SCIENCE AND BIO-MEDICINE

Doctoral Thesis

CORRELATION BETWEEN LIVER HISTOPATHOLOGY AND HEPATITIS B VIRAL (HBV) ANTIGEN EXPRESSION IN HEPATOCYTES IN CHRONIC HBV INFECTION IN RELATION TO THE PHASE OF VIRAL INFECTION.

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1. Introduction

Chronic hepatitis B is a major cause of cirrhosis and liver cancer world wide despite the dramatic progress in controlling the spread of hepatitis B virus (HBV) by mass vaccination of newborns , the general improvement in hygiene and socioeconomic status and in the use of antiviral therapy . This progress has lead to rapid changes in the epidemiology of HBV infection . For example, in western countries the proportion of HBeAg positive chronic hepatitis B fell during recent years and the age of the patients has increased significantly. On the other hand, most of the patients detected to have HBV infection in the Asian regions , where childhood immunisation against HBV is not mandatory, are younger with positive HBeAg. Conversion from HBeAg positive status to negative status is usually synonymous with immunoclerance ; however HBV DNA is detectable in the serum of these person albeit in low titre suggesting low level of viral replication. During the phase of immunotolerance , the serum HBV DNA titre are high , but the disease activity in the liver is very low often with normal transaminase value. There are many reports in the literature underlying the importance of immunohistochemistry along with histopathology to evaluate the stage of chronic HBV infection which are not necessarily obtained on conventional serological assays of HBV antigen and by assessment of HBV DNA. Moreover the expression of HBV core (HBcAg) and surface (HBsAg) antigen in the hepatocytes varies according to the phase of infection. So in this study , the correlation between the liver disease activity (HAI) and the expression of HBcAg and HBsAg antigens in the hepatocytes of persons with chronic HBV infection in relation to the phase of viral replication was explored.

2. History

The first recorded case of “serum hepatitis “appear to be those that following the administration of smallpox vaccine containing human serum to shipyard workers in Bremen in 1833.(1) In the early and the middle part of the century , serum hepatitis was repeatedly observed after the use of contaminated needles and syringes, after plasma administration for immunoprophylaxis and transfusion of blood (2,3,4,5) One of the most important discoveries leading to the rapid advancement of the knowledge of the viral aetiology of serum hepatitis, its epidemiology and disease spectrum , occurred in 1965 when Blumberg and colleagues (6) fortuitously found an antigen in the serum of an Australian aborigine and was first named “Australian antigen.”(7). But it took several years of investigations to establish its eventual association with acute hepatitis (8,9,10) and it was then named hepatitis-associated antigen (HAA) and later given the current name , hepatitis B surface antigen(HBsAg)

The discovery of HBsAg and the recognition that it was a viral antigen lead to the appreciation that HBV has a worldwide distribution, and that infection in some parts of the world , such as parts of Asia, Africa, and Oceania, are extremely high(11). Serological testing provided direct evidence that many serum hepatitis cases were associated with HBV infection , that HBV infection could persists for many years, and that HBV was distinct from other virus associated infectious hepatitis cases like HAV and HCV. The name serum hepatitis use for many years indicated that the first recognised common route of HBV transmission was by percutaneous transfer of serum or blood or virus contaminated needles or by injected blood products. It is now clear that HBV is transmitted most commonly by routs that may not involve such direct or overt percutaneous transfer, that is, by sexual contact and by mother to newborn infant transmission

3. The virus

Hepatitis B virus is a member of hepadnavirus –heapa from hepatotrophic and dna because it is a DNA virus family,(12) discovered by Dane in 1970.(13) .It has a spherical form and the virion has a diameter of approximately 42nm. The outer layer or envelope approximately 7 nm in width that contains HBsAg proteins , glycoproteins , and cellular lipids. Enclosed by the envelope is an electron-dense 28nm diameter spherical internal core or nucleocapsid. (FIG 1)

