipt of injections from "informal" health care provider and cesarean section or abortion had been described as risk factors which are significantly associated with seropositivity for hepatitis C (Habib et al, 2001). In an other recent study, nosocomial infections and tattooing were found to be the most important risk factors for transmission of HCV (Muller et al, 2001). Organ transplant recipients are at high risk of acquiring HCV infection. Infection in this setting can derive from recurrence of HCV infection already present prior to transplantation, to transfusion-associated transmission during transplantation, or to the presence of HCV infection in the organ donor. Antibody tests may underestimate the incidence of transmission and the prevalence of HCV infection among immunosuppressed organ recipients, hence HCV-RNA testing may be required to detect those cases who lose or do not develop HCV antibodies

(Genescà et al, 1995).

1.4.1 Parenteral transmission :

The parenteral route of HCV transmission is responsible for almost two thirds of hepatitis C cases and constitutes the most commonly recognized and best characterized transmission mechanism of HCV. Before the implementation of mandatory anti-HCV screening in 1990, there was a wide range of transfusion-associated hepatitis C (TAH-C) incidents in different geographic areas. Screening of blood donors for anti-HCV has practically eradicated TAH-C so that transfusion of screened blood should no longer be considered a primary risk factor for HCV infection. Intravenous drug addiction carries extremely high risk of HCV infection because of repeated exposure to carriers of HCV through shared, contaminated needles. Several other groups have been shown to be at risk. These include haemodialized patients. The high prevalence of HCV infection in haemodialysis patients has been attributed not only to the frequency of blood transfusion but also to increasing years on dialysis, suggesting that HCV may be transmitted between patients in the dialysis unit probably as a result of poor infection control practices (Dussol et al, 1995).

1.4.2 Nosocomial transmission :

Previous hospitalization is an epidemiological risk factor in patients with HCV infection. Since the prevalence of HCV infection among hospitalized patients is rather high (between 2 and 20% depending on the patient setting) (Alter, 1995), nosocomial transmission is likely if desinfection procedures are inadequate and contaminated equipment is shared between patients. It is likely that with the dramatic decrease of transfusion-associated hepatitis C, nosocomial transmission becomes the predominant mode of health care-associated spread of hepatitis C virus (Quer and Esteban, 1997).

1.4.3 Health care :

Transmission of HCV from infected patients to health care workers has been documented, and molecular evolutionary analysis has confirmed this mode of transmission. Health care workers have a higher prevalence of anti-HCV than blood donors. Dentists seem to be at special risk for HCV infection, where

seropositivity correlates with the proportion of intravenous drug user clients, involvement in oral surgery and years of practice. Hence, the prevalence of HCV infection among health care workers appears to reflect that of the general population or the specific high risk group they serve, as well as the risk of accidental percutaneous injuries associated with a specific type of care (Suzuki et al, 1994).

1.4.4 Perinatal transmission :

Early studies on vertical transmission based on antibody detection failed to demonstrate evidence of HCV transmission to newborns, except for children born to mothers with concomitant HIV infection (Esteban et al, 1992). However, other reports have shown that even in the absence of HIV coinfection, perinatal transmission of HCV occurs in 3 to 5% of newborns to carrier mothers and that the risk of transmission correlates with HCV-RNA levels in the mother. No transmission seems to occur when HCV-RNA levels are below 10^6 genome equivalent / ml. Breast-feeding carries no further risk of transmission (Lin et al, 1994).

1.4.5 Community acquired transmission :

The majority of hepatitis C cases cannot be accounted for by past blood transfusion, or indeed an identifiable source of parenteral exposure to this virus. The disease is prevalent in many parts of the world where the transmission is probably not caused by blood transfusion or intravenous drug abuse. The precise mechanism of most cases of transmission of community-acquired disease is uncertain, but transmission by close person-to-person contact from carriers of HCV, is the most plausible method of explaining transmission in these societies. Sexual transmission seems certainly possible, albeit a relatively inefficient and infrequent means. Transmission by saliva (or saliva containing blood) and by a human bite has been reported (Dusheiko et al, 1990) ; (Wang et al, 1991).

1.5 Diagnosis of HCV infection

Subjects come to diagnostic evaluation for hepatitis C either because they are blood donors who have tested positive on a screening assay or because they are patients with physical complaints suggestive of hepatitis. Diagnosis is achieved through a process of testing and physical examination for symptoms, but an asymptomatic donor whose test results are confirmed by immunoblotting has a high probability of an HCV infection. Symptoms and the finding of elevated liver enzymes add to this probability, and a liver biopsy is indicated as follow up (Decker and Troonen, 1997).

Current serological tests for antibodies to hepatitis C, unlike tests for hepatitis A and B, are unable to differentiate between acute, chronic or past infections. Another unique problem in diagnosing acute hepatitis C is that seroconversion to anti-HCV reactivity is delayed until well after the acute phase. Even with third generation tests, the time from acute illness to the appearance of the antibodies can range from 3 to 6 weeks. Several studies have reported detecting IgM antibodies to HCV proteins as early markers in acute-phase illness (Lau et al, 1994).

While these antibodies can be found in some patients early on, they are not invariably present earlier than IgG antibodies, and signals may be relatively modest. In practice, when hepatitis C is the suspected acute illness, it is customary to perform ELISA testing at regular intervals until a diagnosis is made. At the present time, HCV-RNA is the only viral marker to be observed with good frequency before and during symptoms. Diagnostic testing for HCV-RNA by RT-PCR or other methods is expensive and is presently called for only in cases of confirmed chronic hepatitis C. Often one finds that antibody serology, liver function, and HCV-RNA vary independently of each other; not all acute or chronic hepatitis C patients will have elevated ALT, and HCV- RNA cannot be detected in a portion of patients who are antibody-confirmed positives (Decker and Troonen, 1997).

1.5.1 Detection of HCV antibodies:

The first commercially available diagnostic tests were based on antibodies to an expressed protein (c100-3) derived from the NS3 / NS4 region. This antigen represents 363 amino acids of viral sequence from the NS4 region (4% of total viral protein), and included a fusion protein (superoxide dismutase or SOD) for expression in yeast. Most of the initial sero-epidemiological and diagnostic studies of hepatitis C were initially based on the prevalence of antibodies to c100-3. Current immunoassays for anti-HCV are based on detection of antibody to additional translation products of HCV genes. While the majority of blood donors with typel HCV infection are positive for anti-c100-3, a third of blood donors may not react to this NS4 antigen present in first generation assays (Nagayama et al, 1993).

Other antigens have also been expressed in yeast or E coli, including the 22KDa core protein of HCV, and a second series of non-structural antigens, including c33, and c200, from the NS3, NS4 and NS5 regions. These antigens are included in second and third generation solid phase enzyme linked immuno-assays for antibodies to HCV, which considerably improve the sensitivity of diagnosis.The majority of patients with chronic hepatitis C are anti-C22 positive, indicating a strong anti-capsid response. It had been reported that anti-E2 antibodies are present in the majority of viraemic carriers (Ralston et al, 1993).

It is suspected, but unproved, that antibodies to the envelope glycoproteins are neutralizing. IgM antibody tests have also been developed, but at present, there are no antibody patterns which differentiate persistent viraemia from an episode of resolved viraemia. The presence of HCV-RNA in the absence of anti-HCV antibodies may reflect lack of immune reactivity, particularly in immuno-suppressed and haemodialysis patients. HCV circulates in unconcentrated serum at a concentration below the level of detection of antigen by standard immunoassay. Recently a test for HCV-core antigen has been developed. The availability of an anti-core antigen monoclonal antibody allowed development of an ELISA detecting and quantifying total HCV-core antigen in peripheral blood of HCV-infected patients which is an acurate, precise and specific indirect marker of HCV

replication (Bouvier-Alias et al, 2002).

Supplemental antibody tests:

Initial surveys of antibody to hepatitis C in blood donors indicated a high rate of false positive tests. This has necessitated the development of 'supplemental' assays for confirmation of a positive anti-HCV result. The most widely used supplemental test is the recombinant immunoblot assay (RIBA) in which four HCV antigens are fixed to a nitrocellulose filter, or a Matrix assay, in which HCV antigens are fixed in a matrix pattern along with control proteins. The four antigens comprise one structural (c22) and three non-structural antigens (c33,c100-3,5-1-1). Although important for confirming the specificity of an anti-HCV test in blood donors, confirmatory tests are almost invariably positive in anti-HCV positive patients with chronic hepatitis. Supplemental testing may occasionally be required to confirm an anti-HCV positive assay in an HCV-RNA negative patient in whom IFN-α therapy is contemplated (Dusheiko, 1997).

1.5.2 HCV-RNA testing:

Since the antigens of HCV are present in very low titers, direct tests for viraemia in HCV have relied on the detection of HCV-RNA in serum. RNA detection necessitates an amplification of the circulating HCV-RNA. Sensitive assays for HCV-RNA have been developed, based on the polymerase chain reaction (PCR).

The molecular mechanism of PCR mimics *in vivo* DNA replication resulting in the selective amplification of a particular DNA region (target). The polymerase chain reaction proper consists of three thermal treatment steps: 1) denaturation of double stranded DNA; 2) annealing of primers to complementary DNA (cDNA); 3) extension of primers. The repeat or cyclic process of denaturation, annealing, and extension performed automatically under rigorous thermal cycler controlled conditions of time and temperature leads to the exponential amplification of target DNA. Detection of HCV-RNA requires prior reverse transciption (RT) into cDNA by so-called RT-PCR. PCR primers for the 5′ non-coding region are superior to those from NS3 and core in sensitivity and specificity, because of the relative conservation of this region (Bukh et al, 1992). RNA is detected in the majority (60-80%) of anti-HCV positive patients, and is also detectable in a percentage of anti-HCV negative patients with chronic NANB hepatitis

(Takahashi et al, 1990).

Improved methods of quantitating HCV-RNA are being increasingly used. Limiting dilution assays for HCV-RNA and competitive PCR have been used. One commercially available method uses oligonucleotide probes complementary to the 5′ UTR and core regions to capture HCV-RNA, and then branched oligonucleotides to subsequently amplify the signal. Measurement of transcripts of different genotypes indicate that all of the known genotypes are within the range of assay. The detection limit of this assay is 350,000 copies per ml. Differences in serum levels between type 1,2 and 3 in the first version of this assay were due to lower efficiency of measurement of types 2 and 3. However, the test has been improved, and the second version of the branched DNA (bDNA) assay measures types 1 to 6 with equal efficiency. Other quantitative methods have been developed, including a biotinylated colorimetric assay, which uses an internal standard; the detection limit of this assay is 500 copies per ml (Dusheiko, 1997).

Serological diagnosis of hepatitis C:

Anti-c100-3 appears in the circulation after a mean interval of 15 weeks from the acute illness and first elevations of the aminotransferases (ALT). Although roughly one third of seroconversions take place early in the acute phase of the disease, sometimes as early as two weeks, seroconversion can be delayed for a year or longer (Lim et al, 1991). The average time from transfusion to seroconversion is of the order of 11-12 weeks with the first generation tests, and 7-8 weeks with the second generation tests. Seroconversion occurs much less frequently, and in lower titer, in acute self-limiting infections compared with those that progress to chronic infection.Thus, tests for anti-HCV are of limited benefit in diagnosing acute hepatitis C (Dusheiko, 1997).

During the early phase of primary HCV infection, serum HCV-RNA is the only diagnostic marker of infection, and RNA testing therefore remains the only means of diagnosis in seronegative patients. Serum HCV-RNA has been detected within one to three weeks of transfusion in patients with hepatitis C, and usually lasts less than 4 months in patients with acute self-limited hepatitis C, but may persist for

decades in patients with chronic disease (Farci et al, 1991).

At present there are no antibody patterns which differentiate persistent viraemia from an episode of resolved viraemia, as the antibody patterns are frequently not dissimilar. Anti-HCV antibodies persist in the majority of patients with chronic post-transfusion NANB hepatitis; the development and maintenance of current diagnostic antibodies to hepatitis C virus therefore appears to reflect concomitant virus replication, and consequently a high potential for infectivity. A proportion of patients may improve spontaneously, but the number of patients who do so is unclear. These patients lose antibody after follow up of at least 5 years, and usually develop normal serum aminotransferases (Tanaka et al, 1991). Other patients may have a decline in anti-HCV titer with time (Alter et al, 1989).

HCV-RNA usually persists in patients with abnormal serum aminotransferases and anti-HCV. However, HCV-RNA, and hence viraemia, can also be found in patients with normal liver function tests. HCV antigens can be detected in liver biopsy preparations in chronic carriers (Dusheiko, 1997).

1.6 The natural history of hepatitis C virus infection

Hepatitis C virus, a positive-stranded RNA virus, is the principle causative agent of parenterally transmitted and community aquired NANB hepatitis. HCV is a major health problem, and it is estimated that HCV affects 170 million people worldwide and more than 10% of the population in some countries. Infection with HCV results in subclinical chronic hepatitis in about 85% of the infected individuals. These individuals carry an increased risk of developing liver disease complications including cirrhosis and hepatocellular carcinoma (stated in Tseng and Klimpel, 2002).

The incubation period of hepatitis C varies between 6-12 weeks. Approximately 5-20% of patients may recover and clear the virus, however the great majority (80-95%) of infected patients develop persistent infection. The natural history of hepatitis C that includes both acute and chronic liver disease, and its associated manifestations and long term consequences are not known with certainly, and hence, remain to be established. A major concern in chronic hepatitis C is the possibility of progression to significant liver disease including cirrhosis and hepatocellular carcinoma (HCC) (Gérard and Delwaide, 1997). Approximately 30 million people worldwide are estimated to have cirrhosis associated with HCV (Tarao et al, 1999). Even if severe liver disease appears to develop in a relatively small proportion of patients, the high number of individuals infected suggests that the number of individuals at risk for developing clinically relevant disease is huge. Because a large proportion of HCC develops from cirrhosis especially from those associated with viral infection, patients with HCV associated cirrhosis have a high risk of HCC and HCC actually develops in 6-8% of all patients with HCV-associated cirrhosis every year in Japan (Tarao et al, 1999). HCV associated cirrhosis is now the first indication for liver transplantation in adults in Europe and in USA (Lam, 1999).

Disease progression is very variable and none of the available tools allow to predict who will develop serious liver disease, meaning that the status of patients persistently infected with HCV must be regularly reviewed. Progression when it

develops is generally a long term (10-30 years) and clinically indolent process even when serious liver damage has already developed. A complex set of poorly understood interacting viral and host related factors appear to impact long-term progression. Host factors include sex, age, age at infection, duration of infection, source of infection, alcohol intake, degree of liver damage at initial biopsy and host immune status. Viral factors responsible for oncogenesis in HCV infections are poorly understood (Gérard and Delwaide, 1997).

Kato et al (1993) reported that the level of HCV-RNA in serum (as an indicator of HCV replication) is directly related to the presence of hepatocellular carcinoma. However, these finding have not been confirmed (lau et al 1993). More recently, Tarao et al (1999) demonstrated that patients with HCV-associated cirrhosis with persistently high levels of ALT (which represents the inflammatory necrosis of hepatocytes) were at a high risk of developing HCC. They also found that most of the patients in the high ALT group developed HCC within 5 years of the initial histologic diagnosis of cirrhosis, in contrast to patients in the low ALT group, most of whom developed HCC after 5 years from the histologic diagnosis of cirrhosis. They suggested that suppression of the rise in serum ALT levels by treatment with anti-inflammatory drugs may prolong the interval from the diagnosis of HCV-associated cirrhosis to the development of HCC. Subsequently, it has been suggested that certain genotypes of HCV may be associated with more severe liver disease and possibly with development of hapatocellular carcinoma (Mahaney et al, 1994).

1.7 Mehanism of fibrosis in hepatitis C infection : role of TGF-β in hepatic fibrosis

Tissue is made up of organized groups of cells attached to an extracellular matrix and surrounded by a network of blood vessels. Tissue homeostasis is maintained by coordinating cell growth and proliferation with the production and turnover of the extracellular matrix. Cells achieve this coordination by constant signaling to themselves (autocrine activity) and each other (paracrine activity) by means of polypeptides called cytokines. Cytokines regulate all aspects of tissue remodeling whether planned (as in embryogensis and development) or unplanned (as in carcinogenesis and tissue repair after injury) (Sporn and Roberts, 1992).

Two mechanisms are involved in liver cell injury : necrosis and apoptosis. The role of necrosis in hepatocyte injury has been recognized for long time. Necrosis is a pathological form of cell death resulting from acute cellular injury. More recently, another mechanism has been described in human hepatocytes: apoptosis. The cellular changes observed in apoptotic cells strongly contrast with those related to necrosis. Apoptosis usually occurs in isolated cells, without inflammation. It is characterized by nuclear degradation and cell fragmentation resulting in membrane-bound fragments called apoptotic bodies. Liver disease activity is defined by inflammation and hepatocyte necrosis. Knodell's Score mainly takes into account disease activity, particularly hepatocyte necrosis (Biron et al, 1998).

TGF-β is a prototypical, multifunctional cytokine that was initially discovered as a growth factor for fibroblasts that promoted wound healing. However, it also has considerable antiproliferative activity and acts as a negative regulator of immunity and hematopoiesis. TGF-β is produced by many cell types, including activated macrophages and T lymphocytes. Humans express at least three forms of TGF-β called of TGF-β1, 2 and 3. The TGF-β1 gene is upregulated in response to tissue injury and TGF-β1 is the isoform most implicated in fibrosis. Other cytokines involved with TGF-β1 in tissue remodeiing after injury are platelet-derived growth

factor, basic fibroblast growth factor, tumor necrosis factor and IL1 (Border and Noble, 1994).

In the very early stage of acute liver injury, following aggregation of platelets, immuno-localization of TGF-β1 is observed in injured area. And at the cell migration stage, the infiltration pattern of inflammatory cells was characterized by an ordered progression of inflammatory cells, beginning with platelets and followed by polymorphonuclear leucocytes and macrophages. Following recruitment of inflammatory cells to the necrotic area, it appears that TGF-β could be produced and activated by these inflammatory cells, resulting in the intensification of active TGF-β distribution in the injured area. At the fibrosis stage, TGF-β could also be produced by Ito cells, endothelial cells and hepatocytes at the periphery of the necrotic area, and may play an important role in the promotion of production and accumulation of extracellular matrix components in the injured regions. In many kinds of acute liver injury, fibrogenesis is usually considered to be transient, and the injured liver cells are able to nearly recover in order. On the other hand, in chronic liver injury, fibrosis often progresses and liver cirrhosis results from over production of extracellular matrix (Inuzuka et al, 1994).

Shirai et al (1994) reported higher levels of plasma TGF-β1 in the patients with hepatocellular carcinoma (HCC) than in patients with chronic hepatitis and cirrhosis and suggested that plasma TGF-β1 might be a candidate for a noval tumor marker for HCC. In the liver, TGF-β1 is primarily responsible for activation of fat-storing cells, which are the main source of extracellular matrix proteins. Their deposition play a key role in the development of liver cirrhosis.

Tsushima et al (1999) reported that plasma TGF-β1 levels were significantly correlated with TGF-β1 content in liver tissue in chronic hepatitis C patients before IFN-α therapy. Plasma TGF-β1 in chronic hepatitis C patients was significantly higher than that in controls and significantly correlated with the degree of fibrosis. Liver fibrosis and TGF-β1 levels were significantly decreased in sustained responders as well as non-responders at the end of therapy. Also

urinary TGF-β1 levels can be used as a marker for hepatic fibrogenesis. Higher urinary TGF-β1 levels correlated with more severe liver disease (Tsai et al, 1997).

In hepatitis C patients, progressive hepatic fibrosis and cirrhosis develops in 20% to 30% patients. Host genetic factors influencing fibrogenesis may account for some of the variability in progression of this disease. A statistically significant relationship had been reported between inheritance of higher TGF-β1 and angiotensinogen (AT) producing genotypes and the development of progressive hepatic fibrosis. Patients who inherited neither of the profibrogenic genotypes had no or only minimal fibrosis. Knowledge of these polymorphisms may have prognostic significance in patients with chronic HCV and may direct more aggressive therapy towards those patients with an increased risk of disease progression (Powell et al, 2000).

1.8 Hepatitis C virus: extrahepatic manifestations

Hepatitis C virus (HCV), as RNA virus first identified in 1989, is a major cause of both transfusion-associated and sporadic non A, non B hepatitis (Choo et al, 1989). Persistent infection occurs in approximately 50 percent of patients and may result in chronic active hepatitis, cirrhosis and hepatocellular carcinoma (Malnick and Schmilovitz-Weiss, 1997).

In Italy and other countries where HCV is prevalent, a significant proportion of patients who present with features of autoimmune hepatitis (AIH) are found to have chronic HCV infection (Garson et al, 1991). On the other hand, chronic HCV infection has also been associated with several extrahepatic syndromes including mixed cryoglobulinemia, polyarteritis nodosa, porphyria cutanea trada and a Sicca like syndrome that resembles Sjogren's Syndrome (Manns and Rambusch, 1999). Hepatitis virus infection may also be associated with immunologically mediated renal disease such as membranoproliferative glomerulonephritis (McMurray and Elbourne, 1997). It had also been mentioned that the high prevalence of thyroid dysfunction in female patients with chronic hepatitis C strongly suggests that HCV contributes to the occurrence of thyroid autoantibodies and in some cases initiates autoimmune diseases such as Hashimoto's thyroiditis (Tran et al, 1993).

Coroneos et al (1997) reported a patient with fibrillary glomerulonephritis (FGN) associated with HCV infection who underwent partial clinical remission of her renal disease after treatment with IFN-α. This represents the first reported case of a potentially treatable cause for FGN, a renal disease with undefined cause and poor response to any currently applied therapy, which rapidly leads to end stage renal disease. The rare combination of hepatitis C and panarteritis nodosa has still not been confirmed. Although an epidemiological association of hepatitis C with lichen planus neuropathies and other diseases has been observed, the aetiological role and the pathogenic involvement of HCV infection remains unclear. Furthermore, the question of whether these extra-hepatic diseases are autoimmune has not been clarified. In the clinical setting, the presence of these diseases should

suggest hepatitis C infection and hepatitis C antibodies should be tested for and if positive, HCV-RNA is indicated. If there is any evidence of an aetiological association of replicative hepatitis C infection and the above extrahepatic diseases, antiviral treatment should be considered.(Manns and Rambusch, 1999).

On the other hand, a significant proportion of HCV patients had detectable anticardiolipin compared to healthy controls. The presence of these antibodies in HCV patients and whether they may or may not be associated with the development of antiphospholipid syndrome is a matter of controversy (Mc Marry, 1998); (Dalekos et al, 2000). HCV has been linked to B-cell lymphoproliferation and autoimmunity and has been localized in several tissues. Devita et al (2000) reported gastric mucosa as an additional extrahepatic localization of HCV in hepatitis C infected patients with chronic gastritis and gastric low-grade B cell non-Hodgkin's lymphoma (NHL).

HCV And Autoimmune Hepatitis Disease (AIH):

Viral infections are considered a possible trigger of autoimmune disease. In autoimmune liver diseases the hepatotropic viruses especially HCV have received particular attention as possible etiological agents. HCV is reported to be a major factor in AIH type 2 (AIH-2), which is characterized by autoantibody against the microsomal antigen of liver and kidney (LKM_1) (Czaja, 1997). LKM antibodies recognize the microsomal Protein Cytochrome P45011D6, which is a cytochrome P450 mono-oxygenase. One of the possible mechanisms for the induction of LKM_1 antibodies in HCV infected patients may be via molecular mimicry between the HCV polyprotein and human microsomal Protein Cytochrome P45011D6 (Michitaka et al, 1994).

It was reported that the occurrence of LKM autoantibodies in viral hepatitis may indicate an increased risk for treatment with interferons (Manns and Rambusch, 1999). Jurado et al (1997) reported that markers of hepatitis C virus are often detectable in patients suffering from chronic hepatitis with LKM_1 antibodies. In previous studies, approximately 50% of patients from Germany (Michel et al, 1992) and France (Lunel et al, 1992) and more than 80% of patients from Italy

(Lenzi et al, 1991) with AIH-2 had been reported to be infected with HCV. Antibody to hepatitis C virus (anti-HCV) positive and anti-HCV negative patients with AIH-2 differ clinically. Anti-HCV negative AIH represent more typical autoimmune liver disease, whereas anti-HCV positive patients with AIH-2 are older, more frequently men and present with milder disease activity (Michitaka et al, 1994). On the other hand, an extremely high association has been reported with HLA-DR7 antigen in the first group (HCV positive) patients and HLA-DQ2 antigen in the second group (HCV negative) patients. Hence, different genetic profile in these two patients groups may be suggested (Jurado et al, 1997).

HCV genotype and HLA typing in AIH:

Michitaka et al (1994) evaluated the genotypes of HCV in AIH-2 patients' sera obtained from two geographical areas, Germany and Italy, where the association of HCV infection with AIH-2 varies significantly. For patients from Germany, there was no difference in the ratio of the genotype between the LKM-positive and negative groups of patients with HCV infection. Genotype 2 is dominant in both groups. On the other hand, the rate of genotype 3 is significantly higher in the LKM-positive group of patients from Italy compared with the LKM-negative group of patients from Italy. These results indicate that genotype 3 may have some etiologic relation with AIH-2 in patients from Italy. Gerotto et al, (1994) reported that HCV genotype 1 which is associated with more severe forms of chronic liver disease and resistance to IFN, may more easily induce anti-LKM_1 antibodies compared with other HCV genotypes.

Czaja et al (1993) studied the frequency and genetic predispositions of concurrent immunological diseases and immuno-serological markers in autoimmune hepatitis and chronic viral hepatitis. They reported that human leucocyte antigen DR4 (HLA-DR4) was associated with the concurrence of immunological diseases in both autoimmune and viral hepatitis. This finding suggests that autoimmune hepatitis and viral CAH do have a common genetic predisposition for the expression of immunological disease and that this predisposition is linked to HLA-DR4 (Czaja et al, 1993).

The occurrence of both chronic hepatitis C and AIH probably relies on the prevalence of HCV infection and a genetic predisposition. The tendency to autoimmunity could be genetically determined. HLA-A1, B8 and DR3 antigens have been associated with type 1 AIH and in addition DR4 antigen was recognized as a secondary but independent risk factor. Possession of DR3 and DQ2 antigens seems to predispose patients with HCV infection producing anti-LKM_1 (Garcia-Buey et al, 1995).

On the other hand, Meyer Zum Buschenfelde et al (1995) reported that there is a significant difference in HLA typing between patients with autoimmune hepatitis and patients with chronic hepatitis C. So, they cannot definitely answer the question of whether autoimmune markers such as antinuclear antibodies (ANA), antismooth muscles antibodies (ASMA), soluble liver antigen antibodies (SLA) in patients with viral liver diseases occur more frequently in patients with the above mentioned HLA types, predisposing to autoimmune hepatitis, as reported by Czaja et al (1993).

1.9 Chronic hepatitis C and autoantibody production

Tage-Jensen et al (1980) described NANB chronic liver disease patients positive for ANA and ASMA as "Non viral hepatitis with autoimmune manifestations". In 1989, hepatitis C virus was described for the first time (Choo et al, 1989). In 1993, Abuaf and his colleagues mentioned that antibodies to HCV were detected by ELISA in chronic active hepatitis patients' sera with ASMA or with anti-LKM_1. However, since false positive results in ELISA were suspected that time due to a component in these patients' sera, more specific assays were required to confirm the presence of anti-HCV antibodies (Abuaf et al, 1993).

Results from Spain (Esteban et al, 1989), Italy (Garson et al, 1991) and France (Lunel et al, 1992) indicated an association between non-organ specific autoantibodies and anti-HCV antibodies in chronic active hepatitis C patients, which suggest that HCV infection induces autoantibodies. On the other hand, Meyer Zum Buschanfelde et al (1995) reported a low incidence of actively replicating HCV infection in patients with AIH. Their data showed that HCV infection induced autoantibodies in a significant number of patients but with low titer (1:40 and 1:80 for ANA and ASMA respectively). Very few patients had titers of 1:160 or 1:320 respectively. They also found that the coexistence of LKM autoantibodies with chronic hepatitis is rare and less common than low ANA and ASMA titers in viral liver diseases. It had been suggested that geographical or genetic factors might influence the presence of these autoantibodies (Lenzi et al, 1991).

In 1997, Czaja mentioned that high titer autoantibodies (titers > or = 1:320) are uncommon in chronic viral hepatitis as are multiple concurrent autoantibodies. These findings reflect an autoimmune-predominant disorder in which the viral infection may be coincidental or facilitative. Concurrent immunologic disorders may be viral antigen-driven and associated with immune complex deposition (cryoglobulinemia, glomerulonephritis, cutaneous vasculitis and polyarteritis) or autoantigen-driven (autoimmune thyroiditis and Sjogren's Syndrome) and associated with host rather than virus-specific factors. Genetic predispositions

influence immunologic expression (stated in Czaja, 1997). Moreover, Cassani et al (1997) reported that in chronic hepatitis.C, serum autoantibodies are common, but their sub-specifications are distinct from those occurring in AIH. ANA with the homogeneous pattern (ANA-H) and ASMA with anti-actin specificity (SMA-AA) existed at a lower prevalence and had a lower median titer. Whereas, the absence of ANA-H and /or SMA-AA does not exclude AIH, the characterization of ANA and SMA may help to discriminate between the two conditions.

Garrido-Palma et al (1999) reported that the prevalence of antimitochondrial antibodies (AMA) in chronic hepatitis C is 2%, and titers of AMA are usually low (<1:40). The prevalence decreases to 0.5% when the results are verified by determination of the M2 subtype. In patients in whom both HCV and AMA are present, the therapeutic decision to give IFN-α is complicated, because AMA may be "real", and if it reflects primary biliary cirrhosis, cholestasis can be triggered or exacerbated. They concluded that testing for antibodies to sub-mitochondrial particles (M2, M4 and M8 subtypes) and M2-immunoreactive epitopes in patients with chronic hepatitis C, and AMA titers > 1:160, facilitate the diagnosis of primary biliary cirrhosis, and are a contraindication to treatment with IFN-α despite the presence of HCV infection (Garrido-Palma et al, 1999).

1.9.1 Implication of hepatitis C virus as a cause of the Anti-phospholipid Syndrome:

Antiphospholipid antibodies have been implicated in the occurrence of thrombocytopenia and thrombotic events and have been described in autoimmune disorders and diverse viral diseases. Lupus anticoagulant and anticardiolipin antibodies belong to this family of autoantibodies with an affinity for anionic phospholipids. Antiphospholipid antibodies have been reported in systemic lupus erythematosus (SLE) and other autoimmune disorders but are being increasingly detected in patients without apparent underlying autoimmune disease. These autoantibodies are strongly associated with recurrent spontaneous abortions, thrombocytopenia and thrombotic events including deep venous thrombosis with or without pulmonary embolism, portal or hepatic vein thrombosis, large vessel arterial occlusions, myocardial infarction, fixed or transient cerebral ischemic

events, retinal arterial and venous occlusions, ischemic optic neuropathy, Degos disease and non-bacterial thrombotic endocarditis (Pope et al, 1991).

The combination of persistently positive antiphospholipid test results with any of these clinical manifestations is known as the antiphospholipid antibody syndrome (APLS). This syndrome, which can be part of the clinical spectrum of systemic lupus erythematosus (SLE), can also occur in patients with no connective tissue disease, and for these cases the term primary antiphospholipid syndrome has been suggested. Antiphospholipid antibodies of the immunoglobulin IgG isotype show the strongest association with symptomatic disease. Anticardiolipin antibodies have been detected in a variety of infectious diseases, particularly of viral origin, such as human immunodeficiency virus infection, mumps and hepatitis A (McNeil et al, 1991).

Prieto et al (1996) studied the prevalence of anticardiolipin antibodies in chronic HCV infection and the prevalence of HCV markers in patients with no liver disease and history of thrombotic disease. Their data indicated that HCV infection is associated with the occurrence of antiphospholipid antibodies and that this virus should be considered as one of the possible causes of the antiphospholipid syndrome. Prieto et al (1996) reported that in chronic hepatitis C, no relationship was found between antiphospholipid antibodies and other autoimmune markers except for the fact that patients with positive anticardiolipin test exhibited higher prevalence of ASMA than subjects who tested negatively. They also reported that in patients with chronic HCV infection, positivity for anticardiolipin antibodies was associated with the existence of cirrhosis-related portal hypertension, with the presence of thrombocytopenia and with a history of thrombotic episodes. The significant association of a positive anticardiolipin reaction with the occurrence of portal hypertension in cirrhotic patients might suggest that the risk to develop anticardiolipin antibodies in chronic hepatitis C increases with the progression of the disease (Prieto et al, 1996).