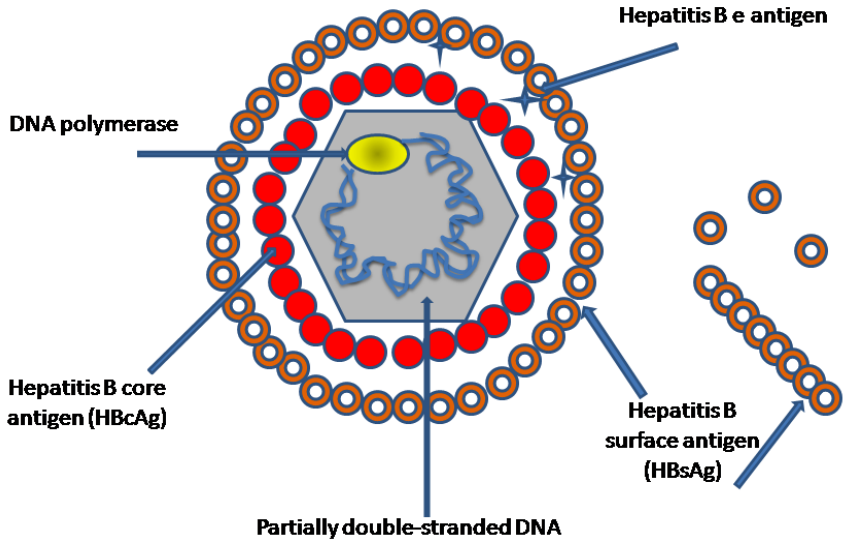


Fig 1. Hepatitis B virus.

HBV contains among the smallest genomes of all known animal viruses. It consists of an approximately 3200 base pair circular DNA molecules that contains a single stranded region of different length in different molecules reflecting the fact that DNA molecules are packaged into virions before viral

DNA replication is complete(14,15). The HBV genome has four long open-reading frames in the complete or long(minus) virion DNA strand.(16) The C (core or nucleocapsid) gene encode the major viral core or nucleocapsid polypeptide and when assembled into the core particles , it manifest hepatitis B core antigen (HBcAg) specificity. A truncated form of this protein with a mass of approximately 17,5000 dalton possesses hepatitis e antigen (HBeAg) specificity(17). This open- reading frame includes a short pre C (precore) sequence delineated by an in-frame initiation codon. In cells, translation is initiated at the first or pre-C initiation codon , suggesting that the pre C region act as a signal sequence. When the translation is initiated at the second or C initiation codon, the full length of C polypeptide is synthesized and assembled into core particles in the cell. In case of HBV mutants , which result in a stop codon in the pre C sequence , infect cells and replicates but do not express HBeAg (18,19) because the HBeAg precursor protein initiated at the first (pre-core) initiation codon can not be made, although HBcAg is expressed.

The S (surface or envelope) gene , including pre-S1, pre S-2 and S regions encodes the viral surface antigen(HBsAg) reactive polypeptide in the virion envelope and in the incomplete viral forms found in the liver and serum of the infected individuals (20,21).

The P (pol or polymerese) gene , which encompasses three fourths of the viral genome and envelopes a portion of C gene , the entire S- gene , a portion of X gene , encodes a basic polypeptide with DNA polymerase or reverse transcriptase activity and ribonuclease H activity. It also serves as a protein primer for DNA minus strand synthesis (22,23,24).

The small X gene encodes a polypeptide of 154 amino acids which can transactivate transcription regulated by HBV (25,26).