The idea of a possible implication of antiphospholipid antibodies in thrombogenesis comes not only from their statistical association with thrombotic

diseases, but also from their ability to interfere *in vitro* with clotting mechanisms (McNeil et al, 1991). Anticardiolipin antibodies have been proposed to mediate platelet destruction by binding to phospholipids in the platelet membrane (Harris et al, 1985). In addition, antiphospholipid antibodies may produce thrombocytopenia and also thrombosis by interacting with endothelial cells and stimulating the synthesis of platelet-activating factor, thereby promoting platelet activation, platelet consumption and thombogenesis (Silver et al, 1991).

The mechanisms leading to the formation of anticardiolipin antibodies in HCV is not known. One hypothesis is that chronic liver infection may induce neoantigens by disrupting liver cell membranes. Antiphospholipid antibodies would be the consequence of recognition of the neoantigens by the immune system (Biron et al, 1998). Alternatively, Rosser and Gores (1995) suggested that, during liver necrosis, hepatocytes and endothelial cells may modify the immune response through expression of adhesion molecules, inducing subsequent development of autoantibodies. It was recently reported that there may be cross reactivity between viral antigens and phospholipids during the immune response to infection, leading to the development of antibodies, or it may simply be an epiphenomenon of a pathological process that occurs as a secondary consequence of an infection (Guglielmone et al, 2001).

It has been shown that antiphospholipid antibodies may, in fact, be directed against lipid-protein epitopes and that the coagulation inhibitor β2-glycoprotein-1 may be the necessary protein cofactor for anticardiolipin antibodies to react with negatively charged phospholipids. This cofactor is a heavily glycosylated glycoprotein of 50 Kd that binds to anionic phospholipids, heparin and DNA. It may function to bind anionic macromolecules that enter into the blood stream as a result of tissue damage or infections and thus diminish unwanted activation of coagulation. Release of procoagulant products from HCV-infected cells might lead to the formation of molecular complexes with β2-glycoprotein-1, which could stimulate the synthesis of anticardiolipin antibodies (Prieto et al, 1996).

Hunt et al (1992) confirmed that binding of anticardiolipin antibodies from patients with infections, unlike that of patients with SLE, is not dependent on β2-glycoprotein. Guglielmone et al (2001) stated that classification of anticardiolipin antibodies into β2- glycoprotein-1 dependent and β2-glycoprotein-1 independent has been applied to patients with different clinical manifestations and helped to distinguish between anticardiolipin antibodies associated with autoimmune disease and those resulting from infections. They also suggested that, in patients with viral infection and positive anticardiolipin antibodies, assessment of the cofactor dependence appears to be a useful tool for estimating the risk of the clinical manifestations observed in the presence of these antibodies (Guglielmone et al, 2001).

Liver disease activity is defined by inflammatory and hepatocyte necrosis. In HCV positive, a direct relationship has been reported between the presence of antiphospholipid antibodies (APAs) and liver fibrosis; however, APA levels did not correlate with disease activity (Biron et al, 1998). On the other hand, Dalekos et al (2000) conducted a study in a well characterized area for epidemiological and prospective studies in the north-western part of Greece to address whether an aetiopathogenesis exists between HCV and antiphospholipid syndrome. They concluded that a significant proportion of the HCV patients had detectable anticardiolipin antibodies as compared to healthy controls. However, the mean titer of these antibodies was significantly lower in HCV patients compared with that seen in APLS patients. Also, the existence of anticardiolipin antibodies in HCV patients was not associated with the development of APLS. On the other hand, none of the APLS patients were positive for HCV. They concluded that testing for HCV patients with APLS or follow up for the possibility of the development of APLS in HCV patients cannot be suggested. More prospective studies of longer duration will be required in order to address whether HCV is involved in the aetiopathogeneis of APLS (Dalekos et al, 2000).

Harada et al (2000) suggested that immunologic disturbances induced either by hepatitis C virus in the liver or tissue damage in other organs as a result of the

extrahepatic manifestations of hepatitis C infection may induce the production of antibodies to various cardiolipin-binding proteins or phospholipids.

1.9.2 Thyroid autoantibodies in chronic hepatitis C patients:

Tran et al (1992) reported two patients with hepatitis C and Hashimoto's thyroiditis. These observations prompted them to carry out a prospective study aimed at detecting the presence of the thyroid autoantibodies: thyroglobulin (TG) and thyroid microsomal or thyroid peroxidase autoantibodies (TPO) in patients with chronic hepatitis C infection before interferon therapy. Their data suggest a high prevalence of thyroid dysfunction in female patients with chronic hepatitis C before the introduction of IFN therapy (Tran et al, 1993). In this study, all female patients with thyroid autoantibodies were found to be HCV viraemic on the basis of PCR findings; the persistence of the virus may have lead to high levels of autoantibodies. They suggest that an infectious agent, such as HCV, could initiate autoimmune thyroid disease by mimicking the structure of some component of thyroid tissue (Tran et al, 1993).

Boadas et al (1995) compared the prevalence of thyroid dysfunction and anti-thyroid peroxidase antibodies in blood donors with hepatitis C virus (HCV) infection (study group) and in seronegative donors (control group). Their results do not show an increased prevalence of thyroid dysfunction or anti-thyroid peroxidase antibodies in blood donors with HCV infection when compared with the control group. They suggest that the difference in geographical distribution, in genetic variability in the populations studied and also in the evolution of chronic HCV infections in the patients studied could explain these discrepancies in relation to the previous findings.

Manns and Rambusch (1999) reported that the existing studies have not answered the question of whether HCV plays a pathogenic role in the development of thyroid dysfunction and autoimmune thyroiditis. There seems to be a genetic predisposition for the manifestations of thyroid disease in the case of hepatitis C infections and interferon therapy. This predominantly affects women of haplotype HLA-DR3. These patients often show antibodies against thyroid

peroxidase and /or thyroglobulin before beginning interferon therapy. Previously, it has been reported that genetic predisposition was of major importance in the tendency to produce thyroid autoantibodies, whereas environmental factors, such as iodine intake or a virus infection, play a major role in the development of clinically recognizable autoimmune thyroid disease (Prentice et al, 1990). In another study to assess the relationship between serological markers of thyroid autoimmunity and chronic hepatitis C, no evidence for an epidemiological association of circulating thyroid autoantibodies and antibodies to HCV was reported (Loviselli et al, 1999).

During IFN-α therapy the induction of thyroid autoantibodies has been observed, mainly against the thyroid peroxidase antigen, in a number of patients with a genetic predisposition to autoimmune thyroid disease. These antibodies are mainly transient. In addition, thyroid function may be altered. Although biphasic alteration of thyroid function (hyperthyroidism followed by hypothyroidism or *vice versa*) has been observed, classical Graves' disease with TSH-receptor stimulating antibodies and hyperthyroidism is extremely rare. In the clinical setting, thyroid autoantibodies and function should be monitored before, during and after IFN-α treatment in HCV-infected patients (Manns and Rambusch, 1999).

Carella et al (2001) reported that the thyroid antibody pattern at the end of treatment is a predictive factor for the different outcomes of IFN-related thyroiditis. The co-existence of TG-Ab and TPO-Ab at the end of treatment is a predictive marker for the presence of thyroid dysfunction, even if subclinical, many years after IFN withdrawal.The positivity for TG-Ab with absence or low TPO-Ab levels are described mainly in the patients with transient and remitting / relapsing thyroiditis. The negativity for thyroid autoantibodies after IFN treatment is a protective factor for the developing thyroid autoimmunity and / or dysfunction in following years. The different behavior observed in these patients could be explained by the hypothesis that genetic predisposition to thyroid autoimmunity is provided by several disease-associated alleles at many genetic loci. When a sufficient number of these alleles are inherited and appropriate environmental events take place, thyroid autoimmune disease develops. They concluded that

IFN-α related thyroid autoimmunity is not always a reversible phenomenon because some patients can develop chronic thyroiditis. Marazuela et al (1996) suggested that thyroid dysfunction is reversible only when it is truly related to IFN-α therapy and is not secondary to the patient's autoimmune predisposition.

The relation between HLA and autoimmune thyroid disorders induced by IFN therapy has been studied in Japan by Kakizaki et al (1999). They described that the frequency of HLA-A2 was significantly elevated among the patients with chronic hepatitis C who developed autoimmune thyroid disorders during or after IFN-α therapy. These results suggest that HLA may be one risk factor for the development of autoimmune thyroid disorders caused by IFN-α therapy.

1.9.3 Hepatitis C virus chronic infection as a common cause of mixed cryoglobulinaemia:

Hepatitis C Virus (HCV) infection has been related to different autoimmune lymphoproliferative diseases such as autoimmune chronic hepatitis (AI-CH) and mixed cryoglobulinaemia (MC). MC is a systemic immunological disorder in which tissue deposition of circulating immune complexes (CIC) is responsible for several manifestations of the disease i.e. cutaneous and systemic vasculitis, glomerulonephritis and peripheral neuropathy. Hepatitis C virus associated cryoglobulinaemic vasculitis is an immune complex mediated vasculitis predominatly affecting small vessels. It typically evolves in patients in the presence of type II mixed cryoglobulinaemia consisting of cryoprecipitating monoclonal IgM [kappa]-RF and polyclonal IgG. This disorder is usually found after years of chronic hepatitis C. Mixed cryoglobulinaemia and the detection of rheumatoid factor (RF) and various autoantibodies are hallmarks of HCV associated cryoglobulinaemic vasculitis. These findings have been attributed to polyclonal activation of B lymphocytes and the subsequent evolution of a so called benign lymphoproliferative disorder with oligoclonal or monoclonal B lymphocyte proliferation. Furthermore, increased serum levels of soluble intracellular adhesion molecule-1 and soluble interleukin-2 receptor have been found in acute and chronic hepatitis C without cryoglobulinaemia (Lamprecht et al, 2001). Liver involvement represents one of the most common complications, which can affect the overall prognosis of the disease. Ferri et al (1994) reported that in a number

of cases chronic hepatitis appears before the typical manifestations of the disease. They discussed that the high prevalence of HCV in MC patients suggests an important role of this virus in the aetiopathogenesis of MC and can explain the high rate of liver involvement observed in this disease. However, HCV is also present in MC patients without any manifestations of hepatitis. This observation suggests that HCV can promote, in genetically susceptible subjects, a polyclonal B cell activation that ultimately yields large amounts of circulating immune complexes, mainly the cryoglobulins and various autoantibodies. It was reported that the selective concentration of HCV-RNA and anti-HCV antibodies in the cryoprecipitate suggest that a specific role in its pathogenesis must be assigned to cold insoluble immune complexes in which hepatitis C virions are bound to specific antibodies cross linked by IgM rheumatoid factor (Sansonno et al, 1995).

Cryoglobulinaemic hepatitis could be the result of different noxious factors: the direct cytopathic effect of HCV and /or virus-induced autoreactions against hepatocytes, and /or circulating immune complexes deposition. The clearance of circulating immune complexes is operated by the mononuclear phagocytic system i.e. liver Kupffer cells, and their activation can promote parenchymal inflammation and fibrosis. Interferons and corticosteroids have been found capable of modulating HCV viraemia and HCV antigen expression in liver tissues (Sansonno et al, 1995). The remote pathogenesis of MC is considered to be a B-cell lymphoproliferation, which in many patients can be complicated by malignant lymphoma (Ferri et al, 1994). Because of the variability of the HCV genome, one might speculate that particular viral variants are responsible for mixed cryoglobulinaemia. Zignego et al (1996) reported that HCV genotype 2a/III had a significantly higher prevalence in HCV-positive patients with mixed cryoglobulinemia than in patients with chronic hepatitis who did not have cryoglobulinemia. On the other hand, the higher prevalence of genotype 2a/III in patients with mixed cryoglobulinemia than in controls, especially in cryoglobulinemic patients with circulating autoantibodies, suggests that type 2a/III might be involved in the pathogenesis of autoimmune lymphoproliferative disorders. The observation that type 2a/III is particularly frequent in Italian patients with anti-LKM_1 autoantibody-positive type 2 autoimmune hepatitis

further support the possibility of a peculiar pathogenetic role for this genotype (Michitaka et al, 1994).

Ferri et al (1993) described that patients with MC have a higher prevalence of HCV infection in peripheral blood mononuclear cells, suggesting that HCV lymphotrophism may play a key role in determining the lymphoproliferative disorder underlying the disease. Zignego et al (1996) confirmed these data and also described the frequent infection of lymphatic cells in chronic hepatitis C patients who do not have cryoglobulinemia. They hypothesized that different viral, genetic or environmental factors in addition to the infection of lymphatic cells, may be involved in the pathogenesis of this disorder. A different combination of the above factors may lead to differing degrees of liver or lymphatic damage, ranging from chronic hepatitis without evidence of immune alterations to mixed cryoglobulinemia without liver involvement. They also added that the exact role of HCV variants, namely 2a/III, which are possibly related to different host immune reactivity or to a greater lymphotropism, should be clarified through deeper virologic analysis, including examination of lymph node and bone marrow specimens from larger series of patients (Zignego et al, 1996). The association of haplotype HLA-B8 and DR3, mixed cryglobulinaemia and HCV infection has recently been demonstrated (Manns and Rambusch, 1999). Interferon-α therapy decreases hepatitis C viraemia and improves the clinical signs and biochemical abnormalities of cryoglobulinaemia although patients often relapse after the end of treatment (Lunel and Cacoub, 1999).

1.10 The immune response to HCV

HCV causes chronic infection in 80-90% of those exposed and persists despite evidence of immune recognition. The immune mechanisms responsible for resolution of viraemia remain poorly understood. Natural immune responses, both cellular and humoral, are not capable of terminating HCV infection in most patients. A role has been suggested for peripheral blood leucocytes in viral persistence and clinical implications as these cells may serve as a viral reservoir and at the same time may be inadequate active participants in antiviral immune reactions. Different cell populations may interact to mount an efficient HCV specific immune response in humans. Antiviral antibodies are present in almost all patients with chronic hepatitis C but do not seem to be virus neutralizing, probably due to high mutation rate of viral envelope proteins (Diepolder et al, 1996).

Natural killer cells (Nk cells) play an important role in the early innate host defence against a number of pathogens. The role of NK cells in host defence against various viral infections has been extensively investigated in humans and in different animals (Biron et al, 1999). *In vivo*, NK cell responses to viral infection can include induction of cytotoxicity, blastogenesis and under certain conditions, IFN-γ production. If activated, the NK cell IFN-γ response clearly contributes to antiviral defence. The NK cell blastogenic response has the potential of promoting NK cell antiviral effects by resulting in NK cell expansion and / or trafficking to new host tissue sites. These NK cell responses are precisely regulated by at least two different classes of cytokines. NK cell cytotoxicity and blastogenesis are the results of virus induced IFN-α/β, whereas NK cell IFN-γ production is dependent upon an IL12 response. Mechanisms are in place to limit or block the IL12 response. In particular, IFN-α/β is regulating IL12 expression and is responsible for the lack of detectable IL12 production during certain viral infections. Once adaptive T cell responses are in place, a variety of mechanisms are able to block further NK cell activity (Biron, 1997).

The possibility that HCV might be able to alter NK cell functions and thus the innate immune response has not been fully explored. Tseng and Klimpel, (2002)

reported for the first time that the major HCV envelope protein E2 has been shown to bind to CD81. This cross linking mediates completely different signals in NK cells versus T cells.These results suggest that one mechanism whereby HCV can alter host defenses and innate immunity is via the early inhibition of IFN-γ production by NK cells (Tseng and Klimpel, 2002).

In chronic carriers, it can be assumed that after the hepatitis C virus has infected the host's hepatocytes and other tissues, intracellular so-called endogenous antigen processing will begin. Finally, processed peptide fragments will be presented to specific cytotoxic T lymphocytes (CTL) in the context of constitutively expressed HLA class 1 molecules. Therefore, efficient *in vitro* and *in vivo* models for acute and chronic human HCV infection have to be established to prove the significance of the CTL responses for viral clearance (Löhr and Gerken, 1997).

Several determinants such as the vigor of the T cell proliferative response to HCV antigens in the acute phase of hepatitis (Missale et al, 1996), the strength of cytotoxic T lymphocyte (CTL) activity against HCV epitopes (Rehermann et al, 1996), the genetic factors of the infected hosts (Aikawa et al, 1996) and the genotype of the virus in association with its quasispecies nature (Simmonds, 1995) have all been suggested as important factors capable of influencing the outcome of acute HCV infection.

Although HCV infection is very common, it rarely presents acutely, as the disease is usually not accompanied by overt jaundice and patients rarely seek medical attention. Consequently, little is known about the specificity and kinetics of the immune response during this period. Lechner et al (2000) indicated that a strong and broadly directed CTL response is observed in persons with acute hepatitis who go on to successfully control HCV infection. Strong cytotoxic T lymphocyte (CTL) responses directed against multiple epitopes located on structural and non-structural HCV antigens had also been reported in other studies (Cerny et al, 1995) ; (Battegay et al, 1995).

In sera from chronic HCV carriers, different virus strains may co-circulate and this "quasispecies" is characterized by high mutation rates and short replication time. Thus, despite strong CTL responses, some viral strains could escape the host's immunological attack. On the other hand, adequate CTL induction may require helping activities from T helper cells (Th) (Simmonds et al, 1993 b).

Lechner et al (2000) speculated that there is a short period of time when CTLs, acting in concert with $CD4^+$ lymphocytes and possibly NK cells, are effective in antiviral control-beyond this; if virus is cleared, they may initially maintain a conventional phenotype, but if virus persists their numbers and effector function may decline. CTL activity is always a two-edged sword, and it is likely that CTLs contribute to hepatic inflammation and damage during acute disease when they have appropriate number, phenotypic markers and lytic function. HCV-core protein had been suggested to play an important role in the establishment and maintenance of HCV infection by suppressing host immune responses, in particular the generation of virus-specific cytotoxic T lymphocytes (Large et al, 1999).

Chang et al (1999) discussed that an effective therapeutic vaccine against HCV must be able to induce a strong multispecific HCV-specific CTL response to eradicate HCV before selection of escape mutants can occur. Furthermore, identification of epitopes restricted by multiple HLA alleles is required for such vaccine to be immunogenic in the general population.

Helper T lymphocytes ($CD4^+$T cells) play a major role in the host defence against viruses and intracellular microbes. T helper lymphocytes (Th) compromise several subsets with different functions. Th1 cells produce IL2, IFN-γ and lymphotoxin and are important for an effective antiviral defense, whereas Th2 cells secrete IL4, IL5, IL6, IL9, IL10 and IL13 and may act as counterparts down-regulating antiviral activity by inhibiting Th1 cytokine secretion. T0 cells synthesize both types of cytokines and are generated in the absence of dominant differentiation signals (Abbas et al, 1996).

$CD4^+$T cells recognize antigens in the form of 8-12 amino-acid peptides bound to autologous HLA class II molecules. Despite a rather low affinity of the T cell receptors (TCRs) for peptide-HLA complexes, specific $CD4^+$T cells can respond to antigen-presenting cells displaying as few as 50 to 100 specific peptide-HLA complexes (Valitutti et al, 1995). However, full T-cell activation has been estimated to require the triggering of approximatly 8000 TCRs (Viola and Lanzavecchia,1996).

Evidence has accumulated that the TCRs are not just an on-off switch but may be able to transmit qualitatively distinct signals into T cells. Minor modifications within the amino-acid sequence of a specific peptide can lead to inactive peptides, weaker or stronger agonists and antagonists or so called altered peptide ligands (Evavold et al, 1993). A high affinity between peptide and MHC or a high antigen dose may promote the differentiation of naive $CD4^+$ cells into Th1 cells, whereas a low affinity between MHC and peptide or a low antigen concentration favors the development of a Th2 cytokine profile (Constant et al, 1995).

Diepolder et al (2001) demonstrated that virus-specific $CD4^+$T cell clones can respond to low antigen concentrations with up-regulation of activation markers and adhesion molecules. A 30 to 100-fold-higher antigen concentration, which corresponds to the triggering of 20 to 30 % of TCRs, is required to induce cytokine secretion or proliferation.

Frasca et al (1999) presented the first evidence that varients of the hypervariable region (HVR1) of the putative envelope 2 protein of HCV can act as powerful TCR antagonists of HVR1-specific $CD4^+$T cells isolated from HCV-infected individuals. The importance of a vigorous $CD4^+$T cell response for a favorable outcome of such infections has been shown in many animal models and can be inferred in humans from the occurrence of opportunistic infections in patients with T helper cell deficiency states (Maloy et al, 2000). On the other hand, an uncontrolled or overshooting T cell response may lead to unnecessary and detrimental tissue damage as seen, for example, in fulminant viral hepatitis or in postinfectious autoimmune disease. It is therefore obvious that a coordinate

regulation of the immune response is required to achieve control of the infection on the one hand and to avoid excessive tissue damage on the other (Diepolder et al, 2001).

A vigorous and multispecific $CD4^+$ proliferative T cell response is maintained indefinitely after recovery from HCV infection whereas it is weak and focused in persistently infected patients. HCV-specific $CD4^+$T cells survive and $CD8^+$T cells are lost after viral clearance while the opposite occurs when HCV persists suggesting the existence of differential requirements for the maintenance of $CD4^+$ and $CD8^+$T cell memory during HCV infection. Furthermore, the relative rarity of circulating $CD8^+$ effector T cells in chronically infected patients may explain the chronic insidious nature of the liver inflammation and also why they fail to eliminate the virus (Chang et al, 2001).

The imbalance of T helper (Th) lymphocyte cytokine production may play an important role in immuno-pathogenesis of persistent hepatitis C virus infection. A preponderance of type II cytokines in chronic hepatitis could contribute to viral persistence by inhibiting T cell proliferation, IL2R expression, MHC expression and reduced antigen-presenting capacities of monocytes (Martinez et al, 1990; De Waal Malefyt et al, 1991).

Woitas et al (1997) studied the cytokine responses and $CD30^+$ induction, a proposed surrogate marker of type 2 cells, in healthy anti-hepatitis C virus seropositive blood donors without viraemia (group A) and in patients with chronic hepatitis C (group B) . They reported marked differences in the cytokine profile of the total T cell population between patients with previous self-limited disease versus patients with chronic hepatitis. They concluded that immuno-dominant HCV core peptides induce preferentially type 1 cytokines in healthy anti-HCV positive blood donors (group A) and $CD30^+$ expression in patients with chronic hepatitis C (group B). However, in both groups, $CD30^+$T lymphocytes produce an intermediate Th0-like cytokine profile. Thus, chronicity in HCV may reflect a lack of type 1 cytokine production (Woitas et al, 1997).

CD30, a member of the tumour necrosis factor receptor superfamily, is expressed on activated T lymphocytes, with stronger more sustained expression on Th2 lymphocytes than on Th1 lymphocytes. Engagement of CD30 by its ligand CD30L is followed by enhanced shedding of CD30, which leads to increased levels of its soluble form, s-CD30. Lamprecht et al (2001) found a significant reduction in s-CD30 and s-IL2 receptor during induction of remission of HCV associated cryoglobulinaemic vasculitis with IFN-α. They concluded that this may indirectly reflect a change in the cytokine balance towards a Th1 profile, with augmentation of the cellular immune response under IFN-α treatment.

Fan et al (1998) reported that the levels of Th2 cytokines (IL4 and IL10) as well as Th1 cytokines (IL2) were significantly increased in chronic hepatitis C infected patients compared with normal controls. However, Th1 cytokine IFN-γ level was not significantly changed during HCV infection. Furthermore, the elevated levels of Th2 cytokines were greater than Th1 cytokines in HCV infection. Thus, their study indicates that enhanced Th2 responses are present during chronic HCV infection, which may partly be responsible for the persistence of HCV infection.

Yao et al (2001) reported that HCV-core induced blockage of intracellular events in T cell activation by a complement-dependent regulatory pathway may play a critical role in the establishment of HCV persistence during the acute phase of viral infection. Cramp et al (1999) studied the HCV specific humoral and cellular immune responses in anti-HCV positive / HCV-RNA negative patients and anti-HCV positive with persistent viraemia. HCV specific IFN-γ production was increased only in the former group. In contrast, the antibody levels were significantly lower and directed at fewer HCV antigens in patients of this group compared with those in the second group.They concluded that patients without viraemia after HCV infection frequently have strong Th lymphocyte response of the Th1 type to multiple HCV antigens many years after the onset of infection, whereas antibody responses are less marked. These results suggest that control of HCV replication may depend on effective Th lymphocytes activation (Cramp et al, 1999).

Recent studies described an association between strong $CD4^+$ Th cell activity to certain HCV antigens and a self-limited course of acute hepatitis C and possibly also a sustained response to treatment with IFN-α (Lasarte et al, 1998). Diepolder et al (1995) described that cellular immune responses to non-structural NS3,4,5 antigens are stronger in patients who resolve their acute infection than patients developing chronic liver disease. Echels et al (1999) used the non-structural protein which is critical to HCV infection and replication, NS3, to study *in vitro* helper T cell responses from infected individuals. Interferon-γ, IL4 and IL10 were secreted in response to stimulation by NS3 antigen, however, IL2 was not. They concluded that the predominance of IL4 and IL10 and the lack of IL2 suggests that *in vitro* response to HCV antigens are biased towards a Th2 phenotype, which may be conductive to viral persistence (Echels et al, 1999).

In the study of Bergamini and his colleages (2001) the hypothesis that the inability to eradicate chronic HCV infection results from either an over growth of Th2 oriented cells or increased production of Th2 type cytokines had not been proven. On the other hand, they stated that it is important to emphasize that the ability of the immune system to mount a protective $CD4^+$ effector response is not only dependent on the number of antigen-specific memory T cells present, but also on the functional phenotype of these cells. They demonstrated that the HCV-reactive $CD4^+$ cells in HCV patients showed a polarized IFN-γ response with a lack of IL2 production. This lack of IL2 production may compromise the overall effector response limiting, for example, recruitment, differentiation and expansion of $CD8^+$ effector T cells or natural killer cells.

The immune surveillance of the host IL12 plays a central role in mounting an effective cellular immune response directed towards elimination of intercellular pathogens. Schlaak et al (1998) suggested that HCV-related cellular immune defect in patients with hepatitis C, can be restored by IL12. Lee et al (2000) studied the effect of IL12 on hepatitis C viraemia in non-responders to previous IFN-α treatment. Their results imply that IL12 exerts only limited antiviral activity against HCV quasispecies *in vivo*. On the other hand, follow up data for patients with chronic hepatitis C treated with IFN-α identified strong persisting NS3 and

NS4 specific Th cell reactivities in those who successfully cleared HCV and not in patients who failed biochemical and virological response (Diepolder et al, 1995). Löhr et al (1998) also reported that IFN-α treatment enhances NS3, helicase and NS4 antigen specific T helper cell responses between patients with viral clearance, whereas viral persistence was associated with increased T cell reactivities against core and NS5 antigens. On the other hand, Lasarte et al (1998) reported that both expansion of HCV-specific Th cell precursors and Th cell recognition of multiple core epitopes seem to be important in the elimination of HCV after IFN therapy.

The humoral immune response to hepatitis C virus: its correlation with disease activity and response to interferon-alpha:

Although the vast majority of patients with chronic hepatitis C developed antibodies to structural and / or non-structural recombinant and synthetic HCV antigens in their sera, the pathogenetic role of the HCV specific humoral immune response is not clear. Neutralizing effects by binding extracellular viral antigens is controversial, but has not yet been proven (Shimizu et al, 1994). On the other hand, viral antigens are expressed on infected hepatocytes, and antibodies bound to those antigens can be recognized by cytotoxic killer lymphocytes, a process named antibody-mediated cellular cytotoxicity (ADCC) (Löhr and Gerken, 1997).

Since the cloning of HCV genome (Choo et al, 1989), a marked correlation was found between the detection of IgG anti HCV-core and the presence of serum HCV-RNA sequences in chronic hepatitis C patients (Yuki et al, 1993). However, the IgG anti HCV-core response does not correlate with HCV replicative states in chronic hepatitis C patients (Yuki et al, 1994). Other studies showed that the response of the IgM anti HCV-core depends on serum HCV-RNA levels, thus indicating distinct virological implications of IgG and IgM immune responses to the HCV-core protein (Yuki et al, 1995). They described that a positive correlation was found between viraemic levels and IgM anti HCV-core levels. Moreover, IgM anti HCV-core levels decreased when serum HCV-RNA was cleared after IFN-alpha therapy and increased in association with reappearance of viraemia. On the other hand, they found no correlation between the occurrence of IgM anti HCV-core and liver disease activity (Yuki et al, 1995).

In chronic hepatitis B virus infection, IgM antibody to hepatitis B core antigen (anti-HBC) has been shown to correlate well with disease activity but not with hepatitis B virus replication. This discrepancy may reflect different mechanisms of liver injury. Yuki et al (1995) also mentioned that HCV viraemic levels did not show apparent relations with serum aminotransferases levels and the histological activity index although serum aminotransferase tend to be high in highly viraemic patients (Yuki et al, 1995). In another study done by Caporaso et al (1994), they concluded that the prevalence of positivity for HCV-core IgM is significantly higher in patients with severe chronic hepatitis than in those with mild chronic hepatitis and that the level of IgM anti HCV-core positivity correlates significantly well with serum ALT levels and is significantly higher in patients with severe chronic hepatitis than in those with mild chronic hepatitis. They also reported that all HCV-RNA positive subjects showing normal values both for laboratory and histological parameters were negative for IgM anti HCV-core (Caporaso et al, 1994).

Gérard et al (1996) described a combination of some serological markers to predict the presence or absence of viraemia in HCV seropositive blood donors. Their results confirm that HCV-RNA detection by PCR in serum is strongly correlated with high titer IgG anti HCV-core (i.e. O.D.≥2), a complete HCV antibody profile in supplemental assay (antibodies against structural and non-structural recombinant HCV proteins), and detectable IgM anti HCV-core. They discussed that they can predict PCR positivity only by combining serological markers and ALT and hence reduce the need for PCR testing prior to donor counselling.

Löhr et al (1996) reported that the detection of IgM anti HCV-core antibodies is associated with a rising disease activity and a negative outcome of antiviral treatment. The follow-up of patients revealed that the humoral response was down-regulated in responders but persisted in patients who showed no clinical improvement. Dentico et al (1999) suggested that IgM anti HCV may serve as a serological marker of HCV infection and a complementary marker of virus replication. Quantitation of IgM-subclass anti-HC 34 antibodies revealed a good

correlation with viraemia level and significantly different IgM antibody levels in non-responder, partial responder and complete responder patients early after therapy onset. Thus, the quantitative monitoring of anti-HC34 core antibody levels may give additional information in the early phase of therapy to allow an earlier prediction of response (Löhr et al, 1996).

Brillanti et al (1992) suggested that during IFN-α treatment, disappearance of IgM antibodies to non-structural (C-100) HCV protein appears to predict a complete remission of disease activity and a sustained response. Tassopoulos et al (1994) reported identical conclusions for IgM antibody response to the structural core rather than to the non-structural C-100. They also suggested that IgM response to structural HCV-core antigen may be an early predictor of successful IFN-α treatment of chronic hepatitis C. These interesting results await confirmation in a controlled study of the long term follow-up of IFN-α treated chronic hepatitis C.

1.11 Treatment of hepatitis C infection

Since their discovery in 1957, interferons (IFNs) have been noted to have protective effects against human viral infections. The use and safety of IFNs in patients with acute or chronic hepatitis B or C infections have evolved over the last 20 years. The most studied IFN for the management of viral hepatitis is IFN-α, but others have recently been evaluated through controlled clinical trials (Zein, 1998).

Although only a few and limited studies have evaluated the use of IFNs in acute hepatitis, treatment appears to decrease the likelihood of chronicity and should be considered. In 1986, Hoofnagle et al first treated patients with chronic non-A, non-B hepatitis with recombinant interferon IFN-α, a molecule with antiviral and immunomodulatory properties (Hoofnagle et al, 1986).