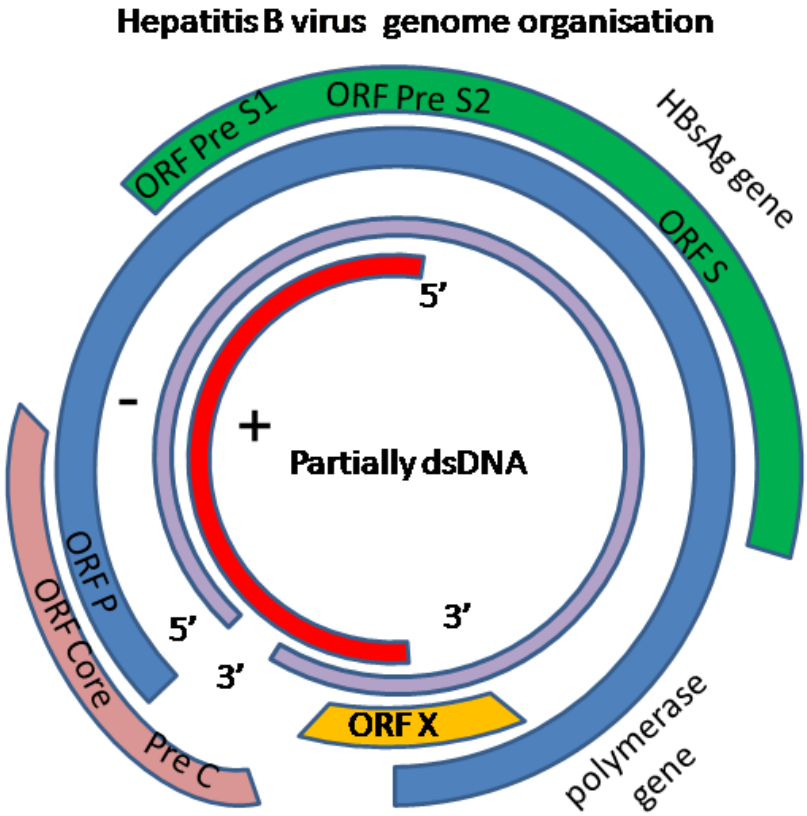


Fig 2. Hepatitis B virus genome.

The virion surface manifests HBsAg specificity , which is contained in the small , middle and large HBsAg proteins that are the principal protein components of the viral envelope (20,21). HBsAg proteins are not only components of the virion envelope but are also released from infected cells as components of small spherical particles . these are heterogeneous in size and appearance , diameter varying from 16 to25 nm and called 22nm particles. They are filamentous or rod-shaped particles and share antigenic determinants (HBsAg) with the surface of virions (12). No nucleic acid has been found in them and they are considered to be incomplete viral envelope particles.

HBsAg is a complex antigen, at least five antigenic specificities are regularly found on HBsAg particles situated in the S region of HBsAg proteins A group specific determinant a is shared by all HBsAg preparations : more than

one antigenic site appease to share this broad specificity , and thus each would be defined as part of the a determinant . Two pairs of subtype determinants have been found -d,y and w,r . and the four major subtypes of HBsAg have been designed adw , ayw , ayr and adr . Several other unusual combinations of HBsAg subtypes also have been reported (27) and are formed during mixed infections .Subtype determinants have been used as viral markers to follow spread of virus among individuals and in populations.

DNA sequence of HBV isolates has shown the existence of 8 viral genotypes A-H and these varies in geographic distribution.(Tab 1) Genotype A is found primarily in North America , Northern Europe , India and Africa. Genotype B and C are common in Asia ; genotype D in Southern Europe , the Middle East and India, genotype E ,in West Africa and South Africa ; genotype F , in South and Central America; genotype G, in United States and Europe (28) Genotype H was recently identified in individuals from Central America and California (29). In China and Japan , some studies have found more severe liver disease to be associated with genotype C compared with genotype B, (30) while other studies have found no such association (31,32) There is some evidence that shows HBeAg seroconversion occurs at a younger age among individuals infected with genotype B (30,32,33). Genotype D has been associated with anti-HBeAg-positive chronic hepatitis B infection in the Mediterranean region (34).

Several genotypes may be associated with the severity of the disease . These genotypes differ by their geographic distribution in populations around the globe. There is evidence that HBV genotypes also differ by their pathogenic properties, including their risk of persistence as chronic infection and their capacity to induce precursor disease or cancer. On the other hand, HBV genes may undergo mutations that become selected during the course of chronic infection and progressive liver disease. The most significant of these mutations in the context of HCC are those occurring in the pre-core (Pre-C) and basal core

promoter (BCP) regions. These mutations may up regulate HBV expression and increase its virulence. These mutations may occur in all HBV genotypes but are more common in genotypes associated with more severe disease and cancer, in particular genotype C (35).