Since HCV has been identified and found to be the main cause of both parenterally acquired non-A, non-B hepatitis and "crypto genetic" chronic hepatitis, a large number of randomized controlled trials of IFN have been done in chronic hepatitis C. The effects of IFN-α may include inhibition of HCV virion production by an effect on viral RNA and protein synthesis, enhancement of immune lysis of HCV infected cells, inhibition of hepatic fibrosis by an effect on TGF-β and an effect on HCV induced carcinogenesis (Thomas et al, 1999).

Schematically, 3 million units of IFN-α three times a week subcutaneously for 6 months induces a biochemical response (BR) characterized by normalization of ALT activity (thought to reflect disease activity) in almost 50% of patients at the end of treatment. However, half of the responders experience relapses within 6 months (shown by an ALT increase). Overall, no more than 25% of patients have sustained BR after a 6 months course of IFN (Tiné et al, 1991). It was recently reported that when IFN is administrated for 12 months or longer, effective cancer prevention will be achieved, even in patients with HCV-related cirrhosis (Ikeda et al, 2001). The advent of molecular biology techniques, such as the polymerase chain reaction (PCR), has meant that HCV-RNA is now widely used to monitor

patients with chronic hepatitis C treated with IFN. A virologic response is defined as the disappearance of serum HCV-RNA detected by PCR. However, discrepancies occur between biochemical and virologic responses. Patients with no BR usually have detectable HCV-RNA at treatment withdrawal, as do most patients who show a BR but experience relapse within 6 months of IFN withdrawal. In contrast, patients with a sustained BR usually remain HCV-RNA negative, but some have detectable HCV-RNA in serum despite repeatedly normal ALT activity. The histologic response to IFN-α therapy can be evaluated by comparing pre-treatment liver biopsies with those several months after treatment ends. Again, discrepancies have been observed between histologic, biochemical and virologic responses (Pawlotsky et al, 1996).

Rates of sustained biochemical and virological responses in the range of 20-40% have been reported for a 12-month regimen of IFN-α. However, this response is transient in the majority of patients and only 10-15% have a sustained response after treatment is stopped. Longer duration (18-24 months) of treatment, higher doses of IFN-α (up to 30 million u/w) and treatment with other types of IFN-α may improve the response, however, many patients still relapse (Liang, 1998).

Shapiro et al (1998) discussed that in addition to its antiviral effects, IFN-α regulates the function of cytokines, their receptor and other molecules of immune importance. They reported m-RNA expression of Th2 (IL4, IL6 and IL10), Th3 (TGF-β) and TNF-α in HCV patients treated with IFN-α while no difference was observed for m-RNA of classical Th1 cytokine (IL2 and IFN-γ) after treatment. They concluded that the cytokine m-RNA profile following IFN-α treatment points to an anti-inflammatory response, which does not appear to be involved in termination of the viral infection. This may explain the failure of the immune system to eradicate HCV chronic infections and suggests that early treatment in the acute phase of disease with agents, which stimulate cytotoxic immune type 1 response, may lead to eradication of HCV infections (Shapiro et al, 1998).

Appasamy et al (1994) reported that in patients with chronic hepatitis C, IFN-α therapy resulted in a significantly decreased number of peripheral blood leucocytes

and, specifically, lymphocytes. This reduction in the number of leucocytes during IFN-α therapy may be due to their migration in vessels or accumulation on liver tissue or other sites of infection. While the number of circulating T and B cells declined during IFN-α therapy, that of activated T cells ($CD3^+$ $CD25^+$) and, particularly, of activated helper-inducer T cells ($CD4^+$ DR^+) selectively increased with IFN-α treatment. The percentage of activated natural killer cells (NK) was also significantly elevated over the pretreatment baseline. In contrast, IFN-α therapy showed no effect on NK activity in peripheral blood mononuclear cells in this study. The absence of a significant increase in NK cells activity in the presence of the increased percentage of activated NK cells observed during IFN-α therapy might represent an attempt at the down-regulation of effector cell function, thus reducing the possibility of liver injury (Appasamy et al, 1994).

Currently, treatment is recommended for persistently elevated aminotransferase concentration, HCV viraemia and findings of fibrosis and moderate inflammation on liver biopsy. For other groups of patients, the indications for treatment are less clear and decisions should be made on an individual basis (Liang, 1998).

Some pretreatment parameters have been suggested as predictive of BR to IFN-alpha including body weight < 86 kg, a low disease activity index and no cirrhosis on liver biopsy, low serum ferritin, normal γ-glutamyl transfersae (γGT) activity, and a low serum bilirubin concentration (Pawlotsky et al, 1996). As for viral factors, Lam (1999) reported that response rates are lower in patients with HCV genotype 1 and serum HCV-RNA concentration higher than one million copies / ml. Patients with genotype 1 HCV and a high viral load require 12 months of treatment to achieve a sustained virological response in approximately 30%, compared to those with genotypes 2 or 3 who achieve a sustained virological response in approximately 65% after 6 months (Sievert, 2002). Finally, some authors have reported that a lack of anti HCV-IgM is predictive of a response to IFN therapy (Pawlotsky et al, 1995). However, the definition of a response (biochemical, virologic or both) and the time of its assessment (during, at treatment withdrawal or several months later) should be considered when talking about sustained response. Sievert (2002) reported that sustained virological

response, defined as undetectable HCV-RNA in blood 6 months after completing antiviral treatment, is the best indicator of a beneficial treatment effect.

Trials of Ribavirin oral antiviral nucleoside-monotherapy conducted in large series of patients have demonstrated that the drug has only transient effects when used alone for chronic hepatitis C. When used alone, Ribavirin was able to normalize ALT in several patients, particularly those infected with HCV-1b, Ribavirin therefore appeared to be more effective than interferon. This biochemical response was not associated with significant changes in HCV-RNA level, however, suggesting that Ribavirin suppressed disease activity without affecting viral replication (Chemello et al, 1995). The potential mechanisms of action of Ribavirin, although not yet fully understood, include inhibition of synthesis of GTP by an effect on inosine monophosphate dehydrogenase thereby limiting viral RNA synthesis, and enhancement of Th1 responses, which may assist viral clearance (Thomas et al, 1999).

When Ribavirin was given with IFN-α, the antiviral effect of IFN-α was significantly enhanced, as evidenced by a reduction in the relapse rate after cessation of treatment. These findings suggest that Ribavirin augments the effect of IFN by acting on some IFN-resistant virus subpopulation or on intracellular reservoirs of HCV not accessible to IFN. The synergism may allow efficient sterilization by clearing any IFN-resistant HCV virions, thus preventing hepatitis reactivation after therapy ceases (Chemello et al, 1995). Combination therapy was also reported to be associated with a significant sustained response in patients with chronic hepatitis C genotype 4 resistant to IFN-α treatment (El_Zayadi et al, 1995 and Koshy et al, 2000). Recently, Dettmer et al (2002) reported that treatment with IFN-α2b plus Ribavirin is effective in approximately 50% of "relapsers" and "non-responders" infected with non-type 1 genotypes of hepatitis C virus and that therapy is only marginally effective in "non-responders" infected with genotype 1a or 1b.

Thomas et al (1999) reported that Ribavirin may have activity at extrahepatic sites of HCV infection, thus explaining the marked reduction in relapse rates with

combination therapy without an appreciable effect on initial antiviral response. In patients who relapse after an initial response to Interferon-alpha, retreatment with IFN-α plus Ribavirin or with a higher dosage of IFN-α is also recommended. New agents under development for use against hepatitis C include viral enzyme inhibitors, ribozyme and antisense oligonucleotides and immuno-modulators (Lam, 1999).

Covalent attachment of a 40-kd branched-chain polyethylene glycol moiety to IFN-α2a results in a compound (peginterferon-α2a) that has sustained absorption, a slower rate of clearance, and a longer half-life than unmodified IFN-α2a. Zeuzem et al (2000) reported that in patients with chronic hepatitis C, a regimen of once weekly administration of peginterferon-α2a is more effective than a regimen of IFN-α2a given three times weekly. The efficacy and safety of peginterferon-α2a in patients with HCV related cirrhosis or bridging fibrosis was examined by Heathcote et al (2000). They reported that 180 microgram of peginterferon-α2a administered once weekly is significantly more effective than 3 million units standard IFN-α2a administered three times weekly in this group of patients. Teoh and Farrell (2004) reported that for chronic hepatitis C patients the most effective therapy is a pegylated (long-acting) interferon with ribavirin. Sustained viral response can be obtained in 40-60% of individuals infected with genotype 1 and in approximately 67% with genotype 4 after 12 months of treatment. Response rates are higher (75-85%) with genotypes 2 and 3 after only 6 months of treatment. This viral cure reverses hepatic fibrosis, reduces the risk of liver failure and of hepatocellular carcinoma.

Recent preliminary data have suggested that Amantadine is effective against HCV infection. Carlsson et al (2000) used Amantadine (200 mg / day) in combination with IFN-α three million units thrice weekly and Ribavirin (1000 mg /day) for 24 weeks in the treatment of non-responders and response / relapsers to previous IFN / Ribavirin therapy. They concluded that the addition of Amantadine to IFN-α and Ribavirin was well tolerated but had little, if any, impact on HCV-RNA eradication in non-responders and response / relapsers to previous IFN / Ribavirin combination therapy (Carlsson et al, 2000). It had also been suggested that priming with IFN-γ prior to the initiation of IFN-α treatment in patients with

refractory chronic hepatitis C can modulate the host immune response and this might contribute to viral clearance (Katayama et al, 2001).

The relapse of HCV infection after IFN-α treatment appears to be associated with the presence of HCV mutation (Finkelstein et al, 1992). A striking association has been demonstrated between mutations in amino acid residues 2209-2248 of the NS5a region of hepatitis C virus and sustained responses to IFN-α in Japanese patients infected with genotype 1b. Therefore, analysis of this sequence has been suggested as a predictor of treatment response (Enomoto et al, 1996). On the other hand, this variation in NS5a (2209-2248) fails to predict IFN responsiveness in American patients infected with the same genotype. This may be explained by difference in treatment regimens, host genetic background or alterations in the interferon signaling pathway induced by surrounding sequences within or outside NS5a (Chung et al, 1999).

Mathematical modeling studies have provided insights into the mechanism of action of IFN-α in chronic HCV. The two-phase plasma HCV-RNA disappearance curve may reflect the presence of an interferon-resistant second site of HCV replication either within or outside the liver. Clinical observations and cerebral magnetic resonance scans provide evidence of functional cerebral impairment in HCV infected patients, raising the issue of the CNS as a site for HCV replication (Thomas et al, 1999). On the other hand, Cribier et al (1999) confirmed that HCV-RNA can be detected in mononuclear cells despite negative plasmatic PCR in patients treated with IFN-α and suggested that the persistence of viral RNA in peripheral mononuclear cells could be a predictive factor of treatment failure.

The natural history of untreated chronic hepatitis C is controversial, and direct knowledge of the long-term clinical and economic outcomes of current IFN-α treatment regimens remains limited. Decision analytic models using available information on outcome probabilities and associated health care costs in the United States have been developed but are available only in abstract form. They suggested that chronic hepatitis C is a life shortening disease and that IFN-α treatment for 6 or 12 months, despite its up-front costs and failure to induce a

prolonged therapeutic response in most patients, increases life expectancy (which nevertheless is still reduced). It does so with a marginal cost-effectiveness well within the acceptable range of medical interventions in the United States. Even empiric therapy, without regard to viral level, genotype and baseline histology, is within an acceptable cost-effectiveness range. Improving the response rate is likely to make treatment even more cost-effective and possibly cost saving. What needs to be kept in mind is that, whether by its natural course or by therapy to suppress liver inflammation or by interferon therapy, the rate of development of hepatocellular carcinoma is reduced if ALT is maintained at low level (Lino, 1999).

Attention should therefore be focused on the development of drugs, which enhance the effect of IFN as well as drugs which suppress liver inflammation. In recent years, there has been a substantial increase in the use of so-called complementary and alternative therapies by patients with liver disease. Silymarin, extracted from the seeds of Silybum marianum or milk thistle is most widely subscribed to as a remedy for liver diseases (Bean, 2002). Silymarin may play a role in the therapy of alcoholic liver cirrhosis. It has metabolic and cell-regulating effects at concentrations found in clinical conditions, namely carrier-mediated regulation of cell membrane permeability, inhibition of the 5-lipoxygenase pathway, scavenging of reactive oxygen species (ROS) of the R-OH type and action on DNA-expression, for example, via suppression of nuclear factor-kappa B. Silymarin has a good safety record and only rare case reports of gastrointestinal disturbances and allergic skin rashes have been published (Saller et al, 2001). An ongoing clinical trial will provide some insight as to whether milk thistle directly affect HCV (Bean, 2002).

In Japan, glycyrrhizin has been used for more than 20 years as treatment for chronic hepatitis. Glycyrrhizin, the active component of licorice root, has been shown to reduce alanine transaminase and aspartate transaminase values in the serum. This protective function has recently been explained as the inhibitory effects of glycyrrhizin on immune-mediated cytotoxicity against hepatocytes and on nuclear factor-kappa B, which activates genes encoding inflammatory cytokines

in the liver (stated in Bean, 2002).

1.11.1 IFN-α therapy in chronic hepatitis with autoantibodies:

Anti-HCV positive chronic hepatitis patients with autoantibodies have been commonly treated with corticosteroids in the past because the presence of anti-HCV was often regarded as a false positive finding and IFN-α treatment was considered potentially dangerous (Nishiguchi et al, 1992). Thus, a short trial of therapy with corticosteroids had been suggested to establish whether disease was caused by an autoimmune process (Papo et al, 1992). Magrin et al (1994) reported that corticosteroids treatment generally increases hepatitis C viraemia among these patients even if improved aminotransferase levels are sometimes observed. Todros et al (1995) reported that hepatitis C patients with anti-LKM may be exposed to an increased risk of an adverse hepatitis reaction while being treated with IFN. However, they found that the extent of the risk was minimal compared with the expected benefits of the therapy. They recommended IFN as the first therapy to choose in these patients and that these patients must be monitored more closely for possible liver dysfunction than the ordinary hepatitis C patients. (Todros et al, 1995).

Cassani et al (1997) performed a study to evaluate the influence of autoantibodies associated with HCV on the profile of the disease. They reported that the presence of autoantibodies did not influence the response to IFN. Wada et al (1997) also discussed that pre-existing or newly developed autoantibodies do not necessarily predict a poor response to IFN-α2. On the other hand, It had also been reported that IFN-α may precipitate or exacerbate autoimmune disease symptoms and that HCV-related autoimmune disease could be treated successfully with corticosteroids, azathioprine and cyclophosphamide, although HCV viraemia persists and may worsen (McMurray and Elbourne, 1997).

Czaja (1997) described that IFN therapy is appropriate for patients with viral antigen-driven process that depend on immune complex deposition and for patients with mild background autoimmune expressions. Corticosteroid therapy should be considered for those unusual patients with predominant autoantigen-driven process since IFN treatment may exacerbate immune-mediated disease.

1.11.2 The role of IFN-alpha in the development of autoimmune diseases in chronic hepatitis C(CHC) patients treated with it:

Different side effects have been reported in patients treated with IFN-α but their incidence and prognosis in the case of adverse reactions remains largely unknown. Several reports have shown that IFN-α treatment can lead to exacerbation of subclinical autoimmune disease. Type-1 interferons were shown to increase the expression of MHC class-1 molecules and may also induce intercellular adhesion molecule (ICAM) and B7.2. Increased expression of MHC class-1 and the respective co-stimulatory molecules ICAM and B7.2 may lead to activation of autoimmune T cells and to increased autoimmune reactivity leading to autoimmune disease (Eibl et al, 2001).

Garcia-Buey et al (1995) reported that prolonged courses of IFN may induce autoantibodies as well as autoimmune disorders and that some of the autoimmune reactions could be directed against the liver causing exacerbation of liver diseases. The incidence of this IFN-induced exacerbation is probably low, but it is unpredictable and may lead to liver failure so an increase in serum ALT levels during IFN treatment for CHC should alert physicians that this could be due to an underlying autoimmune diathesis. IFN induces the over expression of major histocompatibility (MHC) class I antigens on the surface of liver cells and might therefore increase aberrant autoimmune reactions. Female sex was clearly associated with possible latent AIH triggered by IFN therapy and the frequency of association of HLA-DR52 and DQ2 antigens was significantly higher in those patients compared with that in healthy control (Garcia-Buey et al, 1995).

Bayraktar and his colleages (1997) reported that IFN diffusely activates the cellular immune system and can initiate new autoimmune diseases in patients treated with it. Exacerbation or induction of various extrahapatic autoimmune disorders by IFN have been also discussed through several mechanisms including inhibition of T-suppressor cells, enhancement of T cytotoxic and T helper cell function with production of IFN-γ and other cytokines, activation of monocytes and increase of immunoglobulin production. Antibody dependent cellular cytotoxicity or aberrant expression of HLA antigens with subsequent T-cell

cytotoxicity have been proposed with several IFN associated autoimmune disorders such as hypothyroidism, thyrotoxicosis and immune thrombocytopenia.

Carella et al (1995) confirmed that a 12-month IFN-α2a treatment in patients with HCV hepatitis is accompanied with the appearance of anti-thyroid antibodies in a considerable number of patients. This finding is reversible in most cases with the disappearance of antibodies after stopping therapy. They described that the pretreatment positivity for anti-thyroglobulin antibodies (Tg-Ab) was not associated with the subsequent development of thyroid dysfunction; therefore it cannot be considered a risk factor in IFN-α therapy. On the other hand, the appearance in some cases of thyroid peroxidase Ab (TPO-Ab) after stopping treatment suggest that the TPO-Ab could be considered the most useful marker for prolonged follow up of thyroid autoimmunity in previously IFN-α2a treated patients. The persistence in some cases of TPO-Ab after stopping treatment has to lead to prolonged thyroid surveillance in these patients.

Custro et al (1997) reported that patients with hepatitis C were unlikely to develop thyroid dysfunction in the absence of IFN therapy, in spite of being thyroid antibodies seropositive. Moreover, a considerable proportion of sero-negative patients, when IFN treated, developed thyroid autoimmunity and then thyroid dysfunction. Both in seropositive and seronegative patients immediate IFN discontinuation normalized thyroid function and hormone replacement therapy was not necessary.

Carella et al (2002) reported that the addition of Ribavirin to IFN-α therapy for chronic hepatitis C does not modify the thyroid autoantibody pattern but exposes the patients to a higher risk of hypothyroidism, probably as a consequence of direct effects of Ribavirin on Th1- and Th2-like immune responses, with a specific shift favouring the Th1-like activity. In thyroid autoimmune disease, while the Th2 lymphocytes regulate autoantibody production, the Th1 subsets have limited B cell help capacity but function as mediators of tissue damage. Ribavirin could induce hypothyroidism by Th1 dependent activation of $CD8^+$ T lymphocytes which induce cellular destruction predominantly by the perforin pathway. The thyroid cell

damage in Ribavirin-induced hypothyroidism could also be through a complement-mediated injury or induction of follicular cell apoptosis. These authors also concluded that the development of hypothyroidism in patients who develop thyroid autoantibodies during the course of treatment with IFN-α and Ribavirin is significantly associated with the long-term remission of CHC. The Th1-like mechanism shared by both autoimmune hypo-thyroidism and viral clearance, may contribute to this finding. Such a correlation is lacking in patients undergoing treatment with IFN-α alone.

Floreani et al (1998) reported that besides the thyroid gland, pancreatic beta-cells could be a target of autoimmunity during IFN-treatment for chronic HCV hepatitis. Recasens et al (2001) described two cases of adult subjects receiving IFN-α therapy for chronic hepatitis C who developed ketoacidosis soon after reporting hyperglycaemic symptoms. They proposed that in predisposed subjects IFN-α could trigger the autoimmune process that destroys β-cells. They concluded that type-1 diabetes mellitus is a recognized complication of IFN-α therapy in subjects with chronic hepatitis C. In such a case, the onset of the disease may be particularly abrupt with high risk of diabetic ketoacidosis. Betterle et al (2000) described that patients who are initially positive for organ-specific autoantibodies, in particular thyroid and pancreas, and those who seroconvert are at high risk of developing clinical autoimmune disease after treatment with IFN-α. Eibl et al (2001) discussed that as hepatitis C itself and IFN-α have been shown to induce autoimmune mechanisms, autoantibodies specific for type-1 diabetes should be included in the panel of autoantibodies screened before the start of IFN-α treatment. On the other hand, Bosi et al (2001) concluded that type-1 diabetes can occur as a side effect of IFN-α therapy in individuals with a susceptible genetic background, but without detectable autoantibodies prior to treatment. In this example of fulminant type 1 diabetes a pathogenic process unbalanced towards a Th1-mediated autoimmune response is hypothesized. Recently, Weschea et al (2001) reported for the first time that adrenal cortex also has to be considered as a potential target of IFN-α related autoimmunity. A genetic predisposition may be important, though insufficient alone, in the development of IFN-induced autoimmune phenomena.

AIM OF THE WORK

AIM OF THE WORK

CHAPTER 2: AIM OF THE WORK

HCV causes a complex spectrum of diseases. Fluctuating levels of serum alanine aminotransferase (ALT) in some patients, and normal serum ALT concentrations in other patients with detectable viraemia indicate that a complex relationship exists between viral replication, host immune responsiveness and disease pathogenesis. It appears that several aspects of immunodisregulation may play a role in pathogenesis. IgM antibodies to HCV have been found in a variable percentage of patients with chronic HCV infection (Brillanti et al, 1993). The clinical and diagnostic significance of the detection of this antibody remains unclear. However, some authors have proposed that the persistence of IgM anti-HCV in patients with acute HCV infection identifies those who evolve to chronic infection (Quiroga et al, 1991), while others have proposed that the disappearance of this antibody class during the treatment of chronic HCV with interferon-α can predict sustained response (Brillanti et al, 1992). Additionally, a positive correlation between the presence of IgM anti-HCV and the activity of the liver disease has been suggested (Martinelli et al, 1996). On the other hand, viral infections are considered a possible trigger of autoimmune disease. In autoimmune liver diseases the hepatotropic viruses especially HCV have received particular attention as possible etiological agents. HCV is reported to have a major relation to autoimmune hepatitis (AIH) type-2 which is characterized by auto-antibody against the microsomal antigen of liver and kidney (LKM1) (Michitaka et al, 1994). Abuaf et al (1993) indicated that antinuclear (ANA) and antismooth muscle autoantibodies (ASMA) were more frequent in chronic hepatitis C patients than in healthy blood donors. However, there are conflicting reports regarding the frequency of these findings in the patients in Mediterranean countries (Italy, Spain, Egypt) and in Northern and Western Europe.

Lenzi et al (1991) suggested that geographical or genetic factors might influence the presence of these autoantibodies. It was also assumed that HCV genotype may be related to the frequency of an autoimmune response (Michitaka et al, 1994).

Finally, hepatitis C virus infection is associated with a variety of extra-hepatic manifestations including autoimmune features like cryoglobulinaemia and Sjögren's Syndrome and may also be associated with immunologically mediated renal disease such as membranoproliferative glomerulonephritis (Johnson et al, 1993). Tran et al (1993) speculated that the high prevalence of thyroid dysfunction in female patients with chronic hepatitis C strongly suggests that HCV contributes to the occurrence of thyroid autoantibodies and in some cases initiates autoimmune diseases such as Hashimoto's thyroiditis. Anticardiolipin antibodies are frequently found in patients with chronic hepatitis C and in these patients they may be implicated in the occurrence of thrombosis and development of thrombocytopenia. Occult HCV infection is present in a significant proportion of patients with thrombotic disorders. It was suggested that HCV in combination with other infections, environmental and genetic factors can trigger complex immunological disorders with different clinical patterns (Ferri et al, 1994). Clearly the data reviewed leave much to be desired in our understanding of autoimmune phenomena in hepatitis C infection.

The overall aim of the present studies was to analyse aspects of immune status of patients suffering from hepatitis C infection. To this end, the presence of autoantibodies and parameters of cellular immune responses were studied. The presence and the level of autoantibodies have been correlated with the following findings in the same group of patients:

1- Histological characteristics of liver damage.

2- Host immune response to hepatitis C, in particular presence and level of HCV-core IgM antibodies.

3- The response of patients to interferon-α (IFN-α) therapy.

4- Viral load determined by qualitative PCR and hepatitis C virus genotype.

Additionally, parameters of the cellular immunity were analysed in relation to the clinical course of viral infection and the response of HCV infection to IFN-α.

These included the following:

1- Phenotyping of immunocompetent cells, including the quantitation of mature leukocyte subsets ($CD4^+$T cells, $CD8^+$T cells) in the PBMCs of the patients, and the percentage and absolute number of activated lymphocytes ($CD25^+$).

2- Cytokine synthesis by unstimulated and mitogen (Con-A) stimulated PBMCs, using ELISAs. This included an assay for the Th1 cytokine, IFN-γ; Th2 cytokine, IL4 and the down-regulatory cytokine, TGF-β1.

3- Cytotoxicity assay for NK cell activity.

PATIENTS AND METHODS

PATIENTS AND METHODS

CHAPTER 3: PATIENTS AND METHODS

3.1 Patients:

The study cohort comprised of 117 patients (83 males and 34 females), age range 16 – 60 years, median 38 years. One patient was 3 years old. All of them presented to our hospital with high levels of transaminase activity, in particular serum alanine aminotransferase (ALT). All our patients were tested for anti HCV antibody and found to be positive by an ELISA test. Ten patients had a history of blood or blood product transfusions and 5 patients were drug abusers. One patient was HBV seropositive (HbsAg positive) whilst 3 further patients had markers of an old HBV infection (antiHBc and antiHBs positive).

All patients were negative for antibodies to human immunodeficiency virus (HIV). At the beginning of the study, none of the patients with chronic hepatitis C were receiving antiviral treatment. Other causes of chronic liver disease (alcoholism, Wilson's disease, hepatotoxic drugs, haemochromatosis, α1-antitrypsin deficiency, autoimmune hepatitis) had been excluded.

For comparison, a control group of 20 clinically healthy volunteers were included in the study. The age distribution and sex ratio were similar between the control group and the group of patients under investigation. Tawam Hospital Ethical Committee granted approval for the study protocol.

3.2 General design of the study:

Patient sera were initially tested for anti HCV antibodies (UBI HCV EIA 4.0) and positive results were confirmed by line immunoassay (Liatek HCV III, Organon Technika).

The following investigations were undertaken for all the patient / control groups in the study: -

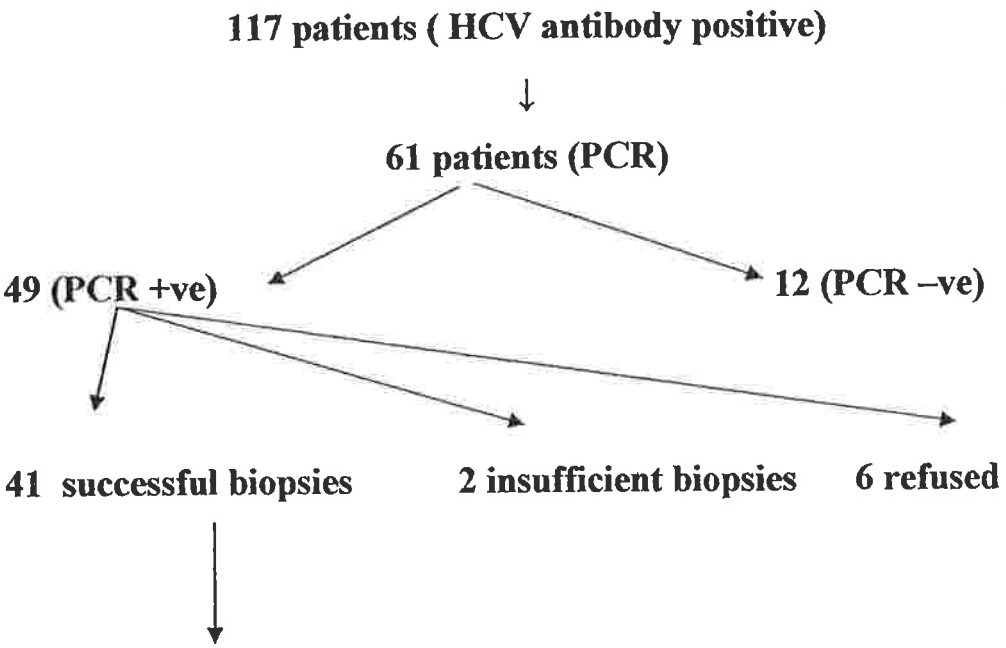
- A full history was taken and the presence of chronic hepatitis C virus infection was established. Confirmatory antibody tests were also employed.

- Blood chemistry and tests of liver function including total protein, albumin, serum total bilirubin, alkaline phosphatase, gamma glutamyl transferase (γGT), serum glutamic oxaloacetic transaminase (AST) and serum glutamic pyruvate transaminase (ALT) were done in most of the patients, however, AST and ALT were included in the patients' analysis in this study.
- Qualitative assay of hepatitis C viral load in the blood of chronic hepatitis C patients was studied.
- The presence of a panel of circulating autoantibodies was analysed: These included antinuclear antibody (ANA), antimitochondrial antibody (AMA), antismooth muscle antibody (ASMA), anticardiolipin antibody, thyroid antibodies (antithyroglobulin and antithyroid peroxidase antibodies), anti-liver/kidney microsomal antibody (LKM_1) and antineutrophil cytoplasmic antibody (ANCA). Sera positive for any of these autoantibodies at screening dilution were titrated out to negative.
- Host immune response to the virus, in particular presence and level of HCV-core IgM antibody production, was also evaluated.
- Relevant parameters of immunocompetence, including T cell subset ratios, INF-γ, IL-4 and TGF-β production by unstimulated and Con-A stimulated peripheral lymphocytes and natural killer (NK) cells were also analysed.
- Serotyping of hepatitis C virus.

The presence and the level of antibodies have been correlated with the following findings in the same group of patients:

- Histological characteristics of the liver damage.
- Host immune response to hepatitis C in particular presence and level of HCV-core IgM antibodies.
- The response of patients to IFN-α therapy.
- Viral load determined by qualitative PCR.
- Hepatitis C virus genotype.

Figure C: The studied groups of hepatitis C patients



(were available for studying the relation between the presence of organ non-specific autoantibodies and disease activity (as shown in Figures 1&2).

3.3 IFN-α treatment schedule:

Out of 117 patients, 61 patients were initially considered suitable candidates for IFN-α therapy. They were tested for qualitative HCV-RNA prior to the antiviral treatment, 49 patients were positive and 12 were negative. Out of the 61 patients chosen initially for IFN-α treatment, 41 patients only started and continued the treatment for 12 months. The 12 HCV-RNA negative patients were excluded due to lack of viraemia. Eight patients out of the 49 HCV-RNA positive patients were not treated either due to high cost or poor patients collaboration (Figure C). Three million units had been given to the above-mentioned patients three times weekly subcutaneously for 1 year.

Patients showing biochemical response characterized by normalization of ALT activity and virological response shown by complete elimination of virus by HCV-RNA test, were classified as responders while those showing neither biochemical response nor virological response were classified as non-responders (Lampertico et al, 1994). Six months after starting IFN-α therapy, the studied patients were grouped as responders and non-responders based on the previously mentioned criteria.

3.4 Laboratory methods :

3.4.1 Assay of antibodies to HCV:

The assay of HCV antibodies was performed using a third generation HCV ELISA kit from Organon Technika (UBI HCV EIA 4.0) which employs synthetic peptides corresponding to the core and nonstructural ($NS_{3,4,5}$) regions of the HCV genome bound to wells of a microplate. During the course of the assay, diluted controls and diluted specimens were added to the wells and incubated. HCV specific antibodies, if present, bound to the immunosorbent. After thorough washing of the wells to remove unbound immunoglobulins and other serum components, a standardized preparation of horseradish peroxidase-conjugated goat antibodies specific for human IgG was added to each well. The conjugate was allowed to react with the

bound antibodies. After another thorough washing of the wells, a substrate solution containing hydrogen peroxide and o-phenylenediamine (OPD) was added to each well. A yellow orange colour developed in proportion to the amount of HCV specific antibodies present, if any, in the serum samples tested. This enzyme-substrate reaction was terminated by the addition of a solution of sulphuric acid. The colour changes that have occurred in each well were then measured spectrophotometrically at a wave length of 492 ± 2 nm. The anti-HCV kit was used according to the manufacturer's instructions. In addition, a more specific test (immunoblot assay) was performed with serum samples that showed HCV antibody activity.

3.4.2 *Confirmation of anti HCV reactive specimens by line immunoassay techniques:*

Specimens reactive for the presence of HCV antibodies were confirmed by line immunoassay (Liatek HCV III, Organon Technika) using antigens from the E_2/NS_1, NS_3, NS_4, NS5 and core proteins coated as discrete lines on a test strip. In addition, the strip includes one antistreptavidin control line and three positive control lines (antihuman IgG and human IgG). Together, strip and sample were incubated in a test trough. Following any specific antibody reaction with the corresponding antigen on the strip, the strip was washed with buffer and antihuman IgG labelled with alkaline phosphatase was added. Following further washing to remove unreacted conjugate and incubation with the substrate (bromochloro-indolylphosphate indimethyl formamide) colour developed on the antigen line where bound antibody was present. The colour reaction was stopped using sulphuric acid (2 mol/l).The test procedures and interpretation of the results was undertaken in accordance with the manufacturer's instructions.

3.4.3 *Assay of hepatitis C viral load in blood:*

Qualitative assay of hepatitis C viral load in blood was performed by using Amplicor HCV, Roche Diagnostic System, Basel, Switzerland. The Amplicor HCV test is a direct DNA probe test that utilizes a nucleic acid amplification technology called Polymerase Chain Reaction (PCR) and

nucleic acid hybridization for the detection of HCV-RNA in human serum and plasma.

Nucleic acids were extracted from 0.1 ml serum, processed within 3 hrs of collection using the Amplicor sample preparation kit (Roche Molecular Systems) which relies on guanidinium thiocyanate (GTC)-mediated lysis of viral particles. After isopropanolol precipitation and ethanol washing, the RNA pellet was resuspended in a dilated buffer. Then, the dilated RNA was added to equal volumes of master mixes with primers of the 5' non-coding region and after a 105 minutes incubation step in the Thermo cycler (GeneAmp PCR system 9600, Perkin Elmer, USA), inactivation of potential carryover amplicons, first-strand cDNA synthesis and PCR amplification had taken place in a single tube.

The Amplicor HCV test is based on three major processes:

- Reverse transcription of target RNA to generate a complementary DNA strand (cDNA) and Polymerase Chain Reaction (PCR) target amplification.
- Hybridization of the amplified product to a specific oligonucleotide probe.
- Detecting the probe – bound amplified product by colour formation.

Selection of the target RNA sequence depends on identification of regions within the HCV genome that show maximum sequence conservation among the various genotypes. Accordingly, appropriate selection of primers is critical to the ability to detect all of these gene families. The 5′-untranslated region of the HCV genome has been shown to have maximum conservation of RNA sequence. The test uses the Primers KY78 and KY80 to define a 244 nucleotide sequence within the highly conserved 5′-untranslated region.

- **<u>Reverse transcription and PCR amplification</u>:**

The downstream, or antisense primer, KY78, was biotinylated at the 5′ end whilst the upstream, or sense primer, KY80, was not biotinylated. The RNA containing sample in the reaction mixture was heated to 60°C to allow the downstream primer to anneal to the target RNA. The thermo-stable recombinant enzyme *Thermus thermophilus* DNA polymerase (rTth)

extends the annealed primer, in the presence of excess deoxynucleoside triphosphates (dNTPs) to form a DNA strand which is complementary (cDNA) to the RNA target. At the end of the reverse transcription step, the reaction mixture was heated to 95°C to denature the RNA: cDNA hybrid; the rTth catalyzes the extension reaction and a second DNA strand was synthesized. The first cycle is a linear copying of the cDNA template. The reaction mixture was then heated to 95°C to separate the double stranded DNA and expose the primer target sequences. As the mixture cools, the primers anneal to their targets. The thermostable rTth polymerase then continues to extend the annealed primers along the target templates to produce a biotinylated DNA sequence termed an Amplicon. This process is repeated for 40 cycles, each cycle effectively doubling the amount of amplicon DNA. This number of cycles theoretically yields more than a billion fold amplification of the target.

- **Hybridization Reaction:**

After the PCR amplication process, the amplicons are chemically denatured to form single strands that are added to a microwell plate coated with the amplicon-specific oligonucleotide probe KY150. This specific hybridization of the amplicon to KY150 increases the overall specificity of the reaction.

- **Detection reaction:**

After unbound material was removed from the microwell plate, an avidin-horseradish peroxidase (AV-HRP) conjugate solution was added to the plate. The avidin binds to the biotin-labelled amplicons captured by the plate-bound probe, KY150. After unbound conjugate had been removed, peroxide and tetramethylbenzidine (TMB) are added and a colour complex was formed following reaction with the conjugate. The reaction was stopped by the addition of weak acid and the optical density is measured at 450 nm in an automated microwell plate reader. The results were classified by comparison with supplied cut off value.

Selective amplification:

Selective amplification of target RNA was achieved by the use of Amp Erase which contains the enzyme Uracil N-glycosylase (UNG), which recognizes and catalyses the destruction of deoxyuridine containing DNA, but not thymidine containing DNA. Deoxyuridine is not present in RNA, but is always present in amplicons due to the use of deoxyuridine triphosphate (in place of thymidine triphosphate) as one of the ddTps in the Master Mix Reagent. Thus, only amplicons contain deoxyuridine. The presence of deoxyuridine in amplicons renders contaminating amplicons susceptible to destruction by AmpErase prior to the amplification of the target RNA. When heated in the first thermal cycling step (at the alkaline pH of the Master Mix), the amplicon's DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. The UNG enzyme, which is inactive at temperatures above 55°C, was denatured by the addition of the Denaturation Solution immediately after the amplification step was completed, thereby preventing the UNG from destroying any "true" amplified products of the test. Test procedures and calculation of the results were performed according to the manufacturer's instructions.

3.4.4 Detection of circulating autoantibodies in chronic hepatitis C patients:

3.4.4.1) Detection of antinuclear antibodies (ANA) in serum:

The antinuclear antibody (ANA) is a term that describes a variety of auto-antibodies against constituents of cell nuclei including DNA, RNA, and various nuclear proteins. The presence of ANA was detected using the indirect immunofluorescence technique, Hep-2 cell ANA kit (The Binding Site Product, UK). Hep-2 cells, an epithelial cell line derived from a human carcinoma of the larynx, are characterized by extremely large nuclei compared to other cell lines enabling easier visualization of cell morphology with a consequent increase in assay sensitivity. Further, their nuclei are actively dividing, exposing antigens not normally expressed in the resting cells. Following incubation of the patient serum with Hep-2 cells, unreacted antibodies were removed by washing before the application of fluorescein isothiocyanate labelled conjugate (FITC conjugated

antihuman IgG). After a further washing step to remove unbound conjugate, the slides were viewed using a fluorescence microscope. Positive samples showed an apple green fluorescence corresponding to areas of the Hep-2 cells where autoantibody has bound. The assay procedures and interpretation of the results are according to the manufacturer's instructions.

3.4.4.2) Detection of antimitochondrial(AMA) and smooth muscle antibodies (ASMA) in serum:

AMA and ASMA were tested by using an indirect immunofluorescence technique employing rat liver, stomach and kidney sections as substrate (Immunofluor, BioScientifica SA, Argentina). Patient and control sera reactive against these tissues were visualized following the addition of FITC conjugated antihuman immunoglobulin. Results were evaluated using a fluorescence microscope as before. The test procedures and interpretation were undertaken in accordance with manufacturer's instructions.

3.4.4.3) Detection of liver-kidney microsomal antibody (LKM_1) in serum:

LKM_1 antibodies were tested in all patients' sera with an ELISA technique using microtiter plates coated with recombinant human cytochrome P450 11 D6 (Varelia LKM_1, ELIAS, Germany). Antibodies specific to cytochrome P450 11D6 present in the patient sample bind to the coated antigen. In a second step, the antigen antibody complex was reacted with an enzyme labelled second antibody (horseradish peroxidase conjugated antihuman IgG) which leads to the formation of an enzyme labelled antigen-antibody sandwich complex. The enzyme label converts the added tetra-methyl benzidine substrate (TMB) to form a coloured solution. The rate of colour formation was proportional to the initial concentration of LKM_1 antibodies in the patient sample. The test procedures and qualitative assay evaluation are according to manufacturer's instructions.

3.4.4.4) Detection of anti-Thyroid Peroxidase (anti-TPO) and anti-Thyroglobulin (anti-TG) in serum:

The Cogent Diagnostics' Autostat II assay (UK) was used for the detection of each of the above-mentioned autoantibodies. Patient serum was added to the Autostat II wells, which were coated with purified antigen. Following washing to remove unbound material, horseradish peroxidase conjugated anti-IgG monoclonal antibody, which binds to the immobilised antibodies, was added to the wells. Following further incubation and washing, tetra-methyl benzidine substrate (TMB) was added to each well. The presence of the antigen-antibody-conjugate complex was indicated by a dark blue colour that became yellow following the addition of the stop solution. The colour intensity was proportional to the amount of autoantibodies present in the original serum sample. The test procedures and calculation of the results were done according to the manufacturer's instructions.

3.4.4.5) Detection of anticardiolipin antibodies (IgG,M,A) in serum:

The Cogent Diagnostics' Autostat II assay (UK) for the detection of anticardiolipin antibodies was used. The test principle is the same as mentioned in anti-TPO. The test procedures and calculation of the results were done according to the manufacturer's instructions.

3.4.4.6) Detection of Anti-neutrophil cytoplasmic antibodies (c,p-ANCA) in serum:

Euroimmun *c* and *p*-ANCA kits (Germany) provide a semiquantitative *in-vitro* assay for human IgG class autoantibodies against proteinase-3 and myeloperoxidase antigens. The test kits contain antigen coated microtiter strips. In the first reaction step, diluted patient and control samples were added to the wells prior to incubation for 30 minutes at room temperature. In the case of positive samples, specific IgG antibodies bound to the antigens. To detect the bound antibodies, a second incubation was carried out using peroxidase-labelled anti-human IgG (enzyme conjugate). Following the addition of an appropriate chromogen / substrate (TMB / hydrogen peroxide), a colour formed which was proportional to the

concentration of the corresponding antibodies. The test procedures and calculation of the results were undertaken in accordance with manufacturer's instructions.

3.4.5 Detection of IgM antibodies to HCV- core (IgM anti HC_c):

HCV-core (IgM) antibodies were detected using the ABBOTT HCV-IgM EIA 2.0, which employs a recombinant HCV structural (core) antigen coating the surface of polystyrene beads. The diluted serum was incubated with the coated beads. HCV specific IgM class antibodies present in the sample bind to the core antigen coated bead during the incubation. After removal of the unbound material by washing the bead, human IgM was detected by incubating the bead-antigen-antibody complex with a solution containing horseradish peroxidase labelled goat antibodies directed against human IgM. After further washing the beads were incubated with the o-Phenylenediamine (OPD) and hydrogen peroxide. A yellow-orange colour developed in proportion to the amount of anti HCV-core IgM bound to the beads. The assay procedures and calculation of the results were performed in accordance with the manufacturer's instructions.

3.4.6 Phenotyping of immunocompetent cells :

Patients with chronic hepatitis C infection exhibit differences in their responses to IFN-α therapy. Six months after starting IFN-α therapy the patients were grouped as responders and non-responders based on the previously mentioned criteria.

Study parameters:

- Phenotypic analysis of the peripheral lymphoid cells including quantitation of $CD4^+$T cells and $CD8^+$T cells using lymphocytes two colour direct immunofluoresence.
- The $CD4^+$ / $CD8^+$ T lymphocytes ratios.
- The percentage and absolute number of (activated) lymphocytes $CD25^+$ (IL-2 receptor expressing cells).

Methodology:

1.5-2 ml of whole blood in EDTA was used for detection of the percentage of mature leukocyte subset ($CD4^+$T cells and $CD8^+$T cells) in the PBMCs of the

patients / controls using the Becton Dickinson Simulset IMK direct immuno-fluoresent lymphocytes two-colour kit. The helper / cytotoxic T lymphocytes ratio ($CD3^+$ $CD4^+$ / $CD3^+CD8^+$) and the percentage and absolute number of activated lymphocytes ($CD25^+$) were determined using FACSort (Becton Dickinson). Samples from patients / controls were used for the enumeration of different subsets of lymphocytes.

Principle of the procedure:

When monoclonal antibody reagents are added to human whole blood, the fluorochrome-labelled antibodies bind specifically to antigens on the surface of leucocytes. The stained samples are treated with lysing solution to remove contaminating erythrocytes. Samples are then washed and fixed prior to flow cytometric analysis. An aliquot of the stained patient sample is introduced into the flow cytometer and passed in a narrow stream through the path of a laser beam. The stained cells fluoresce when excited by the laser beam and the emitted light is collected and processed by the Flow Cytometer.

The use of two fluorochromes permits simultaneous two-colour analysis because each fluorochrome emits light at a different wave length when excited at 488 nm by an argon-ion laser. The fluorescein isothiocyanate (FITC)-stained lymphocytes emit yellow-green light (emission maximum approximately 515 nm) while the phycoerythrin (PE)-stained lymphocytes emit red-orange light (emission maximum approximately 580 nm). The cells also interact with the laser beam by scattering the light. The forward-scattered (FSC) light provides a measure that correlates well with cell size, while the side–scattered (SSC) light is an indicator of cellular granularity. Simultest IMK-lymphocyte software provides a report quantifying immunologically significant lymphocyte subsets as percentage of total circulating human lymphocytes and the helper / cytotoxic T lymphocyte ratio ($CD3^+$ $CD4^+$/ $CD3^+CD8^+$). Specimen preparation and all other procedures were executed in accordance with the manufacturer's instructions.

3.4.7 Cytokine assay from unstimulated and mitogen (Co-A) stimulated PBMCs:

3.4.7.1) Isolation of peripheral blood mononuclear cells (PBMCs):

Patient / control blood samples (15-20 ml) were collected in EDTA blood tubes and a total and differential white cell count performed.

An aliquot of 1.5 ml was separated for immunophenotyping. Plasma was separated from rest of the blood and frozen at -20°C, in 500µl volume aliquots, to be used later for viral load and serological studies.

Following reconstitution with complete medium (RPMI 1640 medium containing 2% L-glutamine, 1% penicillin / streptomycin and 10% heat inactivated foetal calf serum), the blood was double diluted and mono-nuclear cells separated by Ficoll / hypaque density gradient centrifugation. PBMCs from the interface were collected, washed, resuspended in 5ml volume and the viable cells counted using the Trypan Blue dye exclusion technique. These cells were used for the functional assay of immunocompetent cells.

PBMCs were adjusted to $2x10^6$ cells per ml and dispensed into 3 wells of a 24 well plate in 1ml medium volume each. One well was left as control whilst 2.5 µg/ml Con-A was added to each of the two remaining wells. The plate was incubated at 37°C for 72 hrs in a humidified CO_2 incubator (5%). The contents of each well were then transferred to sterile eppendorf tubes and centrifuged at 5000 rpm for 10 minutes. The supernatant from stimulated and non-stimulated cells were transferred to Nunc vials, in 500 ul volume aliquots, and stored at -20°C until used for cytokine assays.

3.4.7.2) IFN- γ assay :-

IFN-γ was measured in supernatant of mitogen stimulated (Con-A, 72hrs) and non-stimulated PBMCs using Inter Test-γ™ Human IFN-γ ELISATest kit (Genzyme Cambridge, MA) according to manufacturer's instructions. In brief, the Inter test-γ Human IFN-γ kit is a solid-phase enzyme immunoassay employing the multiple antibody sandwich principle.

First, a 96-well microtiter plate, pre-coated with monoclonal antibodies specific for human IFN-γ was used to capture IFN-γ present in the sample. Then, a goat polyclonal antibody, which recognizes multiple epitopes on the IFN-γ now bound on the solid phase, was added. Next, a third antibody, biotin-labelled donkey-antigoat Ig, which binds to the goat polyclonal antibody already bound to IFN-γ was then added. Finally, streptavidin-peroxidase complex was added, which binds to the biotin on the third antibody. The peroxidase enzyme reacts with the peroxide substrates hydrogen peroxide and OPD, a chromogen which produces an increased absorbance at 492 nm in the presence of peroxidase activity, indicating the presence of IFN-gamma. Increased absorbance due to bound, immunoreactive IFN-γ was quantitated using an ELISA plate reader. The measured absorbance is proportional to the concentration of IFN-γ present in the original sample. A reference curve was obtained by plotting the IFN-γ concentration of several dilutions of standard versus absorbance. The IFN-γ concentration in experimental samples was then determined by comparison of their absorbances with those obtained from the known amounts of IFN-γ in the standard dilutions. The test procedures were undertaken according to manufacturer's instructions.

3.4.7.3) TGF-β1 assay :

TGF-β1 EASIA (Biosource, Europe S.A) was employed to assay the level of TGF-β1 in the supernatant of Con-A stimulated and non-stimulated PBMCs. The MEDGENIX TGF-β1 EASIA is a solid phase Enzymo-Immuno-assay performed on microtiter plate. A fixed amount of TGF-β1 labelled with horseradish peroxidase (HRP) competes with unlabelled TGF-β1 present in standard or extracted samples for a limited number of binding sites on a specific coated antibody. After 24 hrs incubation at room temperature with continuous shaking, the microtiter plate was washed to stop the competition reaction. The chromogenic solution, TMB, was then added and the plate incubated for a further 60 minutes. The reaction

was stopped with the addition of stop solution and the microtiter plate was read at 450 nm in an ELISA spectrophotometer. The amount of substrate turnover was determined colorimetrically by measuring the absorbance, which is inversely proportional to the TGF-β1 concentration. A standard curve was plotted and TGF-β1 concentration in samples is determined by interpolation from the standard curve. Test procedures were undertaken according to manufacturer's instructions.

3.4.7.4) IL4 assay:

IL4 was measured in the supernatant of unstimulated and Con-A stimulated PBMCs using the Biosource International Cytoscreen ™ Human IL4 ELISA kit according to the manufacturer's instructions. A monoclonal antibody specific for human IL4 (hIL4) had been coated onto the wells of the microtiter strips provided. Samples, including standards of known hIL4 content, control specimens and the tested supernatants of PBMCs are pipetted into these wells, followed by the addition of a biotinylated monoclonal second antibody. During the first incubation, the hIL4 antigen binds simultaneously to the immobilized (capture) antibodies on one site and to the solution phase biotinylated antibody on a second site. After removal of excess second antibody, Streptavidin-Peroxidase (enzyme) was added. This binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove the entire unbound enzyme, a substrate solution (TMB) was added which is acted upon by the bound enzyme to produce colour. The intensity of this coloured product is directly proportional to the concentration of hIL4 present in the original specimen.

3.4.8 Cytotoxicity assay for Natural Killer Cell activity:

Natural Killer activity of PBMCs was tested using MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma) based cytotoxicity assay employing Fen cells as target for NK activity (Hussain 1994). This cell line was originally developed from epithelial carcinoma of bladder and was previously described to be a sensitive target for NK activity.

Briefly, the target cells were maintained as adherent monolayers under standard cell culture conditions at 37°C in 5% CO_2 in air mixture using RPMI 1640 with Glutamex II, containing HEPES (25 mmol/l) (GIBCO-BRL) supplemented with 10% heat inactivated FCS (GIBCO-BRL), 1-glutamine (2 mmol/l), 100 IU/ml Penicillin and 100 µg/ml streptomycin (Sigma). Actively growing target cells were trypsinised using Trypsin /EDTA, and resuspended in complete medium at 10^5 cells/ml. 10^4 target cells (100µl) were dispensed in Nunc 96 well microtiter plates and incubated in a humidified incubator at 37°C for 24± 0.5 hours. The medium from targets cells was replaced with 200µl/well of effector cell (PBMCs) suspension in complete medium achieving the desired Effector: Target (E/T) ratios. All cytotoxicity assays were performed at four different E/T ratios ranging from 5:1 to 50:1, replicating each E/T ratio at least four times and by incubating for 16 ± 0.5 hours. At least 10 replicates of target cells per plate were used for baseline optical density of the target cells alone and were fed with fresh medium (200µl/well) only.

In order to remove both non-adherent effector and dead target cells the microtiter plate was washed five times with 200 µl of wash medium (RPMI-1640 containing 2% FCS, antibiotics and glutamine) per well. The adherent monolayer of remaining target cells was assayed by incubating with freshly prepared and filter sterilised MTT (5mg/ml in PBS) at 10µl/well/100µl of complete medium for 3.5 ± 0.25 hours.

After removing the MTT+ medium the reduced formazan crystals were dissolved in acidified β-isopropanol, (0.04 N HCl) by incubating at 37°C for 30 minutes, and the optical density measured at 570 nm. The optical density of the test wells was compared with optical density of the target cells plated alone and percent cytotoxicity calculated:

$$\text{Percent cytotoxicity} = 100 - \left(\frac{\text{Optical density of test}}{\text{Optical density of target alone}} \right) \text{X } 100.$$

Results are expressed as percent cytotoxicity and LU /10^6 PBMCs. One lytic unit (LU) was defined as true number of effector cells required to result in 50% lysis of the target cells.

3.4.9 Serotyping of HCV:

The Murex HCV serotyping 1-6 Assay was used for the detection of antibodies to serotypes 1,2,3,4,5 and 6 of HCV in human serum or plasma. In brief, diluted samples are incubated, in the presence of heterologous competing peptides, with microwells coated with serotype-specific antigens of HCV. During the course of the first incubation any serotype-specific anti HCV antibodies in the samples will bind to the immobilised antigens. Following washing to remove unbound material, the captured anti- HCV antibodies are incubated with peroxidase-conjugated monoclonal antihuman IgG. During the course of the second incubation an antigen-human antibody-antihuman antibody / enzyme complex will be formed in those microwells which contained samples with antibodies to a specific serotype of HCV. After removal of excess conjugate, bound enzyme was detected by the addition of a solution containing TMB and hydrogen peroxide. A purple colour develops in those wells that contained anti HCV reactive samples. The enzyme reaction was terminated with sulphuric acid (2 mol/l) to give an orange colour that is read photometrically at 450 nm. The amount of colour producing conjugate bound in the wells is directly related to the concentration of specific antibody in the samples.

3.5 Statistical Methods

For bivariate analysis of continuous dependent variable, e.g., platelet count and nominal independent variables, e.g. disease status, the difference between means of groups was assessed using a two-tailed Student's *t* test.

For bivariate analysis of nominal dependent variable, e.g., the prevalence of autoantibodies and nominal independent variables, e.g. disease status, Chi-square test was used to assess the difference between groups (e.g. patients and control; responders and non-responders). Fisher's Exact test (one-Tail) was used when the number of observations was less than 10 in any cell of the 2×2 table.

For multivariate analysis of continuous dependent variable, e.g. auto-antibodies count and nominal independent variables, e.g., liver disease activity, the difference between the means was assessed using one-way ANOVA test.

The null hypothesis was rejected when the p value was equal to or less than 0.05.

RESULTS

RESULTS

CHAPTER 4: RESULTS

4.1 Autoantibodies in hepatitis C patients :-

In this study, sera from 117 patients with chronic hepatitis C have been examined for the presence of a panel of autoantibodies. These patients presented with high levels of transaminases, in particular serum alanine aminotransferase (ALT), and were examined for hepatitis C antibody reactivity by using an ELISA method which was confirmed by line immunoassay method. For comparison, a control group of 20 clinically healthy volunteers were recruited into the study. The age and sex ratios were similar in the control group and the group of patients under investigation.

The autoantibodies tested in the patients and control groups were anticardiolipin, antismooth muscle antibodies (ASMA), antimitochondrial antibodies (AMA), anti-thyroid peroxidase (TPO), anti-thyroglobulin (TG), antinuclear antibodies (ANA), antineutrophil cytoplasmic antibodies (*c* and *p*-ANCA) and liver kidney microsomal antibodies (LKM1). The results are summarized in Table 1.

Anticardiolipin antibodies were positive in 1 of the control group (20) however, the level was relatively low (16-25u/ml). Anticardiolipin antibodies were significantly higher in the patient group compared with control group (p=0.0007). In the patient group, 48 of 114 (42%) were positive for anticardiolipin antibodies. Seven patients (14.3%) had low level (16-25 u/ml), 39 patients (81.3%) had moderate level (26-40u/ml) and 2 patients (4.1%) had high level (>40u/ml) anticardiolipin antibodies. Antismooth muscle antibodies (ASMA) were positive in 4 control subjects with a titer (1:40) and in 65 out of 113 (57.5%) of the patients group (p =0.0018). Of the patients who were positive for ASMA, the majority (61) had a low titer (1:40) while only 4 patients had high titer (1:160) ASMA.

Antimitochondrial antibodies (AMA) were significantly higher in patient group than control group (p =0.0067) (Table 1). AMA were positive in 2 out of control group (20) with titer (1:40), while 40% of patient's sera were positive for AMA with the vast majority having a titer of 1:40; only two patients had a high titer (1:160). Anti-thyroid peroxidase antibodies (TPO) were positive in one subject out of the 20 control subjects with low level (25 IU/ml) (normal value < 20 IU/ml) and in 20 out of 64 (31%) in patients group with a low level of average 27 IU/ml (p=0.013). Anti-thyroglobulin antibodies (TG) were not detected in any of the control sera but were positive in 6 out of 64 (9.4%) of patient group with average level 400 IU/ml in 5 patients and high level in only one patient (1580 IU/ml) (normal value < 275 IU/ml).

Antinuclear antibodies (ANA) were positive in 3 out of the control group (20) with a low titer (1:40) and in 30 out of 103 (29%) of patient group. The prevalence of ANA in patient group is not significantly higher than that seen in the control group. An analysis of the pattern of the immunofluoresence pattern of ANA positive patients and controls is shown in Table 2. None of the subjects of the control group was positive for *c* or *p*-ANCA while 2 out of 56 (3.4%) of patient group were positive for *c*-ANCA ; none of patient group was positive for *p*-ANCA. LKM1 was screened in 95 patients and was not detected in any of the patient or control groups.

The frequency of different autoantibodies in the patients group (n=95) and control groups (n=20) were analysed; these data are shown in Table 3. As expected, only a minority of control subjects exhibited one or two autoantibodies, all of them at a low titer. In contrast, in the patient group less than 17% were free of the presence of autoantibodies, whilst in more than half of the patient groups one or more non-organ specific autoantibodies were found. The difference between the patient and control groups was highly significant (p <0.0001).

Table 1: Autoantibodies in chronic hepatitis C patients

Autoantibodies	Patients +/total	Control +/total	*p value
Anticardiolipin ab.	48/114	1/20	0.0007
ASMA	65/113	4/20	0.0018
AMA	46/115	2/20	0.0067
TPO	20/64	1/20	0.013
TG	6/64	0/20	NS
ANA	30/103	3/20	NS
c- ANCA	2/58	0/20	NS
p-ANCA	0/58	0/20	–
LKM1	0/95	0/20	–

* p value < 0.05 is significant (Fisher's Exact one-Tail)

Table 2: Different ANA patterns in patients and controls

ANA pattern (I.F.)	Patients (30/103)*		Control (3/20)	
	1:40	1:80	1:40	1:80
Homogeneous	16	2	3	0
Nucleolar	10	1	0	0
Speckled	1	0	0	0

* Indicates that homogeneous and nucleolar patterns were most frequently seen in the patients.

Table 3: Non-organ specific autoantibodies* in hepatitis C patients and control group

Total	Autoantibody number				
	**None	1	2	3	4
95 patients	16(16.8%)	25(26.3%)	32(33.7%)	15(15.8%)	7 (7.4%)
20 controls	14(70%)	2(10%)	4(20%)	0	0

* Autoantibodies tested are: anticardiolipin ,ANA ,AMA and ASMA .

** p value is < 0.0001 (Fisher's Exact one-Tail)

4.2 The histopathological status of the liver and autoantibodies in chronic hepatitis C patients: -

Out of 117 patients, 61 patients were initially considered suitable candidates for IFN-α therapy. They were tested for qualitative HCV-PCR prior to the antiviral treatment, 49 patients were positive and 12 were negative (Table 4). Out of those 49 patients with PCR evidence of viraemia, 43 agreed to undergo percutaeneous liver biopsy; in 41, biopsy was successful whilst 2 had insufficient biopsies.

The degree of liver pathology according to Knodell's Score was as follows: 4 patients with normal histology, 13 patients with mild chronic hepatitis, 11 patients with moderate / severe chronic hepatitis (one with severe form and 10 with moderate form of chronic hepatitis) and 13 patients with liver cirrhosis.
The relation between the presence of different autoantibodies and the histopathological status of the liver in the patient group was analysed. The results are summarized in Table 5.

There was no significant difference regarding the presence of different autoantibodies between chronic hepatitis and cirrhotic patients (Table 5). Also, no significant difference was found between mild chronic hepatitis and moderate / severe chronic hepatitis patients for the presence of different autoantibodies (Table 6).

In 29 patients (2 of normal histology, 10 of mild CH, 9 of moderate / severe CH and 8 with liver cirrhosis) semiquantitation of the non-organ specific autoantibodies was performed (Figure 1). The levels of ANA, AMA and ASMA were evaluated by scoring the intensity of fluoresense on a scale of 1 to 4 depending on the end point dilution positivity : 1:40 (+1), 1:80 (+2), 1:160 (+3) and >1:160 (+4).

Anticardiolipin antibodies were evaluated according to their concentration in serum as mentioned in the work protocol.16-25 u/ml (+1), 26-40 u/ml (+2), 40-60 u/ml (+3) and >60 u/ml (+4). The level of autoantibodies increased in moderate / severe CH and liver cirrhosis when compared with the mild CH and healthy carrier groups although these differences did not reach statistical significance (p=0.2 ANOVA between groups) (Figure 1).

We then tested the number of autoantibodies in our patients with different histological status (normal histology, mild CH, moderate / severe CH and liver cirrhosis). Out of the 29 patients, 26 showed presence of one to four types of autoantibodies, while in control group (20) only 6 individuals showed one or two types of autoantibodies (Figure 2). It also appears from Figure 2 that the histological findings directly associated with the presence of autoantibodies (p=0.0001 ANOVA between groups).