Table 1.Genotype of HBV and Geographic Distribution

A	Africa, India, Northern Europe, United States
B	Asia, United States
C	Asia, United States
D	India, Middle East, Southern Europe, United States
E	West and South Africa
F	Central and South America
G	Europe, United States
H	Central and South America, California in United States

HBV has a reported mutation rate of 10 times greater compare with other DNA viruses. These mutations can occur naturally as well as due to selective pressure from antiviral therapy. There are five clinically relevant HBV types: wild-type HBV, precore mutants, core promoter mutants, tyrosine-methionine-aspartate-aspartate (YMDD) mutants induced by lamivudine treatment, and asparagine to threonine (rtN236T) mutants recently identified in patients with adefovir treatment. In a study carried out in the United States, the precore varia HBV has a reported mutation rate of 10 times greater compare with other DNA of HBV was rarely found in association with genotype A, but it was found in almost 50% of those with genotype C and in >70% of individuals with genotype D. Those with precore variant and core promoter mutations had higher HBV DNA levels in sera than those persons without these mutations. It is observed that flares in chronic HBV have been associated with increases in concentrations of precore mutation in proportion to wild-type HBV. Exacerbations have been thought to subside with time when the genetic

heterogeneity disappears and patients become exclusively infected with pre core HBV(36). During the treatment of chronic hepatitis B by lamivudine, drug resistance may develop, which is mediated by point mutations with the YMDD motif at the catalytic centre of the viral reverse transcriptase. With increase in the mutant viral load, patients can sustain further liver injury. The YMDD mutant level will decrease after stopping lamivudine. Viral mutation also occurs in patients on adefovir treatment during their second year therapy at the rate of 2.5%. The mutation has been reported as asparagine to threonine mutation (rtN236T), downstream of the YMDD motif.

3.1. Viral forms in the blood

The concentration of incomplete viral forms in the serum usually greatly exceed the concentration of complete virions or Dane particles. Filamentous HBsAg particles concentrations are commonly 100 fold lower and virion or Dane particles when present are found in even lower concentrations. HBcAg and core particles are not presenting the blood in a free form, but are present only as internal components of Dane particles. Very few Dane particles are necessary to infect a susceptible human .It is interesting to note that some HBsAg carriers circulate only incomplete HBsAg particles and not infectious virions (37,38).

3.2. Virus in cells and in Liver

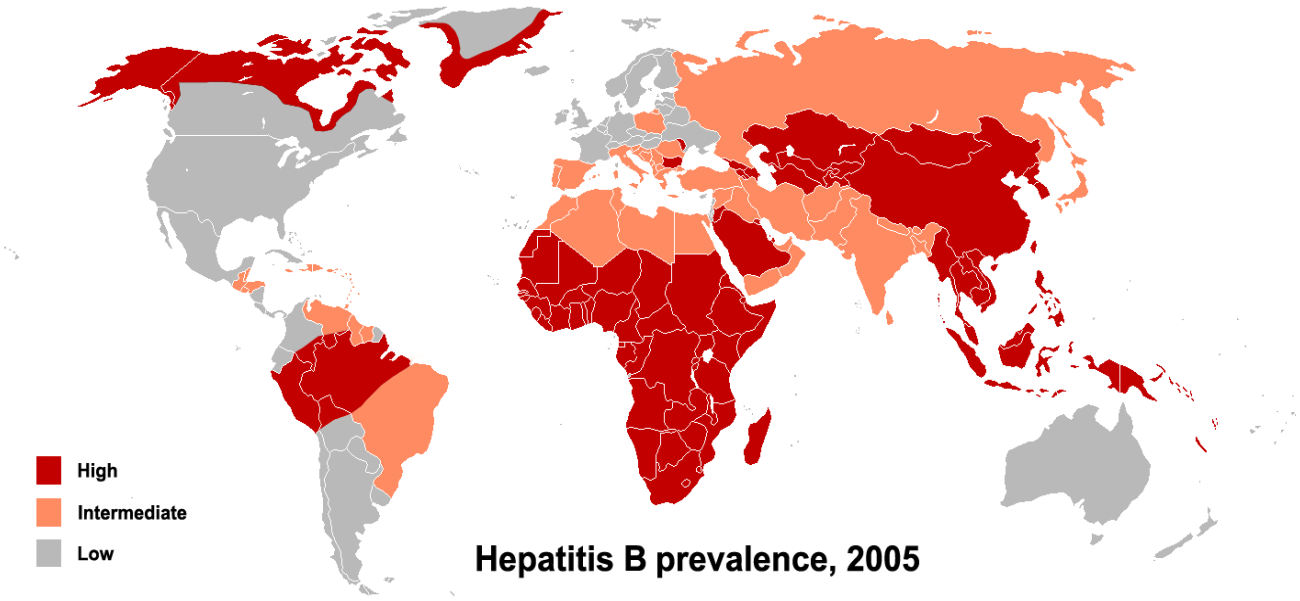
When hepadna virus infect and enter cells , the relaxed circular virion is converted to closed circular DNA (cccDNA), which serves as a template for viral transcription. The resulting transcripts include a longer than genome length RNA with a terminal redundancy and smaller transcripts that serves as mRNA