Table 4: Qualitative HCV- PCR in patients group

Total Patients	Positive	Negative
61	49	12

Table 5: The histopathological status and autoantibodies in chronic hepatitis C patients

Autoantibody	Chronic Hepatitis (n = 24)	Autoantibody % Positive	Liver Cirrhosis (n = 13)	Autoantibody % Positive	*p value
Anticardiolipin ab.	10/23	43.5	7/13	53.8	NS
ASMA	15/23	65.2	10/13	76.9	NS
AMA	11/23	47.8	6/13	46.2	NS
ANA	7/23	30.4	4/10	40	NS
TPO	5/17	29.4	3/8	37.5	NS
TG	2/17	11.8	1/8	12.5	NS
***c* - ANCA**	1/12	8.3	1/5	20	NS
***p* - ANCA**	0/12	0	0/5	0	–

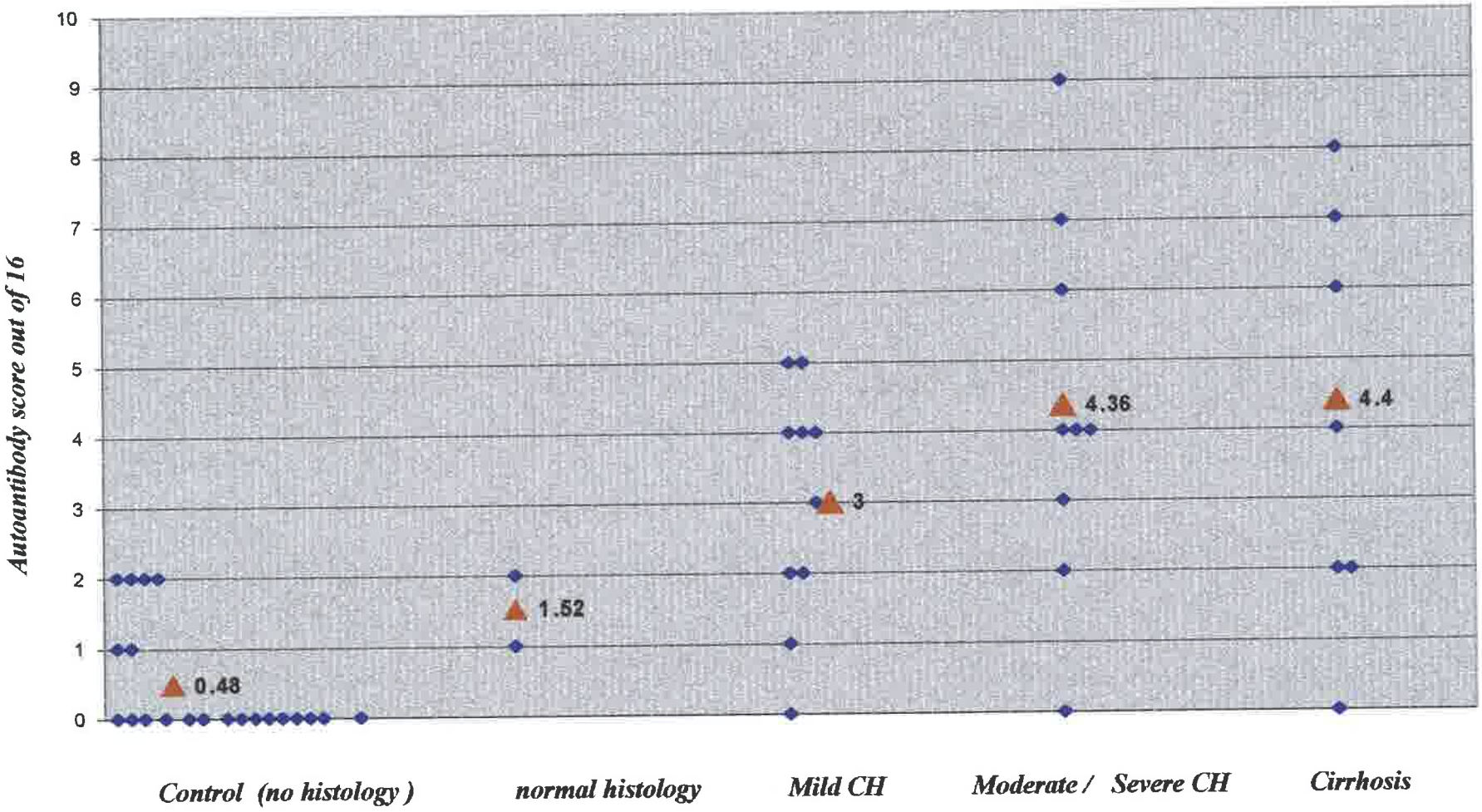
* Fisher's Exact one-Tail test

Table 6: Autoantibody production in mild and moderate chronic hepatitis C patients

Autoantibody	Mild CH (n = 13)	Autoantibody % Positive	Moderate CH (n = 11)	Autoantibody % Positive	*p value
Anticardiolipin ab.	5/13	38.5	5/10	50	NS
ASMA	9/13	69.2	6/10	60	NS
AMA	4/13	30.8	7/10	70	NS
ANA	4/12	33.3	3/11	27.3	NS
TPO	3/10	30	2/7	28.6	NS
TG	1/10	10	1/7	14.3	NS
***c* - ANCA**	1/6	16.7	0/6	0	NS
***p* - ANCA**	0/6	0	0/6	0	–

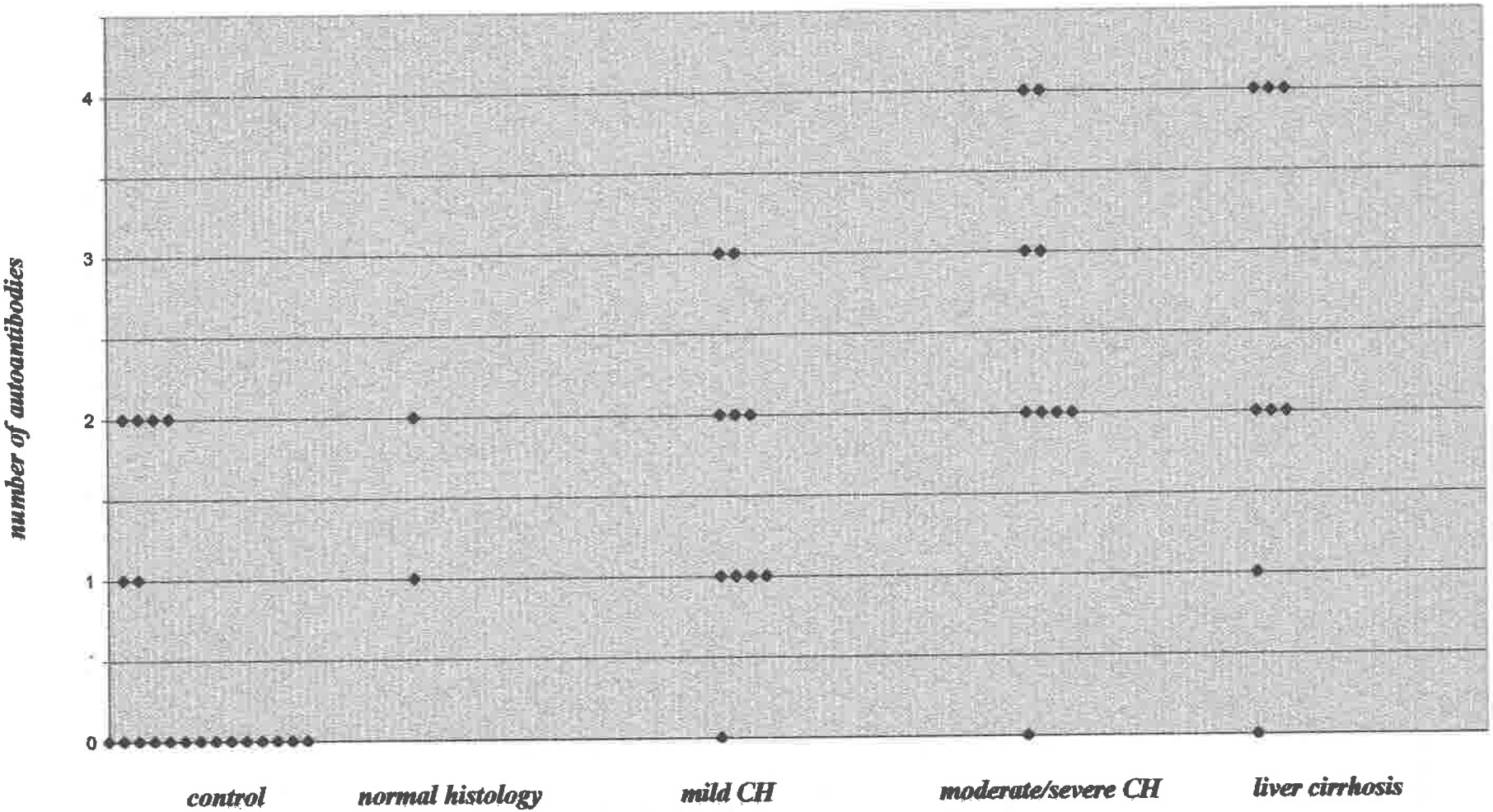
*Fisher's Exact one-Tail test

Figure 1: Relation between liver disease activity and autoantibody production (29 patients)



p = 0.2 (ANOVA between groups)
p < 0.05 is significant

Figure 2: Relation between number of autoantibodies and histological changes in hepatitis C patients



p = 0.0001 (ANOVA between groups)
p < 0.05 is significant

4.3 Virus specific IgM response and autoantibodies in hepatitis C patients: -

Sera from 115 patients were tested for the presence of HCV specific IgM and the frequency of autoantibodies in both IgM positive and negative groups of patients was analysed. Seventy-eight of the 115 patient were positive for anti- HCV-core IgM.

The frequency of autoantibodies in HCV-core IgM positive and negative patients are summarized in Table 7. All autoantibodies were more frequently detected in HCV-core IgM positive patients except for ANA and antithyroglobulin antibodies. Anticardiolipin, AMA, ASMA and anti-thyroid peroxidase antibodies were more frequently found in patients where positive HCV-core IgM was detected. However, statistical significance was only achieved with AMA ($p < 0.005$).

The frequency of autoantibodies in the 61 patients of HCV-core IgM positive group and in 34 patients of HCV-core IgM negative group were compared; the results are summarized in Table 8. When the number of autoantibodies was compared with the presence of anti HCV-core IgM, the result was non-significant. However, when the patients with auto-antibodies were divided into two subgroups (less than 2 and 2-4 autoantibodies), the result was significant ($p=0.018$). Sixty six percent of anti HCV-core IgM positive patients showed the presence of 2-4 autoantibodies whilst 59% of anti HCV-core IgM negative patients had only one or no autoantibodies (Figure 3).

Further we have checked the oocurance of most frequently detected autoantibodies in our patient group shown in Table (1). Hundred and nine patients were tested both for presence of anticardiolipin and antismooth muscle autoantibodies. It was found that ASMA was positive in 34 out of 44 (77%) anticardiolipin positive patients but only in 30 out of 65 (46%) anticardiolipin negative patients. This difference is statistically significant

($p< 0.001$). Platelet count was checked in 31 anticardiolipin antibody positive patients and 34 anticardiolipin antibody negative patients. The mean platelet count was 126.9±51.19 cells/μL in the first group and 188.4±69.75 cells/μL in the second group. The difference between the platelet counts in the two groups was not significant (Table 9).

The relation between anticardiolipin antibody positivity and the degree of liver impairment has also been studied. Out of 26 patients showing different degrees of chronic hepatitis, 13 patients were positive for anticardiolipin antibodies (50%) and 13 patients were negative (50%). In the group of patients showing evidence of liver cirrhosis (13 patients), 7 were anticardiolipin antibody positive and 6 patients were negative. The difference between both groups was non-significant (Table 10).

The relation between the presence of thyroid autoantibodies and the sex of patients was examined in the study group. Sixty four patients were studied for the presence of anti-thyroid peroxidase antibodies and anti-thyroglobulin antibodies (20 females and 44 males). Twenty patients were positive for the presence of TPO: 6 of 20 females (30%) and 14 of 44 male patients (31.8%). The difference between the male and female positivity for anti TPO was not statistically significant. Anti-thyroglobulin antibody was positive in 6 out of 64 patients : 3 out of 20 female patients (15%) and 3 out of 44 male patients (6.8%). The difference between male and female regarding anti-thyroglobulin antibody positivity was also non-significant (Table 11).

Table 7: Relation between the presence of HCV-IgM and different autoantibodies.

Autoantibody	Anti-HCV core IgM +ve			Anti-HCV core IgM -ve			*p value
	Total	+ve	% +ve	Total	+ve	% +ve	
Anticardiolipin ab.	78	36	46.2	36	12	33.3	NS
ASMA	76	44	57.1	37	21	56.8	NS
AMA	78	39	50	37	7	18.9	0.003
ANA	68	19	27.9	35	11	31.4	NS
TPO	43	16	37.2	21	4	19	NS
TG	43	3	7	21	3	14.2	NS
c-ANCA	38	1	2.6	20	1	5	NS
p-ANCA	38	0	0	20	0	0	-

* $p < 0.05$ is significant (Fisher's Exact one-Tail)

Table 8: Non-organ specific autoantibodies in hepatitis C patients

	Total	Autoantibodies (% positive) *				
		0	1	2	3	4
HCV coreIgM + ve	61	9(14.8%)	12(19.7%)	22(36.1%)	14(23%)	4(6.6)
HCV coreIgM -ve	34	7(20.6%)	13(38.2%)	10(29.4)	1(2.9%)	3(11.3)

*Antibodies studied were ANA, AMA, ASMA and anticardiolipin antibodies.

p value is > 0.05 (Chi-square test)

Table 9: ASMA and platelet count in anticardiolipin positive and anticardiolipin negative patients with chronic hepatitis C.

Laboratory parameters	Anticardiolipin +ve	Anticardiolipin -ve	p value
ASMA (n =109)	34/44	30/65	<0.001*
Platelet count (cells/µL) (n = 65)	126.9±51.19 **(n =31)**	188.4±69.75 **(n =34)**	NS**

*Chi-square test $p < 0.05$ is significant

**Student's *t* test

Table 10: Anticardiolipin antibodies and degree of liver impairment

Liver biopsy	No.	Anticardiolipin +ve	Anticardiolipin -ve	*p value
**Chronic hepatitis	26	13	13	NS
cirrhosis	13	7	6	NS

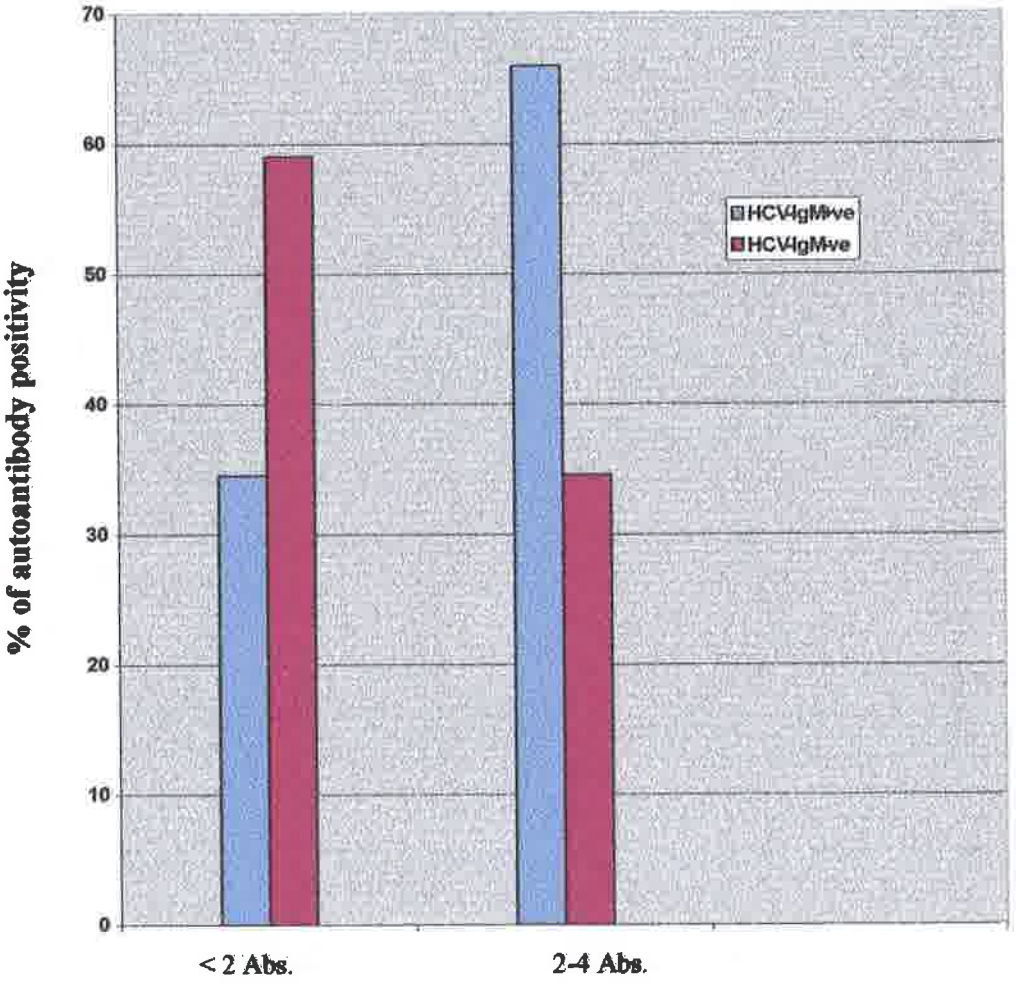
* Fisher's Exact one-Tail ** mild, moderate and severe hepatitis

Table 11: Relation between sex of patients and thyroid antibodies

Thyroid Antibody	Female (n =20)	Male (n =44)	*p value
TPO(+) (%)	6 (30%)	14 (31.8%)	NS
TG(+) (%)	3 (15%)	3 (6.8%)	NS

*Fisher's Exact one-Tail

Figure 3 : Organ non-specific autoantibodies in hepatitis C patients



p = 0.018 (Fisher's Exact one-Tail)

4.4 The relation between qualitative HCV-RNA hepatitis C virus specific core IgM and autoantibodies:

The relation between the presence of virus specific IgM antibodies as a marker of active infection and the HCV-RNA positivity had been studied in 61 patients (12 HCV-RNA negative and 49 HCV-RNA positive). Within the HCV-RNA negative patients, 8 patients had anti HCV-core IgM negative and 4 patients were positive for the core-IgM antibodies while in the HCV-RNA positive group (49 patients), 39 patients were positive for HCV-core IgM and 10 patients were negative. The relation between the presence of HCV-core IgM and the positivity for HCV-RNA was highly significant (Table 12).

Of the patients tested for HCV-RNA (61 patients), 47 patients were tested for the presence of 4 non-organ specific autoantibodies (ANA, AMA, ASMA and anticardiolipin antibodies). The relation between number of autoantibodies and HCV-RNA positivity was studied. Within the HCV-RNA positive group (36 patients), 7 patients (19.4%) had no antibodies, 10 patients (27.8%) had one autoantibody, 13 patients (36.1%) had two autoantibodies, 4 patients (11.1%) had three autoantibodies and 2 patients (5.6%) had four autoantibodies. On the other hand, within HCV-RNA negative group (11 patients), one patient (9.1%) had no autoantibodies, five patients (45.5%) had one autoantibody, three patients (27.3%) had two autoantibodies, one patient (9.1%) had three autoantibodies and one patient (9.1%) had four autoantibodies. The difference between HCV-RNA positive and negative groups in the total number of autoantibodies was not significant (Table 13).

The presence of autoantibodies of different specificities in both HCV-RNA positive and negative patients had been studied. Results are summarized in Table 14. No significant difference between the HCV-RNA positive and negative groups for the presence of these autoantibodies had been found in our patients.

Table 12: Correlation of qualitative HCV- RNA and anti HCV-core IgM antibodies

	Anti HCV-core IgM		Total
	-ve	+ve	
HCV-RNA (- ve)	8	4	12
HCV-RNA (+ ve)	10	39	49
Total	18	43	61

p =0.004 (Fisher's Exact Test) $p < 0.05$ is significant

Table 13: HCV-RNA and autoantibodies in chronic hepatitis C patients

HCV-RNA (n=47)	*Autoantibodies (% positive)				
	0	1	2	3	4
HCV-RNA (+) (n=36)	7 (19.4%)	10 (27.8%)	13 (36.1%)	4 (11.1%)	2 (5.6%)
HCV-RNA (-) (n=11)	1 (9.1%)	5 (45.5%)	3 (27.3%)	1 (9.1%)	1 (9.1%)

$p > 0.05$ (Chi-square test)

*ANA,AMA,ASMA and anticardiolipin antibodies.

Table 14: Relation between qualitative HCV-RNA and autoantibodies production in chronic hepatitis C patients

Autoantibody	HCV - RNA (+ve)		HCV - RNA (-ve)		*p value
	+/total	+ve %	+/total	+ve %	
Anticardiolipin ab.	17/47	36.2	4/12	33.3	NS
ASMA	25/45	55.6	8/12	66.7	NS
AMA	20/47	42.6	4/12	33.3	NS
ANA	11/41	26.8	5/11	45.5	NS
TPO	13/36	36.1	3/6	50	NS
TG	4/36	11.1	1/6	16.7	NS
***c* - ANCA**	2/28	7.1	0/4	0	NS
***p* - ANCA**	0/28	0%	0/4	0	_

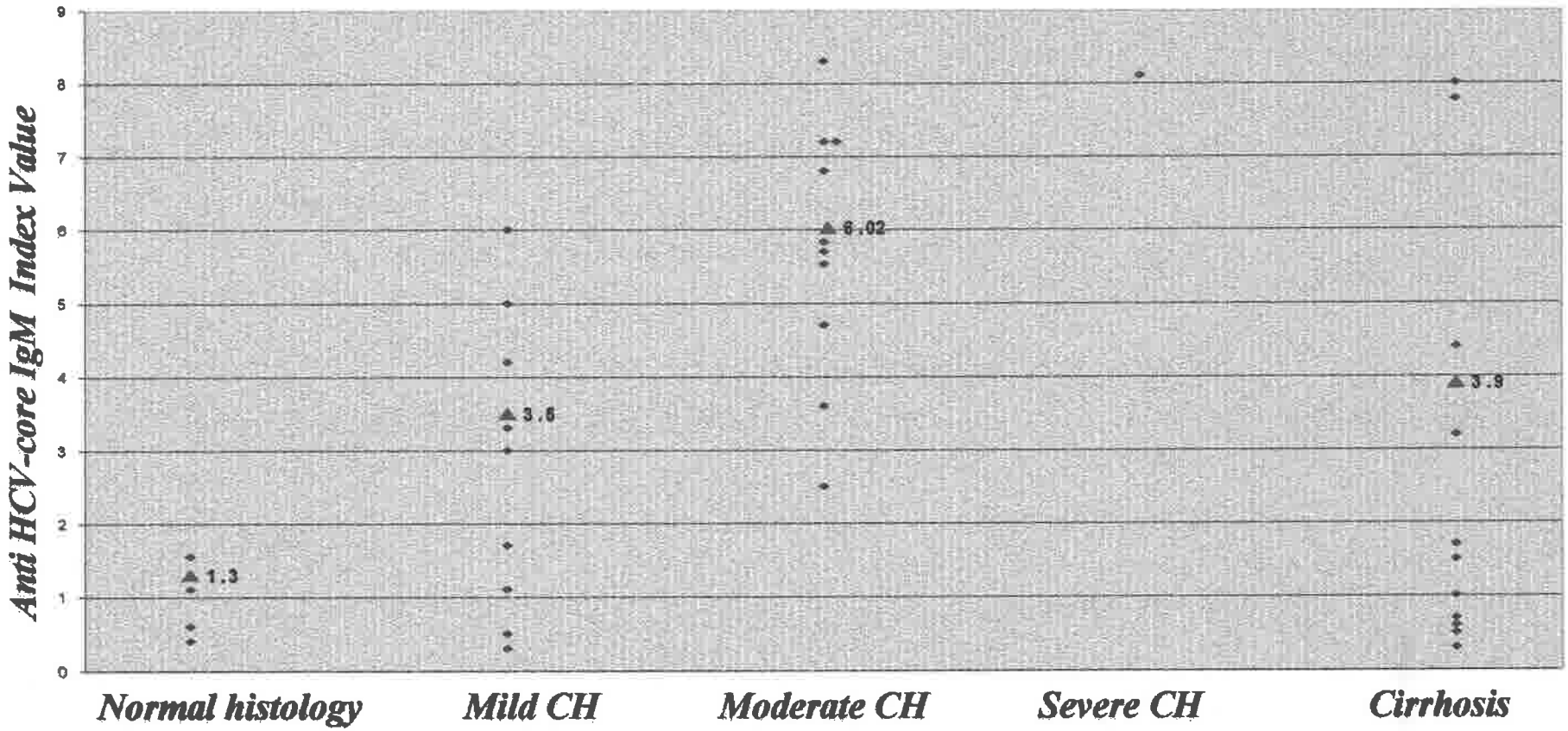
*Fisher's Exact one-Tail

4.5 The relation between HCV-core IgM antibodies and the histological manifestations of the disease:

As mentioned in section 4.2, 41 patients underwent successful liver biopsies. Of these 41 patients, 27 patients were positive for anti HCV-core IgM: 2 patients with normal histology, 7 patients showed mild CH, all moderate/ severe CH patients (n=11) and 7 showed liver cirrhosis.

Anti HCV-core IgM Index Values were calculated for these patients (IgM Index Value = sample OD / mean of cut off value). The correlation between HCV-IgM Index Value and the histopathological damage of the liver was then analysed (Figure 4). Of the 4 patients with no histological findings, 2 did not have HCV-core IgM in their sera while the other 2 exhibited low titer of HCV-core IgM. Most of patients with mild hepatitis (7 out of 9) showed variable levels of HCV-core IgM in their sera, while all patients with moderate / severe hepatitis produced significant amount of HCV-core IgM. In patients with liver cirrhosis, almost half of the patients tested did not have HCV-core IgM in their sera. Statistical analysis of these results revealed that the presence of HCV-core IgM correlated with the severity of hepatitis. The level of HCV-core IgM was significantly higher ($p < 0.005$) in patients with moderate / severe hepatitis when compared with mild form of HCV induced hepatitis. Interestingly, patients with cirrhosis had either very high titers of HCV-core IgM while the remainder showed none at all (Figure 4).

Figure 4: Relation between liver disease activity and anti HCV-core IgM Index value (IgM/cut off value)



Normal histology *versus* mild CH	p= 0.0081
Mild *versus* moderate/severe CH	p= 0.005
Moderate/Severe *versus* Cirrhosis	p= 0.09

$p < 0.05$ is significant (Student's *t* test)

4.6 IFN-α therapy, parameters of disease progression and immunodisregulation in chronic hepatitis C patients :-

We studied the relation between some pretreatment parameters and response to IFN-α therapy including pretreatment serum transaminases, the disease activity index on liver biopsy, level of serum autoantibodies, viral genotype and presence or absence of HCV-core specific IgM .

4.6.1 Serum transaminases and liver biopsy score in responders and non-responders to IFN-α therapy: -

Liver transaminases in particular serum glutamic oxaloacetic transaminase (AST) and serum glutamic pyruvate transaminase (ALT) and the histopathological status of HCV patients, represented as biopsy Knodell's Score in the responder and non-responder groups of patients before starting IFN-α treatment are shown in Table 15. The mean of liver biopsy score in the non-responders was significantly higher than that in the responder group of patients (Table 15).

On the other hand, there was no significant difference in the mean level of serum ALT and AST between the responders (20 patients) and non-responders (21 patients) before starting IFN-α treatment (Table 15). After the end of IFN-α treatment, there was a significant drop in the level of liver enzymes (ALT, AST) in both groups but the level of liver transaminases had reached the normal level only in the responder group of patients (Table 17). By comparing the mean level of serum transaminases in the two groups after the end of IFN-α therapy, the non-responders showed significantly higher levels than the responders as shown in Table 16.

Table 15: The biochemical and histopathological status of HCV patients before starting IFN-α therapy

Parameter	Responders (n =20)	Non-responders (n =21)	**p value
ALT	129.6 ± 64.6	113.6 ± 46.5	NS
AST	97.3 ± 47.3	65.7 ± 18.3	NS
Biopsy Score*	4.38 ±1.8	7.77 ± 3.2	0.007

*Biopsy Score was evaluated in 13 non-responders and 8 responders

**$p < 0.05$ is significant (Student's *t* test)

Table 16: The biochemical status in responders and non-responders after the end of IFN-α therapy

Serum transaminases	Responders (n=20)	Non-responders (n=21)	*p value
ALT	43.4 ± 11.5	83.9 ± 35.2	0.026
AST	17.5 ± 4.7	41.7 ± 4	0.0076

*Student's *t* test

Table 17: The biochemical status in responders and non-responders before starting and after the end of IFN-α therapy

Patients		Before treatment	After treatment	*p value
Responders	ALT	129.6 ± 64.6	43.4 ± 11.5	<0.0001
	AST	97.3 ± 47.3	17.5 ± 4.7	<0.001
Non-responders	ALT	113.6 ± 46.5	83.9 ± 35.2	<0.001
	AST	65.7 ± 18.3	41.7 ± 4	0.006

* Student's *t* test

4.6.2 Presence of autoantibodies and responsiveness to IFN-α therapy:-

The incidence of the various autoantibodies in responder and non-responder groups of patients is detailed in Table 18. A strong correlation between non-responsiveness and the presence of AMA antibodies (p=0.008), anticardiolipin antibodies (p=0.008) and TPO antibodies (p=0.023) was noted.

4.6.3 Presence of anti HCV core IgM and response to IFN-α therapy: -

In these patients, the relation between HCV-core IgM antibody and the response to IFN-α therapy had been studied (Table 19). Fourteen of 18 (78%) non-responders developed HCV-core IgM antibody compared to 9 out of 17 (53%) individuals of the responder group of patients. This difference between responder and non-responder groups was statistically significant (p=0.05).

4.6.4 Total autoantibody production in the responders and Non-responders to IFN-α therapy: -

The number of non-organ specific autoantibodies in our patients was checked before starting IFN-α therapy (Table 20). When the relation between the number of autoantibodies and patients'responsiveness to IFN-α therapy was studied, the result was not significant. However, when the subjects were divided into two sub-groups based on the number of autoantibodies seen (less than 2 and 2-4 autoantibodies), the result showed strong trend towards higher number of autoantibodies in the non-responder patients (p =0.06).

As shown in Figure 5, 11 of 19 (58%) of the responders have less than 2 autoantibodies while only 5 out of 18 (28%) of the non-responders have less than 2 autoantibodies and 72% have 2-4 autoantibodies.

4.6.5 Autoantibody production before starting and after the end of IFN-α therapy in the responders and non-responders:

The presence of organ specific and non-specific autoantibodies in the sera of a limited number of our treated patients (8 out of 20 responders and 12 out of 21 non-responders) was studied after stopping IFN-α therapy. The

prevalence of these autoantibodies after stopping the IFN-α therapy was compared with that before starting the treatment. The results are summarized in Table 21. The difference between the prevalence of these autoantibodies before starting and after stopping the IFN-α therapy was not significant in either group.

Table 18: Autoantibody Profile in responders and non-responders to IFN-alpha therapy

Autoantibodies	Responders (n= 20)	% +ve	Non-responders (n= 21)	% +ve	*p value
AMA	5	25	14	66.7	0.008
ANA	6	30	5	23.8	NS
Anti-TPO **	1	5.9	8	38.1	0.023
Anti-TG**	1	5.9	3	14.3	NS
Anticardiolipin	5	25	14	66.7	0.008
ASMA	7	35	11	52.4	NS

* p value < 0.05 is significant (Fisher's Exact-one Tail)

**only 17 responders were tested for anti-thyroid peroxidase and thyroglobulin antibodies.

Table 19: Relation between anti HCV-core IgM and non-responsiveness to IFN-α treatment.

	Responders (n=17)	Non-responders (n=18)
HCV-core IgM (+ve)	9	14
HCV-core IgM (-ve)	8	4

p = 0.05 (Fisher's Exact one-Tail)

Table 20: Total autoantibody production in responders and non-responders HCV patients to IFN-α therapy.