from which viral proteins are translated. The long transcript and pol gene product (reverse transcriptase) are encapsidated in core particles in which new viral DNA synthesis takes place by reverse transcription of the long transcript(39).Viral DNA replication by reverse transcription is a unique mechanism for DNA virus. HBcAg detected by immunofluorescent staining and core particles detected by electron microscopy have been found almost exclusively in hepatocyte nuclei of HBV infected liver (40, 41,42). HBsAg is detected by immunofluorescent staining as acytoplasmic antigen on cell surfaces (40,41,42).

During persistent HBV infection , a variable number of liver cells contain viral antigen detected by immunofluorescent staining , from less than one percent to virtually all hepatocytes, in different patients (40,42 ,43). The pattern of viral antigen expression appears to be different in different cells of the same chronically infected liver .Commonly, most positive cells stain for HBsAg; fewer have only detectable HBcAg; and even fewer cells contain both HBsAg and HBcAg. At least some cells expressing only HBsAg may contain only integrated viral DNA. In the liver of some chronic carriers HBsAg is the only detectable viral antigen. In such carriers with no detectable virions or HBeAg in serum , liver cells with replicating virus and expressing HBsAg , may have been eliminated by a cytopathic effect of replicating virus or by an immune response directed at HBcAg ; HBsAg may be expressed in remaining infected cells with only integrated virus. In all chronic carriers producing relatively high concentration of virions and HBeAg, significant number of HBcAg positive cells can be found in the liver. The different patterns of viral antigen synthesis in individual cells of the same chronically infected liver indicate that individual viral genes are expressed differently in different cells.

4. Epidemiology and public health burden.

Approximately one third of the world's population has serological evidence of past or present infection with HBV and 400 million people are chronically infected and contributes to an estimated one million death worldwide each year. It has been estimated by the Centres for Disease Control and Prevention (CDC) in 2007 that there are approximately 43,000 new HBV infection per year in USA



High >8%: Intermediate2-7%: Low<2%.

Fig. 3. Hepatitis B prevalence in the world as of 2005

However, the official number of reported hepatitis B cases are much lower. Many people don't know they are infected or may not have symptoms and therefore never seek the attention of medical or public health officials. In the United States, an estimated 800,000 to 1.4 million persons have chronic hepatitis B viral infection .Most are in young adults and approximately one –fourth are associated with acute icteric disease. More than 100,000 patients are

hospitalized with hepatitis B each year , and 300 die with fulminant hepatitis B. Between 6 -10% fail to resolve primary infection and become HBsAg carriers. The life time risk of HBV infection is estimated to be 2% for the whole US population , but for certain high risk group it can reach up to 100%. The primary method of transmission reflects the prevalence of chronic HBV infection in a given area. In low prevalence areas such as USA and Western Europe , where less than 2% of population is chronically infected , injection drug abuse and unprotected sex are the primary methods , although other factors may be important (44). In moderate prevalence areas which includes eastern Europe ; Russia and Japan 2-7% of population is chronically infected , the disease is predominantly spread among children. In high prevalence are a such as China and South East Asia , transmission during child birth is most common, although in other areas of high endemicity such as Africa , transmission during childhood is a significant factor(45). The prevalence of chronic HBV infection in areas of high endemicity is at least 8%.HBV infection is diverse and variable , ranging from a low viremic inactive carrier state to progressive chronic hepatitis which may evolve to cirrhosis and hepatocellular carcinoma (HCC) HBV end stage liver disease or HCC are responsible for over I million deaths per year and currently present 5-10% of cases of liver transplantation (46,47,48,49). Host and viral factors can affects the natural course of HBV infection as well as the efficiency of antiviral strategies.

Chronic HBV may present either as hepatitis B e antigen (HBeAg) positive or HBeAg negative CHB. HBeAg positive CHB is due to the so called “wild type” HBV: It typically represents the early phase of chronic HBV infection. HBeAg negative CHB is due to the replication of naturally occurring HBV variants wit h nucleotide substitutions in the pre core and /or basic core promoter regions of the genome and presents a later phase of chronic HBV infection. The prevalence of HBeAg negative form of disease has been

increasing over the last decade as a result of HBV- infected population aging and represents the majority of cases in many areas, including Europe (50,51).

Morbidity and mortality in CHB are linked to persistence of viral replication and evolution to cirrhosis or HCC. Longitudinal studies of patients with CHB indicates that , after diagnosis , the 5 year cumulative incidence of developing cirrhosis ranges from 8 to 20%. The 5 year cumulative incidence of hepatic decompensation is approximately 20%. With the 5-year probability of survival being approximately 80-86% in patients with compensated cirrhosis (48,52,53). The patients with a decompensated cirrhosis have a poor prognosis with a 14-35% probability of survival at 5 years. The world wide incidence of HCC has increased, mostly due to HBV and HCV infections; presently it constitute the fifth most common cancer , representing around 5% of all cancers. The annual incidence of HBV related HCC in patients with CHB is high ranging from 2% to 5% when cirrhosis is established(53.)However the incidence of HBV related HCC appears to vary geographically and correlates with the underlying liver disease.

Population movements and migration are currently changing the prevalence and incidence of the disease in several low endemicity countries in Europe and elsewhere. Substantial healthcare resources will be required for control of the worldwide burden of disease (54).

5. The course of the Hepatitis B viral infection.

Studies of natural HBV infections in humans and in experimental infections in humans and chimpanzees have defined several patterns of infections with HBV virus .Most primary infections in adults are self- limited and completely resolve within 6 months of onset. Most infections also appear to be sub-clinical and are detected only by serological testing and other methods. A fraction of infection fail to resolve , become persistent and may continue for many years. A unique feature of infection with HBV is the presence of viral forms continuously in the blood during active infection almost in all patients. These forms are most commonly detected by their antigenicity. Tests for HBsAg essentially detect the incomplete viral forms, the 22nm particles and filaments, which greatly outnumber complete virions in most patients. Although most persistently infected patients appear to have complete infectious virus in the blood as well as incomplete viral forms , some of these patients appear to have no infectious virus. The presence of virus in the blood that can be detected by the serological testing and other methods , and the immune response to the viral antigens offer markers that can be used to follow the course of HBV infection. Several patterns of infection that define the spectrum of responses of this virus have been described.

5.1.Self- limited HBsAg – positive primary infection.

This pattern , in which HBsAg can be detected transiently in the blood, is the most common pattern of primary HBV infection in adults. HBsAg is the first viral marker to appear in the blood after HBV infection (Fig 5). The