Patients	No. Tested	Number of Autoantibodies + (%)				
		0	1	2	3	4
Non-responders	18	2 (11%)	3 (16.7 %)	7 (38.9 %)	3 (16.7 %)	3 (16.7 %)
Responders	19	5 (26.3 %)	6 (31.6%)	6 (31.6 %)	2 (10.5 %)	0

P > 0.05 (Chi–square test)

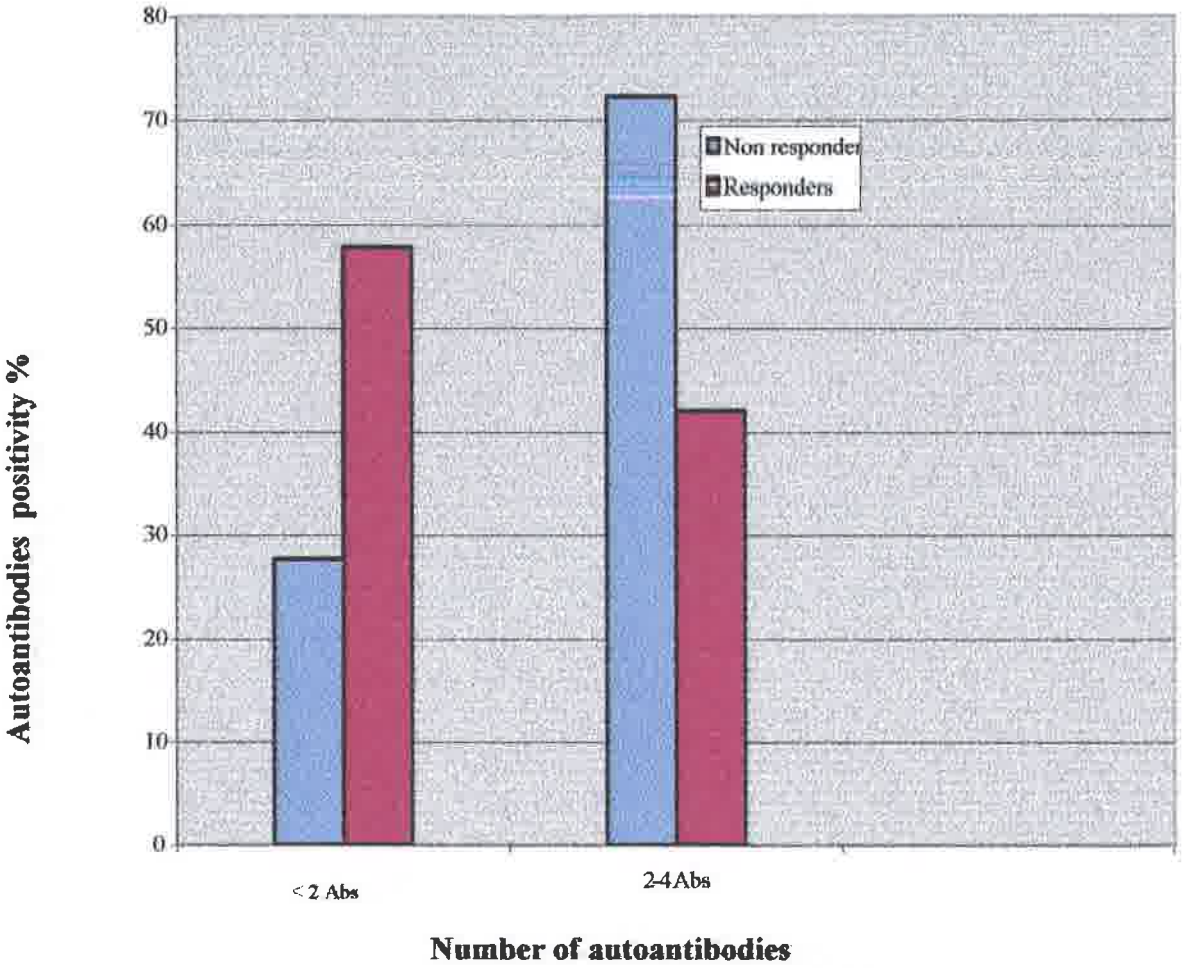
- Only the results of patients in which all autoantibodies are tested are presented.
- Autoantibodies tested are ANA ,AMA ,ASMA and anticardiolipin abs.

Table 21: Autoantibody production in the responders and non-responders before starting and after stopping IFN-α therapy.

Autoantibodies	Responders (n=8)			Non-responders (n=12)		
	Before treatment	After treatment	p* value	Before treatment	After treatment	p* value
AMA	3/6	2/6	NS	2/10	3/10	NS
ANA	2/7	2/7	NS	3/9	2/9	NS
Anti-TPO	1/7	0/7	NS	3/9	4/9	NS
Anti-TG	1/7	0/7	NS	1/9	3/9	NS
Anticardiolipin	1/8	1/8	NS	5/10	4/10	NS
ASMA	3/7	3/7	NS	6/12	4/12	NS

* $p > 0.05$ (Fisher's Exact one-Test)

Figure 5: Total autoantibody production in responders and non-responders HCV patients to IFN-α therapy



p =0.06 (Fisher's Exact one-Tail)

4.6.6 Evaluation of HCV-core IgM as a parameter of responsiveness to IFN-α therapy: -

Patients undergoing IFN-α therapy were classified into two groups, 20 responders and 21 non-responders. HCV-core IgM Index Value in 10 patients under therapy (5 responders and 5 non-responders) are shown in Figures 6,7. The IgM-Index Value showed significant decrease in the responder group at the end of IFN-α treatment ($p = 0.05$) (Figure 6), whilst the change in the IgM Index Value after stopping IFN-α treatment in the non-responders was non-significant ($p > 0.05$) (Figure 7).

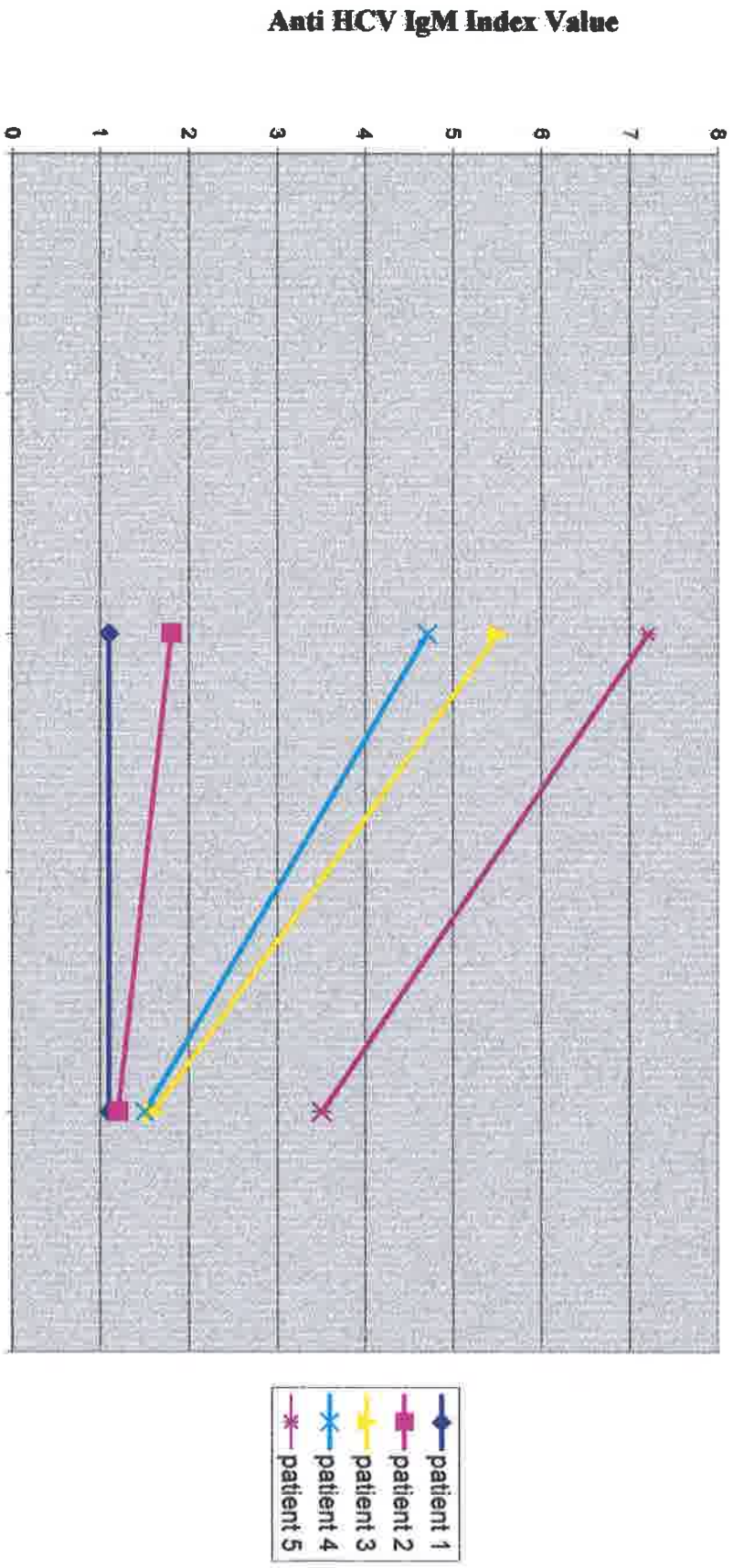
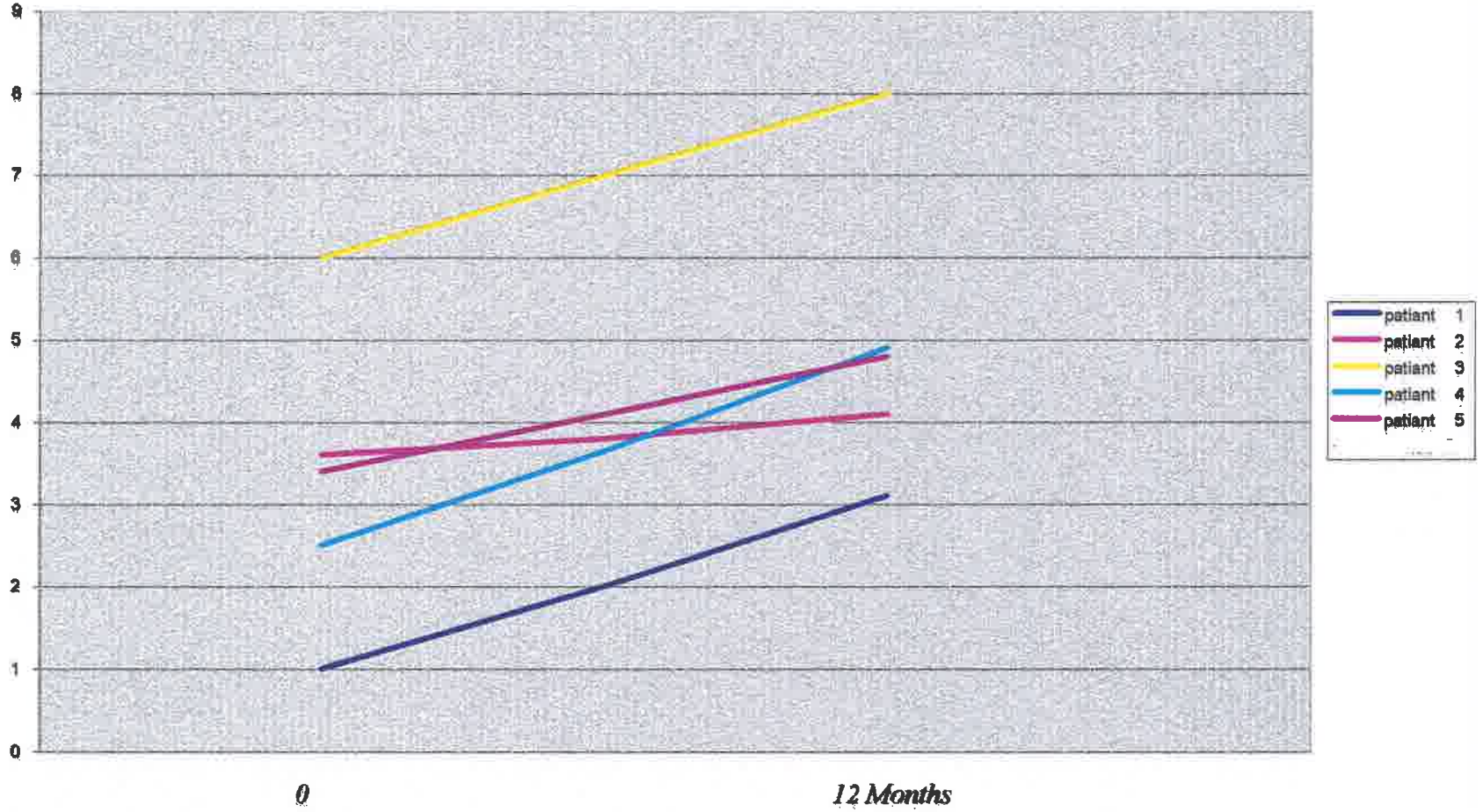


Figure 6: Relation between response to treatment and change in IgM Index Value in the responders

IgM Index value before treatment (n=5)	IgM Index value after treatment (n=5)	*p
4.06 ± 2.6	1.78 ± 0.98	0.05

*p ≤0.05 is significant (Student's *t* test)

Figure 7: Relation between response to treatment and change in IgM Index Value in non-responders



IgM Index value before treatment (n=5)	IgM Index value after treatment (n=5)	*p
3.3 ± 1.8	4.98 ± 1.83	>0.05

*p ≤ 0.05 is significant (Student's *t* test)

4.7 Viral genotype, response to IFN-α therapy and parameters of immunodisregulation in chronic hepatitis C patients: -

4.7.1 HCV genotype distribution: -

The presence of antibodies to variable antigenic regions from non-structural (NS4) protein of HCV types 1-6 had been tested in a subgroup of patients (35 patients) who received IFN-α therapy. The prevalence of different serotypes is reported in Table 22. In this study, HCV serotype 4 was identified as the dominant serotype (60%), 1 in 20%, 3 in 14% and each of serotype 5 and 2 in 3% of patients.

4.7.2 Viral genotype, production of anti HCV-core IgM and response to IFN-alpha therapy: -

Table 23 shows the prevalence of HCV specific IgM in the different genotypes . The distribution of viral genotypes were similar in patients with sera tested positive for HCV-core IgM and those that were HCV-core IgM negative. The success of IFN-α treatment in HCV-core IgM antibody positive and IgM core free patients infected with HCV virus of different genotypes is shown in Table 24.

In patients with serotype 4, a strong association was detected between the presence of HCV specific IgM antibodies and non-responsiveness to IFN-α therapy. Conversly, there was a strong association between serotype 1 and non-responsiveness to IFN-α therapy (6 out of 7 patients i.e. 86% having serotype 1 are non-responders) (Table 24).

4.7.3 Autoantibodies production in different genotypes :-

The prevalence of different autoantibodies in serotype 4, the dominant serotype in this patient group, was compared with the antibody response in the other serotypes (non-4 serotypes). There was no statistical difference between the two groups for the presence of different autoantibodies (Table 25).

Table 22: HCV serotypes in a subgroup of 35 patients

Serotype (n =35 pts.)	Positive (%)
4	21 (60%)
1	7 (20%)
3	5 (14%)
5	1 (3%)
2	1 (3%)

Table 23: The presence of HCV- core IgM in different serotypes in a subgroup of 35 patients

Serotypes (35 patients)	Number	HCV-core IgM (+ ve)	HCV-core IgM (- ve)
Serotype 4	21	15	6
Serotype 1	7	4	3
Serotype 3	5	2	3
Serotype 5	1	1	-
Serotype 2	1	1	-

Table 24: The relation between HCV-core IgM and response to IFN-α therapy in different HCV serotypes.

Serotype	No.=35	Responders		Non-responders**	
		IgM +ve	IgM -ve	IgM +ve	IgM -ve
Serotype 4	21	5	5	10*	1
1	7	1	–	3	3
3	5	2	3	–	–
5	1	1	–	–	–
2	1	–	–	1	–

* P = 0.05 (Fisher's Exact -one Tail)

** P =0.04

Table 25: Comparison between serotype 4 and non-4 serotype for the presence of different autoantibodies.

Autoantibodies	Serotype 4		Non-4 serotype		p value*
	+	-	+	-	
Anticardiolipin (n=35)	10	11	6	8	NS
ASMA (n = 34)	7	13	8	6	NS
AMA (n = 35)	7	14	4	10	NS
ANA (n = 32)	9	9	5	9	NS
TPO (n = 32)	8	13	1	10	NS
TG (n = 32)	2	19	0	11	NS
***c*-ANCA (n = 12)**	1	8	1	2	NS
***p*-ANCA (n = 12)**	0	9	0	3	-

* Fisher's Exact one-Tail

4.8 Phenotyping of immunocompetent cells in HCV patients on IFN-α therapy:

Six months after starting IFN-α therapy, the patients were grouped as responders and non-responders based on the previously mentioned criteria. The relationship between response and variations in their general immune status, as reflected in ratio of T cell subpopulations and expression of activation markers, was examined in the patient groups. Phenotypic analysis of the peripheral lymphoid cells was undertaken for the group of patients eligible for IFN-α therapy including the quantitation of $CD4^+$, $CD8^+$ T cells. The $CD4^+$/ $CD8^+$ T lymphocyte ratio and the percentage and absolute number of activated lymphocytes $CD25^+$ (IL2 receptor expression cells) were also analysed.

$CD4^+/CD8^+$ ratios in both responders (no.=18) and non-responders (no.=9) was compared with that of control individuals (no.=20). The results are shown in Table 26. No significant difference in $CD4^+/CD8^+$ ratio was found between responders and non-responders. However, both responders and non-responders showed significantly lower ratios compared with the control group. The absolute number as well as the percentage of cells expressing IL2 receptors ($CD25^+$) was significantly higher ($p<0.05$) in non-responders than in responders group of patients. The results are shown in Table 27.

Table 26: T cell subsets in HCV Hepatitis ($CD4^+$: $CD8^+$ ratios)

Studied group	$CD4^+ : CD8^+$
Controls (n=20)	1.906 ± 0.79
Responders (n=18)*	1.516 ± 0.53
Non-responders (n=9)**	1.206 ± 0.3

*p <0.05 *versus* controls (Student's *t* test)

**p <0.05 *versus* controls

Table 27: CD 25^+ cells in hepatitis C patients

Patients	CD 25^+ (%)*	CD 25^+/µl**
Responders	17.43 ± 3 **(n=16)**	413.69 ± 152.7 **(n=13)**
Non-responders	27.22 ± 9.5 **(n=9)**	683.85 ±313.4 **(n=7)**

*p < 0.05 **p < 0.02 (Student's *t* test)

4.9 NK cell activity of peripheral lymphocytes in HCV patients on IFN-α therapy:-

A significant increase in the NK cell activity was seen; represented as an increased percentage of cytotoxicity in the responders group (12 patients) compared with the control group (20) at effector:target ratios of 20:1, 10:1 and 5:1 ($p<0.001$, <0.001 and <0.005 respectively), in the absence of any significant difference between the non-responders group (7 patients) and the same control group. The results are shown in Figure 8 and Table 28.

The relation between NK activity in the treated group of patients was analysed in relation to the viral serotype. NK cell activity, represented as percentage of cytotoxicity, in the patients having serotype 4 (10 patients) and serotype 1 (7 patients), the most dominant serotypes in our patient group, were compared. There was a significant increase in the NK cell activity in patients having serotype 4 compared with serotype 1 ($p<0.01$) at the E/T ratio 10:1 (Table 29).

The relation between NK cell activity, represented as the number of lytic units, and HCV serotypes was also studied. The data show a positive correlation between serotype 4 and NK cell activity and a negative correlation between serotype 1 and NK cell activity compared with the whole group of treated patients. The results are shown in Table 30.

Table 28: NK cell activity in patients on IFN-α therapy [Controls (C) vs. Responders (R) and Non-responders (NR)]

	50:1	20:1	10:1	5:1
C vs (R + NR)	N.S.	<0.05	<0.05	NS
C vs R	N.S.	<0.001	<0.001	<0.005
C vs NR	N.S.	N.S.	N.S.	N.S.

P< 0.05 is significant (Student's *t* test)

Table 29: NK Cytotoxicity in HCV serotype 1 and 4

Serotype	*NK Activity (10:1)
Serotype1 (n=7)	26.614±10.33
Serotype 4 (n=10)	55.559±11.72

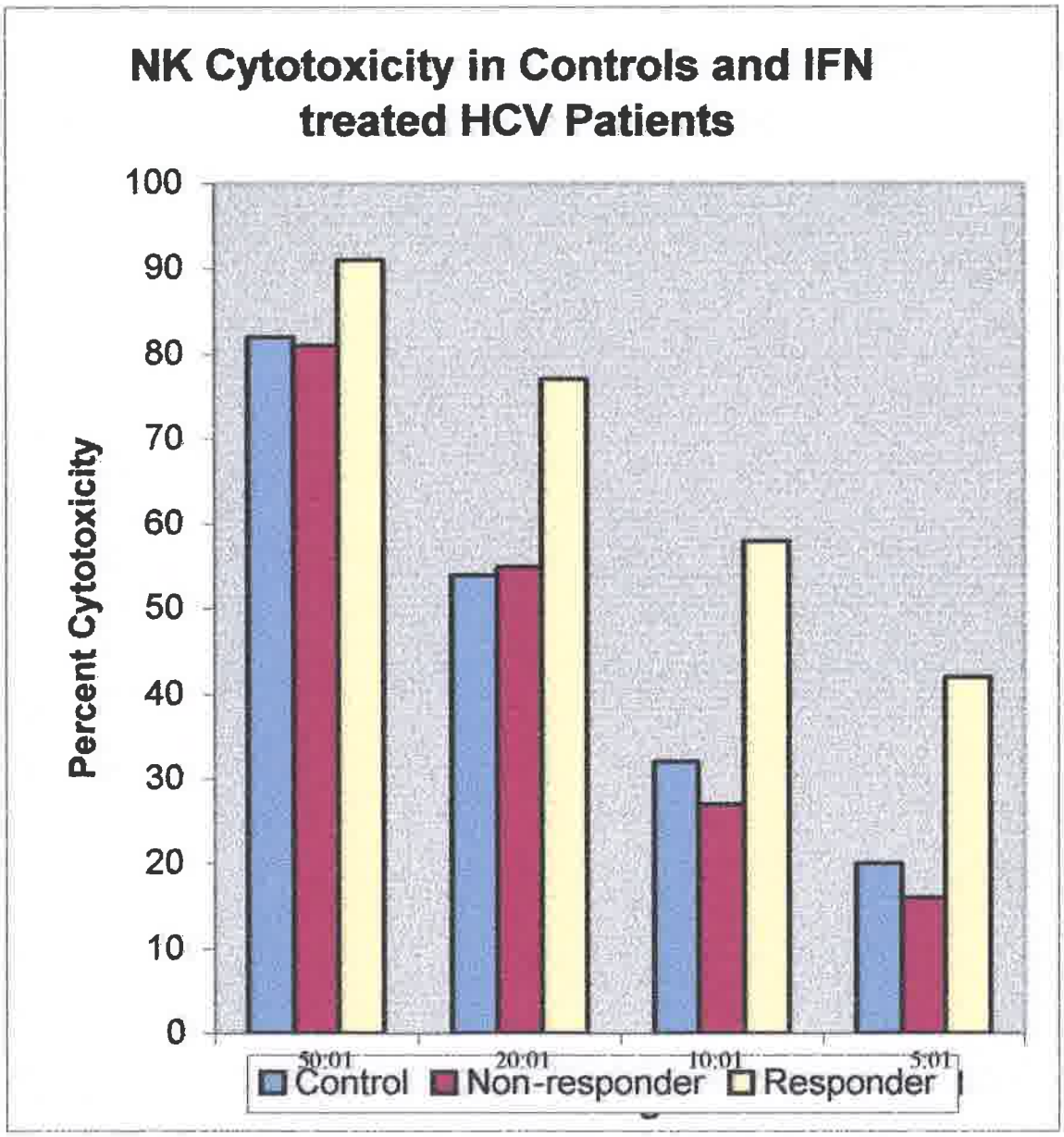
*p < 0.01 (Student's *t* test)

Table 30: HCV Serotypes and NK Activity

HCV serotype	Lytic Units	*p value
Serotype 1 (n=7)	6.45 ± 2.1	0.02
All others (n=12)	15.98 ± 3.8	
Serotype 4 (n=10)	19.47± 5.5	0.038
All others (n=9)	8.11± 1.6	

*Student's *t* test

Figure 8



Effector:Target Ratio	Control	Non-responders	Responders
50:01	82±15	81±31	91±22
20:01	54±10	55±28	77±12
10:01	32±9	27±11	58±14
5:01	20±7	16±9	42±11

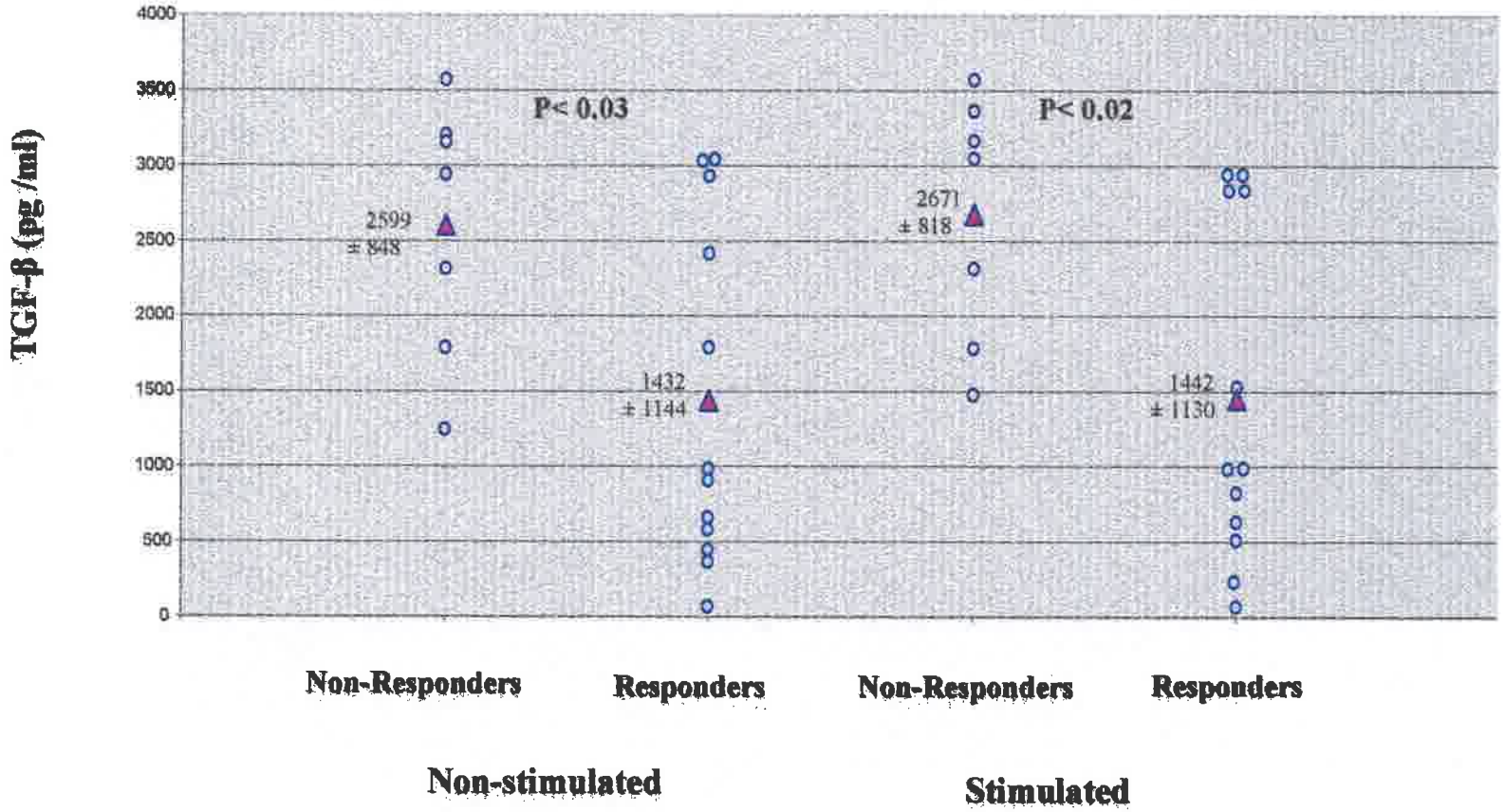
4.10 Cytokines production by PBMCs in chronic HCV patients:

Six months after starting IFN-α the capacity of PBMCs to produce TGF-β after 48 hours stimulation with Con-A was analysed in the group of patients eligible for IFN-α therapy. There was no significant difference in the TGF-β level in unstimulated and stimulated cell culture but there was a significant difference in the level of TGF-β in the responders (12 patients) and non-responders (7 patients) to IFN-α therapy. The non-responder group showed statistically higher level of TGF-β compared with the responder group (Figure 9).

The relationship between the level of TGF-β production and viral serotype was also analysed. High TGF-β production was found in patients infected with HCV serotype 1 when compared with that in patients infected with other serotypes ($p < 0.04$), whereas serotype 4 was associated with low TGF-β production when compared with its level in other serotypes ($p < 0.01$) as shown in Figure 10.

The level of IFN-γ in the supernatant of 24 hr Con-A stimulated PBMCs of the same group of responders and non-responders was also studied. Although the PBMCs from the non-responders (6 patients) and the responders (13 patients) produced constitutively similar level of IFN-γ when cultured *in vitro*, IFN-γ production in the non-responders was higher than that seen in the responders when the lymphocytes were cultured with Con-A. The difference between the two groups is statistically significant (Figure 11).