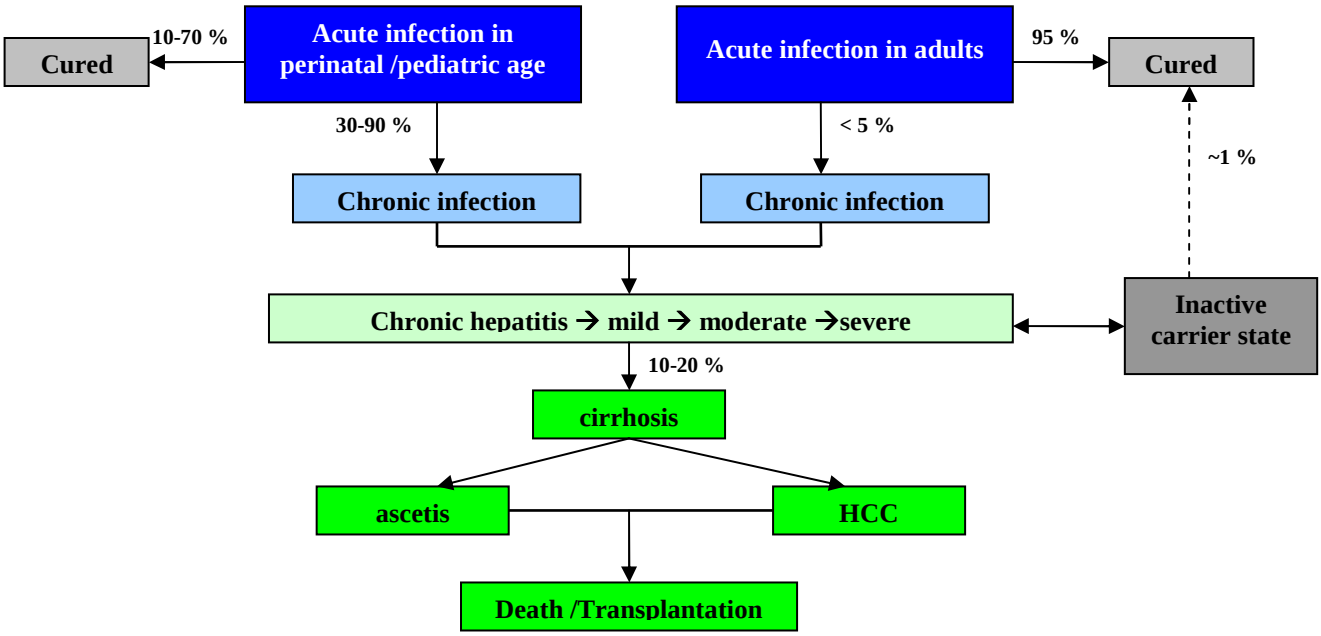


Fig 4.Clinical outcome of HBV infection acquired during perinatal /adult stage of life.

(Vigano M.Personal communication.Agorà in HBV 2009).

presence of this antigen is considered to be synonymous with active infection. HBsAg can be detected as early as first or second weeks ,and as late as 11 or 12 weeks (55,56) after exposure to HBV. Evidence of hepatitis are was found to follow the appearance of HBsAg by an average of 4 weeks (usual range 1-7 weeks)and after al least 3-6 weeks(56) in different studies. In self-limited infections, HBsAg was found to remain detectable in the blood for 1-6 weeks in most patients , although it may persists for up to 20 weeks(56).

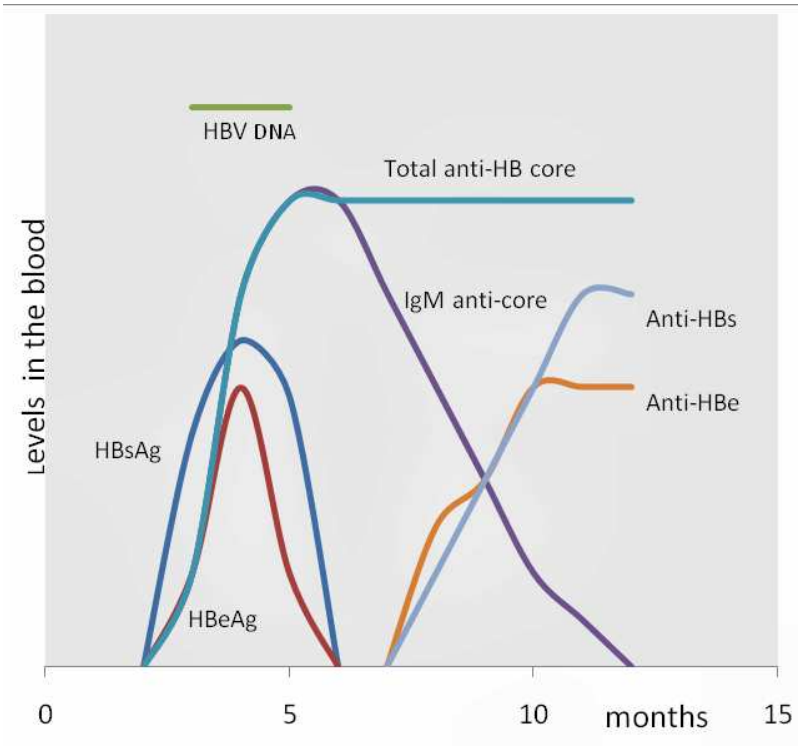


Fig 5. HBV antigen and antibodies detectable in the blood following acute infection