Figure 9: TGF-β production by PBMCs responders and non-responders to IFN-α



*p < 0.05 is significant (Student's *t* test)

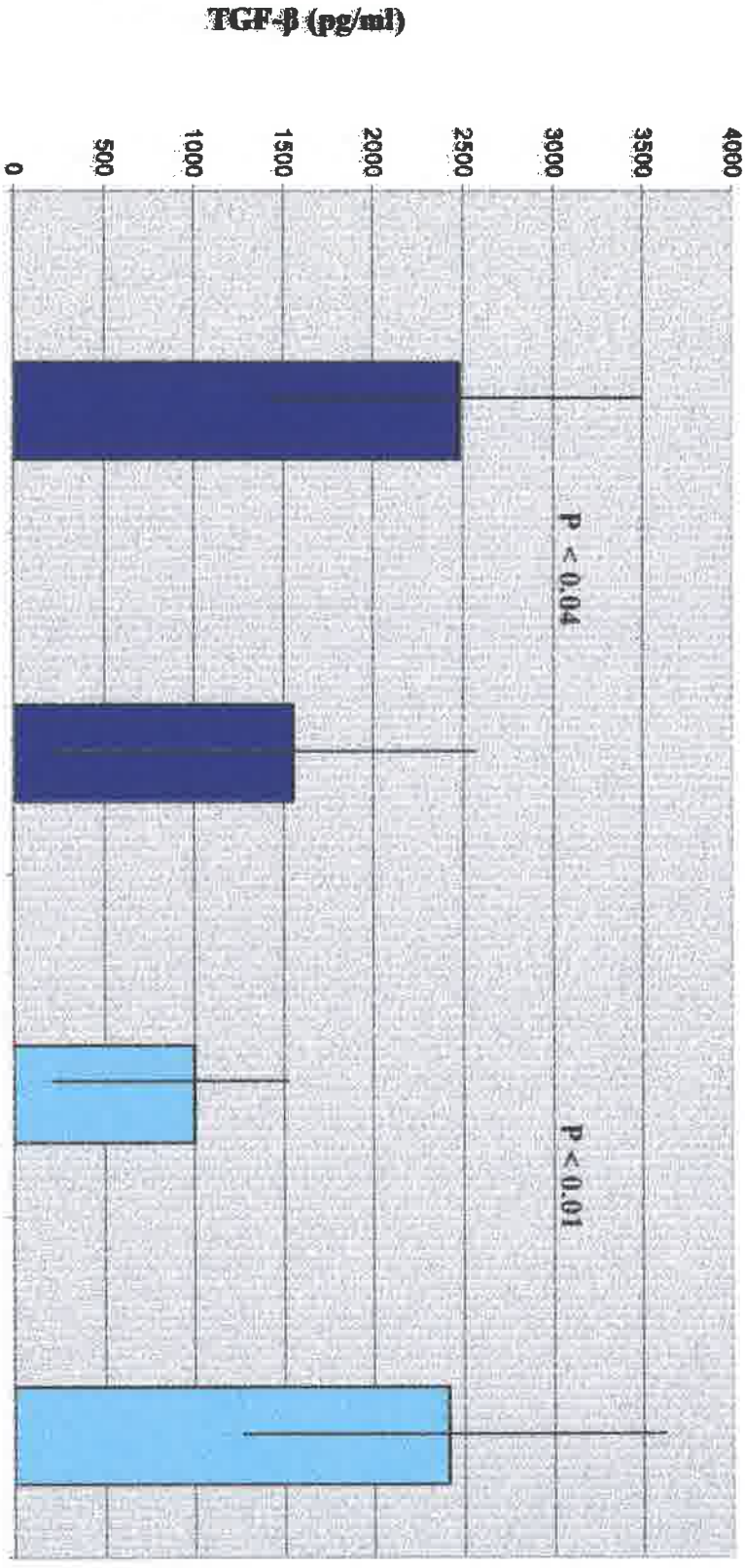
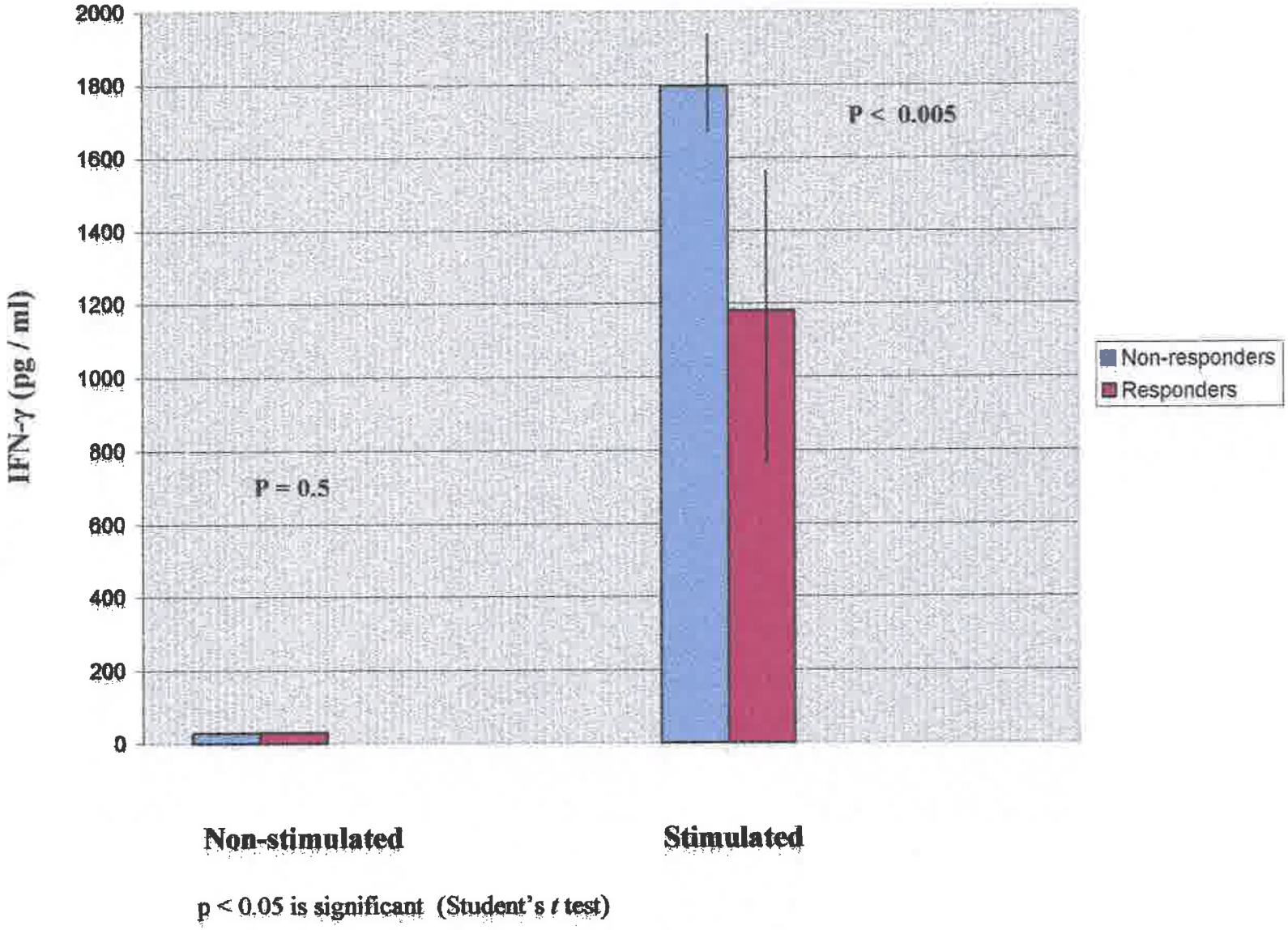


Figure 10: Association between HCV serotype and TGF-β production by PBMCs of HCV patients

P < 0.05 is significant (Student's *t* test)

Figure 11: IFN-γ production by PBMCs of responders and non-responders to IFN-α therapy



p < 0.05 is significant (Student's *t* test)

DISCUSSION

DISCUSSION

CHAPTER 5: DISCUSSION

5.1 Prevalence of autoantibodies in hepatitis C patients:

Hepatitis C infection has been associated with multiple autoimmune manifestations. The immune disregulation in HCV infection encompasses the development of autoantibodies, formation and deposition of immune complexes and cryoglobulinemia complicated by vasculitis and nephropathy (Mc Murray, 1998). HCV infection has been associated with antiphospholipid antibody syndrome (Harada et al, 2000), SLE (Ramos-Casals et al, 2000) and thyroid disease (Tran et al, 1993). Sicca Syndrome also seems to be associated with hepatitis C virus (Manns and Rambusch, 1999) and reports of low-grade lympho-proliferative malignancies have emerged (Ferri et al, 1998).

A recent study demonstrated a "true" Sjogren Syndrome, with similar clinical and histological features to those observed in primary Sjogren Syndrome, may occur in some patients with chronic HCV infection (Ramos-Casals et al, 2001). These associations may be interpreted as being the result of immune modulation induced by the lymphotropism of hepatitis C virus itself or, as in the case of non-organ specific antibodies, a manifestation secondary to the hepatocellular damage favoured by the genetic background of the host (Lenzi et al, 1999).

Several studies had reported an increased prevalence of non-organ specific autoantibodies (NOSAs) in patients with hepatitis C virus related chronic liver disease (Abuaf et al, 1993; Bortolotti et al, 1996; Drygiannakis et al, 2001). The data on the prevalence of different autoantibodies in HCV patients are controversial. Additionaly, the true prevalence of NOSAs in the general populations is still not convincingly established for different Ethenic and racial groups (Lenzi et al, 1999).

Several studies have evaluated the prevalence of antinuclear antibodies (ANA) and antismooth muscle antibodies (ASMA) in patients with HCV chronic

infection as ranging between 6-21% and 14-55% respectively. LKM1 antibodies are more rarely detected (0-5%). This wide range of prevalence reflects both the heterogeneous methodology used in the detection of these autoantibodies and / or different selection criteria of the patient populations studied. The clinical significance of the presence of non-organ specific antibodies in hepatitis C patients remains to be clarified. Nevertheless, higher degree of disease activity in patients with NOSAs during IFN-α treatment, suggesting the possibility that the immunomodulatory activity of the drug may activate an autoimmune reaction and that the presence of NOSAs may be the hallmark of a subclinical autoimmune disease (stated in Lenzi et al, 1999).

It had been suggested in the past that the autoantibodies seen in viral liver diseases (such as hepatitis B and C) during IFN-α treatment appear to be an expression of a generalized immune activation by cytokines (Meyer Zum Buschenfelde et al, 1995).

As we mentioned before, our first target was to assess the incidence of organ and NOSAs in chronic hepatitis C patients. Patients' results were compared with those of a control group of normal individuals (Table 1). As expected, the number of individuals free of autoantibodies in the patient group was significantly less than that in the control group (Table 3).

The results of this study indicate that NOSAs are common in chronic hepatitis C. These antibodies usually exist at low titer (although in some sera they reach levels observed in cases of autoimmune chronic active hepatitis) and, most commonly, one or two autoantibodies were found. In the sera of less than 17 % of the patients studied autoantibodies were not detected while in more than half the patients, one or two NOSAs were found (Table 3).

In the patient group, the prevalence of anticardiolipin, ASMA, AMA and anti-TPO was significantly higher in hepatitis C patients compared with control group (Table 1). Similar findings had been reported by other investigators who described that anticardiolipin antibodies (Prieto et al, 1996), ASMA

(Drygiannakis et al, 2001) and anti-TPO antibodies (Manns and Rambusch, 1999) are frequently found in patients with chronic HCV infection. The major impact of the above autoantibodies in the hepatological area relies on their well-recognized relevance as diagnostic markers of type 1 (ANA and / or ASMA) and type 2 (anti LKM1) autoimmune hepatitis (AIH) respectively.

It must be taken into account that each of the three reactivities does actually include antigenically heterogeneous populations of antibodies. Within ANA and ASMA, subspecificities have been identified, such as ANA with homogeneous immunofluorescence pattern and ASMA with antiactin specificity (ASMA-AA), which are more closely associated with AIH. Other ANA and ASMA subspecificities have been conversely reported in association with viral liver disorders. However, the potential usefulness of the identification of ANA and ASMA subspecificities remains uncertain in the differential diagnosis between AIH and chronic hepatitis C (Cassani et al, 1997).

The relevant target antigen of ANA associated with autoimmune chronic hepatitis is unknown, the available information being that it is different from ds-DNA and "rheumatological" extractable nuclear antigens of "rheumatic diseases" (Cassani et al, 1992). The occurrence of ANA and ASMA in chronic active hepatitis C may be triggered by alteration in cytokine production. The fact that their prevalence is also important in other viral and non-viral liver diseases suggests that the immunogenic process could be the release of immunomodulators (i.e.cytokines) by inflammatory cells at the site of inflammation or the release of intracellular antigens that may be able to modulate the phenotype of liver cells. Hepatocytes, in acute and chronic inflammatory liver diseases, express major histocompatibility complex antigens and surface "accessory" molecules. These immunomodulators, as signaling activating molecules, induce a spectrum of functional responses by the immune cells that could be responsible for specific and non-specific amplification of the immune response (Lunel, 1994).

In this study, the prevalence of ASMA (57%) was higher than that for ANA (29%) (Table 1), in accordance with some previous reports (Meyer Zum Bushenfelde et al, 1995). High prevalence of ASMA (66%) in HCV patients has also been reported by Clifford et al (1995). More than half of the patients (17 out of 30) having ANA showed concomitantly ASMA.

The analysis of ANA subspecificities shows that most of the autoantibodies detected in this study were ANA with a homogeneous pattern (Table 2), in contrast with some previous studies (Lenzi et al, 1999) which reported the speckled pattern as the most predominent pattern. None of our ANA-H reactive patients showed positivity for anti-dsDNA.

From the above results it appears that distinguishing between autoimmune hepatitis and chronic HCV infection on the basis of presence or absence of the "conventional" autoantibodies may be difficult. The positivity of either ANA-H or ASMA although considered signs of marked immune stimulation, does not rule out a coexisting hepatitis C virus infection (Cassani et al, 1992).

Antiphospholipid antibodies (APAs) are a heterogeneous family of immunoglobulins directed against different protein-phospholipid complexes. Among them, anticardiolipin antibodies had been detected in a variety of infectious diseases, particularly of viral origin. An association between thrombotic events and high ACA titers in patients with HCV-related diseases has been reported (Prieto et al, 1996). Biron et al (1998) stated that HCV infection may induce neoantigens by disrupting liver cell membranes. APAs would be the consequence of recognition of these neoantigens by the immune system. In liver disease induced by HCV, APA synthesis may result from overexpression of negatively charged phospholipid on apoptotic cell membrane surfaces leading to immunization against phospholipid-binding proteins. Rosser and Gores (1995) suggested that during liver necrosis, hepatocytes and endothelial cells may modify the immune response through expression of adhesion molecules, inducing subsequent development of autoantibodies.

This study has demonstrated a high prevalence of ACAs in patients with HCV-induced chronic hepatitis. These antibodies were found in 48/114 (42%) of the cases studied. No significant correlation was observed between ACAs positivity and tendency to thrombocytopenia in our studied group of patients (Table 9). These results may support the work of Dalekos et al (2000) who concluded that the existence of ACA in HCV patients was not associated with the development of antiphospholipid syndrome. It is interesting to note that one of the HCV patients studied with positive ACA test had history of repeated thrombotic events. History of myocardial infarction was reported in another four cases. These clinical data are not enough to prove its relation with the ACA positivity.

The clinical significance of autoantibodies against phospholipid antigens remain unclear. APAs are found in several clinical conditions and in asymptomatic persons. APAs may only be passive markers of disease severity, or they may participate in the evolution of disease by a still unknown mechanism (Biron et al, 1998).

It has been previously discussed that β2-glycoprotein1(β2-GP1), a serum protein with high affinity for anionic phospholipid, is required for binding of anticardiolipin antibodies to cardiolipin in autoimmune disease, whereas this requirement is not found in the binding that occurs in association with various infections. In addition, binding of ACA from patients with infections, unlike that of patients with SLE, is not dependent on β2-GP1. Classification of ACA into β2-GP1 dependent and β2-GP1 independent has been applied to patients with different clinical manifestations and helped to distinguish between ACAs associated with autoimmune disease and those resulting from infections (Guglielmone et al, 2001).

Antimicrosomal thyroid antibodies are the hallmark of autoimmune thyroiditis (i.e. Hashimoto's thyroiditis and Graves'disease). The immunodominant antigens inducing these antibodies in autoimmune thyroiditis has been identified as thyroperoxidase, a glycoprotein playing an essential role in thyroid hormone synthesis (Portmann et al, 1985). Anti-thyroid antibodies and dysthyroidism have been described in patients with chronic hepatitis C virus infection treated with IFN-α therapy. Interestingly, anti-thyroid antibodies have also been reported in

sera of HCV patients before IFN-α therapy. The prevalence of anti-thyroid antibodies in these patients ranged from 5-36 % (stated in Peoc'h et al, 2001). Such a remarkable variation may be attributable to the different methods used and /or to the different geography, race, age and sex of the populations targeted in these reported studies (Huang et al, 1999). Loviselli et al (1999) mentioned that there was no evidence of an epidemiological association of circulating thyroid antibodies and antibodies to HCV. Ganne-Carrie et al (2000) concluded that latent autoimmune thyroiditis is more frequent in untreated HCV patients than controls.

Manns and Rambusch (1999) indicated that it is still unclear whether hepatitis C plays a pathogenic role in autoimmune thyroid diseases. They recommended that in the clinical setting, thyroid autoantibodies and function should be monitored before, during and after IFN-α treatment in HCV-infected patients. Marazuela et al (1996) reported that the prevalence of positive anti-thyroid antibodies and hypothyroidism were significantly higher in women and were directly associated with increasing age. However, no significant association was found between chronic hepatitis C and the presence of thyroid autoimmunity in these female patients. The preponderance of women is characteristic of several autoimmune diseases and one possible explanation could be the hormonal status of the female gender. Huang et al (1999) reported that thyroid microsomal antibodies (TMA)-positive female HCV patients had a significantly higher prevalence of antibodies to a putative autoantigen (GOR), which has been considered to reflect HCV-induced autoimmunity. Tran et al (1995) further indicated that HCV patients with thyroid autoantibodies not only had a higher prevalence but also a higher level of anti GOR than HCV patients without thyroid antibodies and suggested that high prevalence of thyroid autoantibodies is a specific autoimmune phenomenon in chronic HCV infection. Alternatively, it is also possible that HCV patients with thyroid autoimmunity are more likely to show other evidence of autoimmunity.The mechanism of such autoimmunity is unknown. One possible explanation is that HCV may share sequence homology in amino-acid residues with those of thyroid autoantigens, and may thus trigge

an autoimmune reaction through the mechanism of molecular mimicry. However, this speculation requires further study (Huang et al, 1999).

Peoc'h et al (2001) described that antimicrosomal antibodies are heterogeneous in HCV patients and that HCV may induce autoantibodies directed to a protein distinct from thyroperoxidase. They recommended further studies to determine whether HCV infection leads to a breakdown of tolerance to a thyroid self-protein other than thyroperoxidase.

This study has assessed the high prevalence of TPO antibody in patients with chronic HCV before IFN-α therapy. These antibodies were found in 31% of the patients (with low titer) and was not associated with clinical manifestations of dysthyroidism before IFN-α treatment. The frequency of TG antibodies showed no significantly elevation compared with the control group (Table 1).

The presence of thyroid autoantibodies in this study was equally distributed between the male and female chronic HCV patients (Table 11). This does not accord with the findings of Tran et al (1993) who showed that thyroid autoantibodies were found predominantly in the female patients.

The presence of AMA has been reported to be the hallmark of primary biliary cirrhosis where it has been found in 95% of patients (Nishio et al, 2000). Grimbert et al (1996) reported that the AMA, present in patients with chronic hepatitis C, may disappear after eradication of HCV suggesting that the prodution of AMA antibodies is linked to the presence of the virus. None of these patients had biological or histological evidence of primary biliary cirrhosis. They also suggested that AMA in patients with chronic hepatitis C do not always recognize the same epitopes as in primary biliary cirrhosis.

In this study, AMA was found in 40 % of the patients and the prevalence was significantly higher than that seen in the control group (Table 1). The prevalence of AMA in this study was also relatively higher than that reported in other studies

(Garrido-Palma et al, 1999 ; Bayraktar et al, 1997) in which the prevalence of AMA in chronic HCV patients was 2% and 4% respectively.

It has recently been discussed that oxidative stress and mitochondrial injury play a role in liver injury in chronic hepatitis C patients. A fraction of the expressed core protein localized to the mitochondria and was associated with a redistribution of cytochrome C from the mitochondrial to the cytosolic fractions. Oxidative injury occurs as a direct result of HCV-core protein expression both *in vitro* and *in vivo* and may involve a direct effect of core protein on the mitochondria. These results provide new insight into the pathogenesis of hepatitis C and provide a rationale for investigation of antioxidant therapy (Okuda et al, 2002).

The presence of organ specific and non-specific autoantibodies in chronic HCV patients may suggest that HCV can promote, in genetically susceptible subjects, polyclonal B-cell activation which ultimately yields large amounts of various autoantibodies. A cross recognition between HCV and host epitopes, immunologic disturbances induced by HCV or prolonged tissue damage in systemic organs as a results of extra-hepatic manifestations of HCV infection may induce the production of antibodies to various autoantigens.

In agreement with the results of Jiron et al (2000), anti LKM1 antibody were not detected in this study group (Table 1). These results are also in agreement with previously published data that the coexistence of LKM autoantibodies with chronic hepatitis C is rare (Meyer Zum Buschenfelde et al, 1995).

Lunel and Cacoub (1999) reported that LKM1 antibodies are present significantly more often in chronic hepatitis C patients than patients with other liver diseases. One of the possible mechanisms for the induction of LKM1 antibodies in HCV infected patients may be molecular mimicry between HCV polyprotein and human microsomal P45011D6 (Michitaka et al, 1994).

Other published reports (Gerotto et al, 1994) suggested that HCV type 1, which has been associated with more severe forms of chronic liver disease and resistance to IFN-α, may more easily induce anti-LKM1 antibodies, compared to other HCV genotypes.

In this study, HCV type 1 represents only 20% of the studied group while the majority (60%) were classified as HCV serotype 4 (Table 22). So, genetic pattern of suseptibility and enviromental factors, which may act as trigger factors, as well as differences in viral genotype may be responsible for these discrepancies in the data concerning the prevalence of anti LKM1 antibodies in chronic hepatitis C patients.

Numerous hypothesis have been proposed concerning the potential role of HCV in autoimmune disorders. The infection of lymphocytes and the persistence of HCV infection may facilitate the expression of immunologic manifestations through a stimulation of mononuclear cells and immunoglobulin production responsible for mixed cryoglobulins and rheumatoid factor activity, a production of cytokines (i.e. interferon) that may enhance the expression of HLA, the presentation of autoantigens, and the activation of T lymphocytes, a molecular mimicry mechanism with host epitopes leading to cross- recognition between autoantigens and the virus, the liver lesion induced by HCV (i.e.hepatocellular necrosis) may modify the expression of host epitopes and thus lead to the production of autoantibodies and immune complexes, a genetic predisposition that could be necessary to trigger the abnormal immune response; or the genetic variability of HCV (Lunel, 1994).

In summary, various genotypes of the HCV have differing immunological responses in the host. So, it seems logical that the development of the HCV subtype-specific immune response may stimulate the formation of antibodies directed to some organs or may be generalized, which are named organ and / or non-organ specific autoimmune disease.Therefore, various types of viral antigens may play an important role during the development of the various types of autoimmune diseases via the host's immune responses (Sanver et al, 2001).

5.2 Autoantibodies and histopathological changes in liver biopsy in chronic hepatitis C patients:

The association between the presence of autoantibodies and the clinical, biochemical and histological picture of HCV related chronic liver disease is still controversial. Several reports have failed to identify the presence of autoantibodies as an untoward factor for chronic liver disease. Cassani et al (1997) showed for the first time, in a prospective series of chronic hepatitis C patients, that patients having autoantibodies exhibited a higher biochemical and histological activity when compared with those without autoantibodies. These observations suggest that the emergence of autoimmune reactions might contribute to a worsening of the liver disease profile. In their study, they also discussed that the only significantly different histological parameter was the portal-periportal necroinflammation, which represents the typical pattern of immunologically mediated liver damage. However, it can not be excluded that the appearance of serum autoantibodies might be the consequence rather than the cause of the disease severity.These results are strengthened by the observations obtained by Lenzi et al (1999) that the presence of NOSAs is significantly associated with the presence of chronic liver disease, with HCV infection in a replicative state, and with higher levels of ALT and γ-GT. However, the hypothetical immune mechanism of liver damage is still not proven (Lenzi et al, 1999).

In this study, the level of ANA, AMA and ASMA were evaluated by scoring the intensity of fluoresence on a scale of 1 to 4 depending on the end point dilution positivity. Anticardiolipin antibodies were evaluated according to its concentration in serum as mentioned before (see the results). By using this semiquantitative method for the NOSAs, the relationship between the presence of these antibodies and the degree of liver damage was assessed. A tendency towards a higher score of NOSAs in parallel with an increasing degree of liver damage was observed but this relationship did not reach statistical significance (Figure 1). However, when the number of NOSAs and their relation to the degree of liver damage was examined, the relationship was statistically highly significant

(Figure 2). These results are in accordance with those described by Cassani et al (1997) and Lenzi et al (1999).

Romero-Gomez et al (2000) reported that anticardiolipin IgG was significantly related to portal hypertension and presence of hepatic cirrhosis complication and concluded that anticardiolipin IgG may play a potential role in fibrosis progression and liver disease in chronic hepatitis C patients. The high prevalence rates of thrombosis together with the relevant clinical sequelae of its occurrence have prompted the search for factors which may perdispose cirrhotic patients to this complication. Recently, attention has focused on the role of APA, in particular ACAs (Mangia et al, 1999). Prieto et al (1996) reported that chronic hepatitis C patients exhibited higher prevalence of ASMA than those who tested negatively. They suggested that there was a greater risk of developing ACA in chronic hepatitis C increases with the progression of the disease.

At a univariate analysis, ACA prevalence was not positively related to the severity of liver damage or the presence of cirrhosis even though they were more frequently detected among patients with ASMA positivity (Tables 9,10). These results are in agreement with these reported by Biron et al (1998) who found no relation between APAs and histological severity assessed by Knodell's Score.

Knodell's Score mainly takes into account disease activity, particularly hepatocyte necrosis. Liver disease activity is defined by inflammation and hepatocytes necrosis. Biron et al (1998) found a direct relation between APAs presence and liver fibrosis whereas APAs levels were not correlated with the disease activity. More prospective studies of longer duration may be required in order to address the relation between anticardiolipin antibodies and liver histopathological changes in chronic HCV patients. Also, no positive association was found between other autoantibodies and liver histopathological status in this study (Tables 5,6).

From the above data we may conclude that HCV can promote a polyclonal B cell activation which ultimately yields large amounts of the various autoantibodies which may participate in liver cell damage. Chronic hepatitis could be the result of virus induced autoreactions against hepatocytes and / or circulating immune complex deposition. The clearance of these immune complexes is undertaken by the mononuclear phagocytic system i.e. liver Kupffer cells and their activation can promote parenchymal inflammation and fibrosis.

5.3 The relation between anti HCVcore-IgM and other disease parameters:

Although the vast majority of patients with chronic hepatitis C developed antibodies to structural and / or non-structural recombinant and synthetic HCV antigens in their sera, the pathogenetic role of the HCV specific humoral immune response is not clear. In the majority of patients with acute hepatitis C, the anti-HCV IgM antibodies were present in serum, however, some patients with chronic hepatitis C were positive for anti-HCV IgM too (Stransky et al, 1996).

The second aim of this study was to assess the role of IgM antibodies to hepatitis C virus-core protein as a marker of chronic HCV infection, the relation between its presence and the production of autoantibodies, to determine whether the positivity for core IgM has an impact on the histological findings in the liver and its role as a predictor of successful IFN-α therapy.

Recent studies showed that the response of the IgM anti HCV-core depends on serum HCV-RNA levels, thus indicating distinct virological implication of IgG and IgM immune responses to the HCV-core protein (Gérard et al, 1996). Core IgM antibodies to hepatitis C virus have been reported to be a serological indicator of viral replication and active disease (Fracassatti et al, 1996). Other investigators reported that IgM anti HCV seems to be a specific index of viraemia in HCV antibodies positive patients who have no clinical and biochemical signs of liver damage (Morisco et al, 1998). Other published articles reported that the prevalence of positivity for HCV-core IgM is significantly higher in patients with severe chronic hepatitis than with mild chronic hepatitis and that the level of IgM

anti HCV-core positivity correlates significantly with serum ALT levels and is significantly higher in patients with severe chronic hepatitis than in those with mild chronic hepatitis (Caporaso et al, 1994). Fabrizi et al (1996) reported that they utilized the anti HCV-IgM to gain information on the infectivity of anti-HCV positive patients receiving chronic haemodialysis treatment.

In this study, 78 out of 115 (67.8%) of patients with anti HCV-IgG sero-positivity showed anti HCV-core IgM antibodies. Picciotto et al (1995) also reported a high prevalence of IgM antibodies (35 out of 62 i.e. 56.4% were positive for HCV-IgM at base line) in chronic HCV patients.

It has been argued that IgM humoral response to core proteins of HCV may reflect the virological status of the patients. One of the suggested explanations for the presence of IgM response to core HCV protein is the association with acute exacerbations of chronic infections. However, the virological implications of IgM anti HCV-core in chronic HCV infection are unclear. Positive correlation has been described by some authors between viraemic levels and IgM anti HCV-core levels (Yuki et al, 1995).

A strong correlation between the presence of HCV-core IgM antibody and HCV-RNA ($p=0.004$) was seen in this study (Table 12). A similar relationship was also observed by other investigators (Yuki et al, 1995).

In this study, the presence of HCV-core IgM antibodies was positively associated with the tendency for production of non-organ specific antibodies (Figure 3). These findings may indicate a positive association between the presence of non-organ specific antibodies, disease activity and continuous viral stimulation. Both HCV specific IgM antibodies and autoantibodies may be indicative of continued viral replication. Here, we report, as a novel finding, a positive association between the presence of HCV-core IgM antibodies and AMA in sera of chronic hepatitis C patients (Table 7). This association is statistically

highly significant (p=0.003). However, no positive association was observed between HCV viraemia (represented by qualitative PCR) and the presence of non-organ specific antibodies (Table 14).

It has been suggested that the liver injury in chronic hepatitis C patients is correlated with the persistance of detectable HCV-RNA in serum. However, the presence of HCV-RNA in asymptomatic individuals indicates that some immunological mechanisms my also be pathogenetically relevant. This may also be explained by the possibility that liver disease in anti-HCV positive patients may be unrelated to HCV but caused by superimposed and unrelated form of liver injury.

In the current study, a positive association was observed between anti HCV-core IgM and the activity of liver disease in patients with biopsy-proven chronic hepatitis C (Figure 4). Therefore, testing for anti HCV-IgM may provide the specific diagnosis of type C hepatitis by identifying anti-HCV positive patients with HCV infection and an active immune response to the virus. The positive association between HCV-core IgM and liver disease activity has also been reported by Tran et al (1997) who described that the mean Knodell's Score of IgM anti HCV-core positive patients was significantly higher than that of the IgM negative patients and concluded that IgM anti-HCV antibodies appears to be a simple serological marker of more severe liver disease.

5.4 IFN-α therapy, parameters of disease progression and immuno-disregulation in chronic HCV patients:

Interferon-α (IFN-α) has become the standard therapy for chronic hepatitis C, inducing biochemical (persistent normalization of alanine-aminotransferase) and virological (sustained negativity for HCV-RNA) response in about 15-25% of treated patients (Shiratori et al, 1999). Currently, IFN-α in combination with Ribavirin is the standard therapy for patients with chronic HCV infection. The protocol for this study, including the treatment regimen, was designed before this combined treatment is considered. This has enabled us to study the effect of IFN-alpha alone as an antiviral therapy and its relation with other disease parameters.

IFN-α is an expensive drug with relatively poor efficacy on viral replication and potentially serious side effect. As a result, it is mandatory to identify which patients are most likely to respond to IFN-α therapy.

Some pretreatment parameters have been suggested as predictive of biochemical response to IFN-α including low disease activity index, low serum bilirubin concentration, low levels of viraemia and lack of anti- HCV-core IgM (Pawlotsky et al, 1995). Absence of cirrhosis and non-1 viral genotype were also described as pretreatment features predictive of a good response to monotherapy with IFN-α (Dusheiko et al, 1994). Sustained biochemical response was defined as normal ALT and sustained virological response was defined as negative HCV-RNA six months after end of therapy (Koshy et al, 2000).

5.4.1 Serum transaminases and liver biopsy score in responders and non-respondersto IFN-α therapy:

In the present work, non-responsiveness to IFN-α therapy in patients with chronic HCV hepatitis is associated with general and viral specific parameters of altered immunological reactivity has been demonstrated. In the patients, the response to treatment could not be predicted by the degree of pretreatment serum transaminases while a positive relationship was found between the final outcome of IFN-α treatment and pretreatment inflammatory activity in the liver

(represented by Knodell's Score) (Table 15). This relation has also been described by Kaserer et al (1998) who reported that non-responders had significantly more advanced fibrosis than the responders to IFN-α treatment and concluded that liver biopsy provides useful information for selection of patients with hepatitis C for IFN-α therapy. These findings may reflect the fact that serum transaminases levels at one point in time are a poor indicator of the long term natural history of HCV infection. In a disease with a long natural history, it is important to consider histological liver and disease severity rather than serum transaminases levels.

Sobesky et al (1999) suggested that spontaneous progressive liver fibrosis in patients with chronic hepatitis C can be estimated by a fibrosis progression rate per year. Therefore, a reduction in this liver fibrosis progression rate could be considered as a primary therapeutic goal. This estimate is probably more clinically relevant than aminotransferase activity or virological end points for evaluation of IFN-α effect because this cytokine has not only an antiviral effect but also an antifibrotic effect. Elevated liver enzymes in both responder and non-responder groups before treatment showed a statistically significant decrease, although liver enzymes reached normal levels only in the responder group after treatment withdrawal (Table 17).

5.4.2 Presence of anti HCV core-IgM prior to treatment and response to IFN-α therapy:

Although a variable percentage of HCV patients are known to have IgM core antibodies to HCV, the exact clinical and diagnostic significance of these antibodies is still being debated. It has been argued that IgM humoral response to core proteins of HCV may reflect the virological status of the patients. One of the suggested explanations for the presence of IgM response to core HCV protein is the association with acute exacerbations of chronic infections. There are, however, reports that persistant IgM-HCV in acute infection identifies those who evolve as chronic active hepatitis patients (Quiroga et al, 1991) and its disappearance during IFN-α treatment of chronic active hepatitis has been linked with sustained response (Brilliant et al, 1992). Additionally, a positive

correlation between the presence of IgM anti-HCV and the activity of the liver disease has been suggested (Martinelli et al, 1996).

In this study, responsiveness to IFN-α was in a positive association with the lack of detectable anti HCV-core IgM before IFN-α treatment (Table 19). This observation is in keeping with previous reports (Tassopoutous et al, 1994 and Tabone et al, 1997). Moreover, the responders with detectable core IgM antibody before treatment showed a significant drop in their level after IFN-α therapy, although the number studied was small (Figure 6). These results support that reported by Picciotto et al (1995) who observed a progressive disappearance of anti HCV-core IgM in patients responsive to IFN-α therapy over the long term and concluded that the loss of anti HCV-core IgM positivity in patients positive at base line can predict the long term response to IFN-α therapy. Taken together these results with the previously reported correlation between the presence of AMA and HCV-core IgM antibody and if IgM to core antigen production is related to virus mutation and therefore the escape from immunological surveillance, it was interesting to study to what extent the presence of AMA may be useful as a predictive and follow up parameter of IFN-α therapy.

5.4.3 Presence of autoantibodies prior to treatment and response to IFN-α treatment:

Several reports have indicated that appearance of serum autoantibodies of various specificity are common in chronic hepatitis C (Cassani et al, 1997). The relevance of these antibodies to pathogenesis of hepatitis C and response to treatment is unclear. Also several reports have shown that IFN-α treatment may trigger autoimmune disease in predisposed individuals. So, hepatitis C itself and IFN-α have been shown to induce autoimmune mechanisms.

As there are insufficient data to evaluate the relation between the presence of these autoantibodies and the response to IFN-α in chronic HCV patients, as an initial step, the number and specifications of autoantibodies in these patients under IFN-α treatment have been analysed. A tendency towards the production

of more autoantibodies in non-responders than responders to IFN-α (Figure 5, Table 20), although this difference did not reach statistical significance. This may support the reports of Cassani et al (1997) that the autoantibody positivity does not influence the response to IFN-α in chronic hepatitis C patients. The production of anticardiolipin, TPO as well as AMA was in direct correlation with non-responsiveness to IFN-α therapy (Table 18). The relation between pretreatment production of AMA in chronic hepatitis C patients and non-responsivness to IFN-α had also been discussed by Garrido et al (1999). In their patients, absence of AMA was accompanied by a favourable response to IFN-α while the presence of AMA was accompanied by an unsuccessful treatment response with biochemical exacerbation of cholestasis. The presence of AMA in chronic HCV patients should be carefully interpreted because the theraputic decision to give IFN-α in such condition is complicated as AMA may be "real" and if it reflects primary biliary cirrhosis, cholestasis can be triggered or exacerbated with IFN-α therapy. For this reason, it had been suggested that the presence of AMA with titers in excess of 1:160 in chronic hepatitis C patients should be a contraindication for treatment with IFN-α therapy (Garrido-Palma et al, 1999). On the other hand, Grimbert et al (1996) suggested that the production of AMA antibodies is linked to the presence of the virus, although no difference in the clinical course and response to IFN-α treatment was reported in chronic HCV patients who had AMA. Whether this correlation suggests that the presence of these autoantibodies can be considered a surrogate marker of progressive HCV infection and / or tissue damage remains to be elucidated.

5.5 Autoantibodies production before and after IFN-α therapy:

Different side effects have been reported in patients treated with IFN-α but their incidence and prognosis in the case of adverse reactions remain largely unknown (Betterle et al, 2000). These side effects include the production of a variety of autoantibodies and occasionly the development of clinically relevant autoimmune diseases. Marazuela et al (1996) reported that IFN-α therapy induced anti-thyroid autoantibodies and thyroid dysfunction *de novo* in patients with chronic hepatitis C without pre-existing thyroid abnormalities. Thyroid dysfunction secondary to

IFN was reversible after discontinuation of therapy. Thyroid abnormalities associated with IFN-α have varied from destructive thyroiditis with hypothyroidism to transient thyrotoxicosis followed by hypothyroidism and rarely hyperthyroidism. Pre-existing autoantibodies or autoimmune conditions predispose to the development of autoimmune disorders during IFN-α treatment (stated in Marazuela et al, 1996). Carella et al (2001) reported that high thyroid autoantibody levels at the end of IFN-alpha therapy for chronic hepatitis C are related to the risk of developing chronic thyroid autoimmunity. The co-existence of thyroglobulin antibodies and thyroid peroxidase antibodies at the end of the treatment is a predictive factor for the development of thyroid dysfunction, even if subclinical, many years after IFN-α withdrawal.

The mechanism by which IFN-α induces autoimmune reactions is unclear but could also enhance recognition of autoantigens, leading to an increased incidence of autoimmune disease. Antigen presentation by macrophages and dendritic cells could be enhanced by IFN in autoimmune disorders. Cytokines such as IFN produced by infiltrating T cells or administered exogenously could not only trigger, but also would perpetuate the disease. In addition, IFN can affect directly both thyroid hormone synthesis and secretion *in vitro*. This could explain the occurrence of hypothyroidism in the absence of antithyroid antibodies. In summary, IFN-α may affect the thyroid function acting both on systemic immune surveillance and at the thyrocyte level. IFN-α is both an antiviral agent and an up regulator of the cellular immune system. The later effect is non-specific. Thus, IFN-α diffusely activates the cellular immune system and can initiate new autoimmune diseases in patients treated with it (Bayraktar et al, 1997).

IFN-α may induce a direct effect on thyroid function by reducing the intrathyroidal organification of iodine in absence of autoimmune thyroid disease (Roti et al, 1996). Dalgard et al (2002) reported that female gender and being of Asian origin were independent predictors of the development of biochemical thyroid dysfunction during IFN-α treatment. In their study, neither the IFN-α dosage nor the virological treatment response was related to the incidence of thyroid dysfunction.

The prevalence of organ specific and non-specific autoantibodies has only been studied in a limited number of the treated patients (8/20 of the responders and 12/21 of the non-responders). Using these limited data, the prevalence of different organ specific and non-specific autoantibodies showed no statistically significant change after IFN-α therapy in either responder or non-responder groups (Table 21) compared with that before starting IFN-α treatment.

5.6 Viral genotype, production of anti HCV-core IgM and response to IFN-α treatment:

It was assumed that defined HCV genotype were confined to specific geographic regions. With increasing sequence data it has become evident that multiple genotypes can co-exist in a given geographic region. It was assumed by many investigators that different genotypes are associated with different course of disease and different response rate to IFN-α therapy (lam, 1999).

In the current study, genotype 4 was the most dominant genotype in the patients (Table 22). The results agree with those in previous reports (Martinot-Peignoux et al, 1995) that genotype 1 was associated with significant tendency to non-responsiveness to IFN-α therapy and that genotype 3 showed high rate of response to IFN-α therapy although the number of patients was too small to draw any firm conclusion. Genotype 4 was associated with good response (about 50%) to IFN-α treatment and again significant number of its non- responders had core-IgM to HCV antigen in their sera before treatment. In this study group, no significant difference between serotype 4 and other serotypes with respect to the production of autoantibodies was seen (Table 25). How HCV genotype may influence the tendency of patients to have a sustained response to IFN-α therapy remains to be determined. Kanazawa et al (1994) have shown that the degree of quasispecies complexity and diversity of the hypervariable regions were associated with poor response to IFN-α therapy. In the presence of a heterogeneous viral population, IFN-α therapy might induce a progressive selection of the virus. Some HCV genotypes might be cleared by IFN-α therapy

before producing escape mutants, whereas others might be associated with a selection of HCV quasispecies IFN-α resistant. Hsich et al (2000) reported that the total frequency of IFN-α induced thyroid dysfunction was higher in patients with mixed HCV subtypes infection than in those with single HCV subtype infection.

5.7 Phenotyping of immunocompetent cells in HCV patients on IFN-α therapy:

Chronic hepatitis C is associated with several phenomena of immuno-disregulation which may be important in the understanding of HCV infection.

T cell modulation is considered to play an important role in both pathology and response to IFN-α therapy. Polyclonal antibody stimulation and alteration in cytokine production are either involved or at least accompany the development of hepatitis C. On the other hand, patients with chronic hepatitis C infection exhibit differences in their response to IFN-α therapy.

Six months after IFN-α therapy, the patients were grouped as responders and non-responders based on the previously mentioned criteria. The relationship between response and variations in their general immune status, as reflected in ratio of T cell subpopulations and expression of activation markers, was examined in the patient groups. Phenotypic analysis of the peripheral lymphoid cells was undertaken for the group of patients eligible for IFN-α therapy including the quantitation of $CD4^+$, $CD8^+$ T cells. The $CD4^+$/ $CD8^+$ T lymphocyte ratio and the percentage and absolute number of activated lymphocytes $CD25^+$ (IL2 receptor expression cells) were also analysed. $CD4^+$/ $CD8^+$ ratios in both responders and non responders were compared with that of control individuals. No significant differences in $CD4^+$/ $CD8^+$ ratios were seen between the two treatment groups. However, both responders and non-responders showed significantly lower $CD4^+$/ $CD8^+$ ratios compared with the control group (Table 26). This may reflect a general state of immunosuppression which accompany chronic infection.

The absolute number as well as the percentage of cells expressing IL2 receptors were significantly higher ($p<0.05$) in non-responders than that in responders group of patients (Table 27). This may be related to continuous viral stimulation in the non-responders. However, it may be also considered that some of $CD4^+CD25^+$ cells are regulatory T cells (stated in Groux, 2003). It has recently been described that human $CD4^+CD25^+$ T cells display anergic and suppressive properties *in vitro*, similar to the corresponding rodent T cells. Human $CD4^+CD25^+$T cells proliferated poorly and were deficient in IL2 secretion when stimulated with polyclonal activators, such as phytohemagglutinin, anti CD3 antibodies or allogeneic cells (Curotto de Lafaille and Lafaille 2002). The anergic condition of $CD25^+$T cells could be reversed by IL2 and / or IL15. In addition, $CD4^+CD25^+$T cells were able to suppress proliferation and IL2 production of $CD4^+CD25^-$T cells in a contact-dependent fashion. There were discrepancies in the description of cytokine production by stimulated human $CD4^+CD25^+$T cells *in vitro* but, in general, these cells were found to produce inhibitory cytokines, such as IL10 and TGF-β and variable or undetectable amounts of IL4 and IFN-γ (Read and Powrie 2001). Xu et al (2003) reported that $CD4^+CD25^+$T regulatory T cells suppress both Th1 and Th2 cells and that these regulatory T cells have a profound therapeutic potential against diseases induced by both Th1 and Th2 cells *in vivo* (Xu et al, 2003).

Although T lymphocyte response to HCV gene products have been demonstrated, the role of such responses in controlling HCV infection is not well defined. Previous studies showed that asymptomatic anti HCV positive subjects who successfully cleared HCV-RNA from serum and PBMCs after a previous episode of acute HCV infection can express vigorous HLA class ll- restricted T cell responses to HCV antigens, even several years after resolution of hepatitis. In these patients the T cell responses were stronger than those in chronically infected patients, raising the question of whether the relative stength of response can represent a crucial determinant of viral clearance or persistence and whether a quantitative difference in the T cell response among patients with different outcomes of disease is also present at the early stages of infection, when the crucial pathogenetic events are likely to occur (Missale et al, 1996). In addition,

several lines of evidence indicate that the pattern of cytokines secreted by $CD4^+$ helper T cells can facilitate eradication of infection when it is dominated by Th1 responses or can favor viral persistence if it is oriented toward Th2 effects (Missale et al, 1996).

An imbalance between helper T cells type-1 (Th1) and type-2 (Th2) cytokine is suggested to play an important role in the pathogenesis of chronic viral infection, but this issue is not resolved in patients with hepatitis C virus infection (Sobue et al, 2001). Yao et al (2001) stated that strong and broad cytotoxic T lymphocytes (CTL) and Th1 responses have been detected in an individual with acute hepatitis C, who subsequently cleared the virus. However, in patients chronically infected with HCV, the frequencies of antiviral CTL are relatively low. In addition, the production of Th1-type cytokines (i.e. IFN-γ and IL2) is dramatically suppressed in peripheral T cells of chronic HCV patients. The presence of inefficient T lymphocyte responses in chronic HCV patients suggests that HCV gene products might be involved in suppressing host immune response.

It is important to emphasize that the ability of the immune system to mount a protective $CD4^+$ effective response is not only dependent on the number of antigen-specific memory T cells present, but also on the functional phenotype of these cells. Bergamini et al (2001) have demonstrated that the HCV-reactive $CD4^+$ cells in HCV patients showed a polarized IFN-γ response with lack of IL2 production. It is possible that the lack of IL2 production may compromise the overall effector response limiting, for example, recruitment, differentiation and expansion of $CD8^+$ effector T cells or natural killer cells. On the other hand, enhanced Th2 responses are suggested to participate in persistance of HCV infection in chronic hepatitis C patients (Fan et al, 1998). However, other recent studies (Bergamini et al, 2001) do not support the hypothesis that the inability to eradicate chronic HCV infection results from increased production of Th2-type cytokines. Osna et al (1997) stated that IFN-γ production deficiency in chronic hepatitis C patients is secondary to blockage by high levels of IL10 and impaired IL12 production.

It has been reported that IFN-α induces IL12 receptors (IL-12R) on Th1 lymphocytes, presumably increasing their response to this cytokine and amplifying the Th1 / CTL response. This is usually important for the clearance of virally infected cells during recovery from an acute viral infection. IFN-α may have an important role in determining the outcome of infection if it influences the balance of Th1 and Th2 (Thomas et al, 1999).

The hypothesis that non-responsiveness to IFN-α was in some way related to T lymphocytes subpopulation was also tested. To this end, the capacity of PBMCs to produce Th1 type cytokine (IFN-γ), Th2 cytokine (IL4) and down-regulatory cytokine (Th3; TGF-β) was examined. In the treated patients, Th2 cytokine IL4 was below the level of detection, while Th1 cytokine IFN-γ production was in a positive association with non-responsiveness to IFN-α therapy (Figure 11). These findings may support the notion of Sobue et al (2001) whose data showed a shift to a Th1 cytokine profile with the progression of liver disease and on the other hand contradict the assumption that chronicity in HCV infection may reflect a lack of type-1 cytokine production (Woitas et al, 1997).

This lack of Th1 cytokine production in the responder group may be explained by the fact that the study was focused on the peripheral blood compartment which may only partially reflect immune events occuring within the infected liver. Compartmentalisation of HCV-specific lymphocytes within the liver of patients with chronic HCV infection has been shown in other studies and could explain the finding of weaker T cell responses from the peripheral blood lymphocytes of chronically infected cases (Cramp et al, 1999). An additional factor to take into account in the interpretation of the present results is that in most studies (Missale et al, 1996; Large et al, 1998), HCV specific proteins were used to study the T cell response whilst in this study mitogen stimulation (Con-A), which reflect the general state of T cell reactivity, was used. Additionally, other authors (Fan et al, 1998) obtained their cytokine profile results in chronic HCV individuals using ELISA testing of sera for cytokine concentrations which produced contrasting

results. These discrepancies are probably the result of methodology drawbacks associated with ELISA measurment of serum samples. Also, the serum levels have been reported to be subject to diurnal fluctuations in immune activity. Moreover, quantification of serum cytokine levels using ELISA may be misleading as specific binding protiens exist for a number of cytokines, including autoantibodies, α2-macroglobulin, heterophilic antibodies and soluble cytokine receptors, all of which would interfere with cytokine detection. Finally, cytokine levels measured by ELISA are the net of protein synthesis, consumption and biodegradation (Bergamini et al, 2001).

However, results from this study (Figure 11) are in accordance with those reported by Bergamini et al (2001) which suggested that IFN-α treatment was associated with a reduction in mitogen-induced Th1 cytokine response in those patients who cleared their plasma HCV-RNA. Thus, the marked decreased in the Th1 response in the patients who responded to IFN-α therapy may be explained by the hypothesis that the clearance of viral load induced by IFN-α abolished the antigenic stimulus to drive a Th1 response. This could also explain the apparent contradiction that IFN-α, which has been reported to promote development of Th1 cells, induced a strong reduction of Th1 cells *in vivo* (Bergamini et al, 2001).

It has been suggested that $CD4^+$T cells respond to specific antigen in a graded manner depending on the antigen concentration (Diepolder et al, 2001). Low antigen concentration induced the expression of T cell activation markers (e.g.$CD25^+$) while high antigen concentration (30-100 fold) was required for the induction of proliferation as well as IFN-γ secretion. This may explain the significantly higher number of $CD25^+$cells as well as the level of IFN-γ in the non-responder patients compared with the responders to IFN-α therapy reported in our study (Table 27, Figure 11).

The overproduction of IFN-γ plus the significant elevation of IL2-receptor cells in the non-responders may support the results of Napoli et al (1996) which showed that there is an increase in m-RNA expression of IL2 and IFN-γ in

chronic hepatitis C which correlates with disease severity and liver damage. In a previous study the expression of messenger-RNA of Th2 cytokines, Th3 (TGF-β) and TNF-α had been observed in HCV patients treated with IFN-α while no difference was observed for m-RNA of classical Th1 cytokines after treatment. This may indicate that the cytokine m-RNA profile following IFN-α treatment points to an anti-inflammatory response, which does not appear to be involved in termination of the viral infection (Shapiro et al, 1998).

It had been reported that hepatic fibrosis and HCV induced carcinogenesis may be due to an effect of TGF-β which has been implicated in both mediating fibrosis and down-regulating immune response (Thomas et al, 1999). Neuman et al (2002) reported that serum TGF-β reflects the progression of inflammation and degree of fibrosis in liver biopsies in HCV patients.

IFN-α treatment was shown to improve the histological assessment scores of inflammation and fibrosis, even in the absence of a sustained virological response. In untreated patients, fibrosis often progress, whereas it is often constant or even slightly reduced in post-treatment biopsies of non-responders and is often reduced in sustained responders (Sobesky et al, 1999). Ishimura et al (1996) reported that the absence of IFN-α receptor m-RNA in the liver was significantly associated with the severity of liver fibrosis. These results indicated that IFN-receptor expression decreases with the histological progress of the disease, suggesting that lower expression of IFN-receptor m-RNA may be partially responsible for the poor IFN-α response in liver cirrhosis. Tsushima et al (1999) reported that the effect of IFN-α on fibrosis may result from inhibition of TGF-β production.

In this study the capacity of PBMCs to produce TGF-β after 48 hours stimulation with Con-A was examined. There was no significant difference in TGF-β level in unstimulated and stimulated cell cultures but there was a significant difference in the level of TGF-β in the responders and non-responders to IFN-α therapy (Figure 9).These data indicated that the production of TGF-β in

the non-responders may be at least partially due to the higher inherent capacity of mononuclear cells in the non-responders to produce TGF-β. These results are in accordence with that reported by Nelson et al (1997) who described that the responsiveness to IFN-α therapy in HCV patients correlates with lower serum TGF-β levels. These results may also be explained by the presence of higher number of $CD25^+$ which has been recently described to be either activated cells or regulatory cells (Treg) inhibiting T cell response by the production of inhibitory cytokines such as TGF-β.

The relationship between the level of TGF-β production and viral serotype was then analysed. The high TGF-β production was found in patients infected with HCV serotype 1 whereas serotype 4 was associated with low TGF-β production. The differences were statistically significant.(Figure 10).

Natural killer (NK) cells play an important role in the early innate host defense against a number of pathogens. NK cell production of IFN-γ and / or recognition of virally infected cells are believed to play important roles in the innate immune response to virus infections. These functions are an important aspect of the immediate response of the host to virus and are believed to control the initial virus infection so that levels of virus are not too high or overwhelming for when the adaptive immune response develops. The production of IFN-γ by NK cells not only directly inhibits virus replication but more importantly, can also skew the Th cell response to a Th1 profile. NK cell function has been shown to be decreased significantly in chronic HCV patients, compared with non-infected individuals, even though the numbers of circulating NK cells were comparable between these two groups of individuals (Corado et al, 1997).

Recently, Tseng and Klimpel (2002) described that the major HCV envelope protein E2 can inhibit, via cross-linking CD81, NK cell production of IFN-γ and the non-major histocompatibility complex (MHC)-restricted cytotoxic activity of these cells. This could have a potentially important impact on the initial host response, the innate immune response, to HCV infection and on the kinetics and magnitude of the T cell B cell immune responses that later develop. The main

effect of IFN-α, may include inhibition of HCV virion production by an effect on viral RNA and protein synthesis and enhancement of immune lysis of HCV infected cells. Both amplification of NK and CTL activity had also been mentioned (Thomas et al, 1999).

These data suggested the presence of a positive correlation between NK cell activity, represented as percentage of cytotoxicity as well as number of lytic cells, and a response to IFN-α therapy (Table 28, Figure 8). On the other hand, infection with the more pathogenic genotype (genotype1) correlates with lower NK cell activity in the studied group of patients (Table 29). These findings suggest that chronic hepatitis C infection may affect the effector function of NK cells and that a more vigorous (possibly genetically determined) cell mediated immunity is correlating with beneficial effects of IFN-α. Furthermore, NK activity appears to be a valid parameter of disease progression and theraputic effect of IFN-α.

Taken together these findings suggest that the higher production of TGF-β as well as the lower NK cell activity support the notion of altered immunoregulation in the non-responders HCV patients. Also, the over representation of HCV serotype 1 in the non-responders to IFN-α support the hypothesis that the viral genotype may be responsible for the observed difference in response to IFN-α treatment.

CONCLUSIONS

CONCLUSIONS

CHAPTER 6: CONCLUSIONS

In this work 117 chronic hepatitis C patients were recruited to study the parameters of immunodisregulation in relation to the disease progression and responsiveness to IFN-α therapy. These parameters included the presence and the level of organ specific and non-specific autoantibodies and their relationship to the following findings in the same group of patients:

1. Histological characteristics of the liver damage.
2. Host immune response to hepatitis C in particular presence and level of IgM production.
3. Response to IFN-α therapy.
4. Viraemia detected by qualitative PCR.
5. Hepatitis C virus serotype (genotype).

The study showed that the prevalence of autoantibodies is common, and usually at low titers with significant presentation of anticardiolipin (42%), antismooth muscle (57%), antimitochondrial (40%) and anti-thyroid peroxidase (31%) antibodies in the patients group compared with the control group of healthy individuals (0-20%). The presence and level of these autoantibodies were not associated with clinically relevant pathological manifestations.
The number of different autoantibodies seen was directly associated with hepatic injury assessed by Knodell's Score of liver biopsy.

In 67% of the patients diagnosed with chronic hepatitis C, anti HCV-core IgM was detected. The presence of HCV-core IgM antibodies correlated with viraemia, as evaluated by qualitative HCV-PCR. Production of multiple autoantibodies as well as increasing liver cell damage (evaluated by Knodell's Score of liver biopsy) was observed in the anti HCV-core IgM reactive subgroup of hepatitis C patients. There was also a positive association between the presence of HCV-core IgM antibodies and antimitochondrial antibodies in the studied group of patients ($p=0.003$). Further, the presence of HCV-core IgM

antibodies was in a positive association with non-responsiveness to IFN-α therapy.

The above data suggest that both HCV-core IgM antibodies and autoantibody production may be indicators of continuous viral replication and that HCV-core IgM may act as a simple serological marker of viral replication as well as a predictive parameter of progressive liver cell damage. Furthermore, the production of antimitochondrial antibodies as well as anti-thyroid peroxidase antibodies and anticardiolipin antibodies correlated with non-responsiveness to IFN-alpha therapy.

In the study group, HCV genotype 4 was the most dominant (60%) genotype followed by genotype 1(20%) whilst the other genotypes (3,5 and 2) form the last 20% of the study group. After six months of IFN-α therapy, genotype 4 patients showed about 50% response rate to treatment and again significant number of the non-responders in genotype 4 group were reactive for core IgM antibodies before starting IFN-α therapy, while 86% of the patients having genotype 1 were non-responders.

Phenotypic analysis of the immunocomptent cells, detection of lymphocyte activation markers ($CD25^+$), levels of cytokines production by PBMCs and cytotoxic activity of NK cells were studied in the treated group of patients six months after starting IFN-α therapy. Significantly higher percentage of $CD25^+$ cells was observed in the non-responders compared with the responder group, perhaps due to continuous viral stimulation.

The cytokine profile seen in PBMCs in chronic hepatitis C patients 6 months after IFN-α therapy showed significantly higher Th1 cytokine (IFN-γ) in the non-responders compared with the responders group of patients. This may reflect strong HCV specific T cell response within the liver cells of the responders and contradict the assumption that non-responsiveness to IFN-α therapy reflects a lack of Th1 cytokine production and / or a shift to Th2 cytokine production.

TGF-β production was higher in the non-responders compared with the responders to IFN-α therapy. This may explain the progression of inflammation and degree of fibrosis in the liver biopsy in the non-responders. Also, non-responsiveness to IFN-α correlated significantly with a reduction in the cytotoxic activity of the natural killer cells (NK cells).

These data suggest that chronic hepatitis C infection affects the effector function NK cells and that vigorous cell mediated immunity is correlated with beneficial effects of IFN-α therapy and suggest that NK cell activity appears to be a valid parameter of disease progression and the therapeutic effect of IFN-α.

To summarize:

1. Autoantibody production is common in chronic hepatitis C and the presence of these autoantibodies may participate in hepatic injury.
2. Both HCV-core IgM and autoantibody production may be indicators of continuing viral replication.
3. HCV-core IgM may act as a simple serological marker of viral replication as well as a predictive parameter of progressive liver cell damage
4. IFN-α responsiveness rate is directly related to NK cell activity, lower TGF-β production and HCV non-1 genotype viral infection.
5. Higher IFN-γ production and higher number of $CD25^{+}$ cells in non-responders to IFN-α therapy further suggest immunodisregulation in hepatitis C virus infection.

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REFERENCES

CHAPTER 7: REFERENCES

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SUMMARY

SUMMARY

CHAPTER 8: SUMMARY

8.1 Introduction and aim of the work:

Viral infections are considered a possible trigger of autoimmune diseases. Hepatitis C infection has been associated with multiple autoimmune manifestations. There is cumulative evidence that some autoimmune phenomena, including the presence of autoantibodies, may be observed in a significant proportion of the patients suffering from chronic hepatitis C infection.

However, there are conflicting reports regarding the frequency of these findings in the patients in Mediterranean Countries (Italy, Spain and Egypt) and in Northern and Western Europe. Given the frequency of chronic hepatitis C patients in the UAE hospitals, we plan to analyse the presence of a panel of autoantibodies including: anticardiolipin, antismooth muscle, antimitochondrial, antinuclear, anti-thyroid peroxidase, anti-thyroglobulin, anti-liver kidney microsomal and antineutrophil cytoplasmic antibodies by using ELISA and /or immunofluorescence techniques.

The presence and the level of antibodies have been correlated with the following findings in the same group of patients:

- Histological characteristics of the liver damage.
- Host immune response to hepatitis C in particular presence and level of HCV-core IgM antibodies.
- The response of patients to interferon-α (IFN-α) therapy.
- Viral load determined by qualitative PCR..
- Hepatitis C virus serotype (genotype).

IFN-α has become the standard therapy for chronic hepatitis C, inducing biochemical (persistent normalization of alanine transferase) and virological (sustained negativity for HCV-RNA) response in about 15-25 % of treated patients. IFN-α is both an antiviral agent and an up regulator of the cellular immune system. The later effect is non-specific. Thus, IFN-α diffusely activates

the cellular immune system and can initiate new autoimmune diseases in patients treated with it.

The relation between the cellular immunity, the clinical course of viral infection and the response of HCV infection to IFN-α has been studied. This includes the followings:

- Phenotyping of immunocompetent cells including the quantitation of the percentage of mature leukocytes subset ($CD4^+$ T cells, $CD8^+$ T cells) in the PBMCs of the patients and the percentage and absolute number of activated lymphocytes ($CD25^+$).
- Cytokine synthesis by unstimulated and mitogen (Con-A) stimulated PBMCs using ELISA technique. This includes the assay of the Th1 cytokine, IFN-γ; Th2 cytokine, IL4 and the down-regulatory cytokine, TGF-β1.
- Cytotoxicity assay for NK cells activity using MTT based cytotoxicity assay employing Fen cells as targets for NK cytotoxic activity.

8.2 The results:

The results of this work can be summarized as followings:

8.2.1 Autoantibody production in chronic hepatitis C patients:

Sera from 117 patients with chronic hepatitis C were examined for the presence of the previously mentioned panel of autoantibodies. For comparison, a control group of 20 clinically healthy individuals were recruited into the study. In the study group of patients, the prevalence of autoantibodies is fairly common, usually at low titer but with a significant presence of anticardiolipin (42%), antismooth muscles (57%), antimitochondrial (40%) and anti-thyroid peroxidase (31%) antibodies in the patients group compared with the control individuals (all less than 20%). ($p= 0.0007$, 0.0018, 0.0067 and 0.013 respectively).

The relation between the presence of different non-organ specific autoantibodies and the histopathological status of the liver in the patients group was analysed.

We observed that the number of non-organ specific autoantibodies was directly related to hepatic injury, as evaluated by the Knodell's Score of the liver biopsy ($p = 0.0001$).

The clinical features of anticardiolipin antibody positive versus negative patients were analysed in chronic hepatitis C patients. There was no significant difference between the two groups regarding the degree of liver cell injury and platelet count although there was a tendency for production of antismooth muscle antibodies in anticardiolipin positive patients. No significant difference was found between HCV-PCR positive and negative groups regarding the total spectrum of autoantibody production.

8.2.2 Virus specific IgM response and parameters of disease progression in hepatitis C patients:

Sera from 115 HCV-patients were tested for the presence of HCV specific IgM and the frequency of autoantibodies in both the IgM positive and negative groups of patients was analysed. Anti HCV-core IgM was detected in 67% of the patients diagnosed as chronic hepatitis C. The production of non-organ specific autoantibodies correlates with the positivety for core-IgM negative patients ($p = 0.018$). A positive association between the presence of HCV-core IgM antibodies and antimitochondrial antibodies was also observed ($p=0.003$).

The presence of HCV-core IgM antibodies correlated with viraemia evaluated by qualitative HCV-PCR ($p=0.004$). Also, the presence of HCV-core IgM correlated with the severity of hepatitis. The level of HCV-core IgM was significantly higher ($p=0.005$) in patients with moderate to severe hepatitis when compared with mild form of HCV induced hepatitis.

8.2.3 Parameters of disease progression and response to IFN-α therapy:

Forty-one patients underwent successful liver biopsies. Out of these patients, 27 patients were positive for anti HCV-core IgM. Lack of detectable HCV-core IgM antibodies correlated with responsiveness to IFN-α therapy ($p=0.05$).

In the responders, a significant decrease in the level of HCV-core IgM antibodies was associated with the responsiveness to IFN-α therapy ($p=0.05$).

The presence of antimitochondrial ($p=0.008$), anti-thyroid peroxidase ($p=0.023$) and anticardiolipin ($p=0.008$) antibodies before treatment correlated with non-responsiveness to IFN-α therapy. The non-responder patients showed more severe liver damage, as evaluated by Knodell's Score of liver biopsy, compared with the responder group ($p=0.007$).

Six months after IFN-α treatment, a significant reduction in liver transaminases activity was observed in both the responders and the non-responders ($p<0.05$) although subsequent normalization of liver enzymes was only observed in the responder group. The presence of antibodies to variable antigenic regions from non-structural (NS4) protein of HCV types 1-6 was examined in a subgroup of patients (35 patients) who received IFN-α therapy. Genotype 4 is the most dominant genotype in our patients (60%). Genotype 1 was found in 20% of the patients and genotypes 2,3 and 5 in the remaining 20% of patients. Genotype 4 was accompanied with about 50% response rate to IFN-α therapy while 86% of genotype 1 group of patients was classified as non-responders. In genotype 4 patients, a strong correlation was observed between the presence of core IgM antibodies and non-responsiveness to IFN-α therapy ($p=0.014$).

8.2.4 Phenotypic analysis of immunocompetent cells, detection of activation markers and cytokine profile of chronic hepatitis C patients:

Six months after IFN-α therapy, the patients were grouped as responders or non-responders based on the previously mentioned criteria (see the introduction).
Phenotypic analysis of the PBMCs of the responders (n=18) and non-responders (n=9) was compared with that of the control group (20 individuals).

The results can be summarized as followings:

- No significant difference in the $CD4^+/ CD8^+$ ratios was found between the responders and non-responders. However, both groups showed significantly lower $CD4^+/ CD8^+$ ratios compared with control group ($p< 0.05$).
- The non-responsiveness to IFN-α was associated with higher production of the Th1 cytokine, IFN-γ ($p< 0.005$) and the down regulatory cytokine, TGF-β ($p< 0.05$) and higher IL2 receptor expression ($p< 0.05$).
- The responders showed significantly more NK cell cytotoxic activity compared with the control group.
- A positive correlation between serotype 4 and NK cell activity together with a negative correlation between serotype 1 and NK cell activity was also seen ($p<0.01$).

- Serotype 1 patients showed significantly higher level of TGF-β whilst in those infected with serotype 4, the level of TGF-β was significantly lower compared with other serotypes.

8.3 Conclusions:

- Autoantibody production is common in chronic hepatitis C and the presence of these autoantibodies may participate in hepatic injury.
- Both HCV-core IgM and autoantibody production may be indicators of continuing viral replication. HCV-core IgM may act as a simple serological marker of viral replication as well as a predictive parameter of progressive liver cell damage.
- IFN-α responsiveness rate was directly related to NK cell activity, lower TGF-β production and a non-1 genotype viral infection.
- Higher IFN-γ production and higher number of $CD25^+$ cells in non-responders to IFN-α therapy further suggest immunodisregulation in hepatitis C virus infection.