(http://en.wikipedia.org/wiki/Hepatitis_)

Patients who remain HBsAg positive for less than 7 weeks rarely appear to develop symptomatic hepatitis (56). The severity of hepatitis as measured by bilirubin elevations has been roughly correlated with the duration of HBsAg positivity in patients with self-limited experimental infections. As symptoms and jaundice clear , the HBsAg titre falls and HBsAg become undetectable in most symptomatic patients several weeks after the resolution of hepatitis. However, in experimental transmission studies, 9 percent of patients had become HBsAg negative even before the onset of symptoms , and 28% were negative by the time symptoms has resolved (56).

HBeAg is another regular and early marker of HBV infection . Higly sensitive assays have demonstrated that HBeAg appears simultaneously or within a few days of the appearance of HBsAg in all or almost all primary infections, and its titre peaks and then declines in parallel with HBsAg

(57,58,59). The prevalence of HBeAg declines constantly over the first 10 weeks after the onset of symptoms. (59). HBeAg disappears just before the disappearance of HBsAg in self limited infections. Patients who remain HBeAg positive for 10 weeks or longer appear likely to become persistently infected (58). Anti HBeAg appear in most patients at the time HBeAg becomes undetectable or shortly thereafter. Anti HBeAg persists for 1-2 years resolution of HBV infection. (55).

The third viral marker in order of appearance is DNA and DNA polymerase-containg virions. These particles detected by their DNA polymerase activity , appear in the blood of most patients soon after the appearance of HBsAg. They rise to high concentrations during the late incubation period of HBV , and fall with the onset of hepatic disease (60).

A fourth marker of infection that appears in virtually all patients and before the onset of hepatic injury in most , is Anti- HBc the antibody directed against the internal antigen of virions. Anti HBc can usually be detected 3-5 weeks after the appearance of HBsAg.in the blood and before the onset of clinically apparent hepatitis (55,56, 61). Anti-HBc titres rise during the period of HBsAg positivity , level off, and eventually fall after HBsAg become undetectable. The highest titre of anti HBc appear in the patients with the longest period of HBsAg positivity.(61). Anti-HBc titre fall three-to fourfold in the first year after infection , and then drop more slowly (62). Anti HBc can still be detected by immunoelectroosmophoresis 5-6 years after acute infection in most patients (55, 62). The high correlation between the prevalence of Anti-HBc and Anti-HBs detected , indicates that these two antibodies persists for a similar time after acute self-limited infections.

Although most of the Anti-HBc activity is in the IgG class, IgM anti-HBc has been found in almost all patients with acute hepatitis B.(63). Anti-HBc IgM was found to decline rapidly after disappearance of HBsAg only in 40% of

cases with self- limited acute hepatitis B ; in the remainder , the decline was slow, with 20% still being positive after 2 years (63).

Antibody to HBsAg (AntiHBs) has been shown to appear before the onset of clinically apparent hepatitis in 10-20% of patients . In most patients with self limited HBV infection , however, Anti-HBs can be detected only after HBsAg disappears from the blood(55,56,61). Anti HBs can not be detected in in many patients immediately after HBsAg disappears. There is a time interval of up to several months between the disappearance of detectabile HBsAg and the appearance of anti HBS in approximately one half of patients with self- limited infections (56). In approximately 10% of patients with transient antigenemia, anti HBs never appears . In patients with measurable anti HBs response , the antibody titre rises slowly during recovery and may still be rising 6-12 months after disappearance of HBsAg (56). In contrast to the Anti- HBc response , the highest titres of anti- HBs appear in those patients with the shortest period of antigenemia. The antibody may persists for years and after HBV infection and is associated with protection against reinfection (64).

In contrast to the extensive studies of viral markers in the blood early in the course of primary HBV infection , only few investigators have examined the state of virus in cells in liver at this early time. During the late incubation period and early during the acute disease , almost all hepatocytes have been reported to be positive for HBsAg and HBcAg (41,42). During the acute hepatitis B , the state of viral DNA in the liver has not been adequately studied , but the forms of virus in the blood and antigen expression in the liver suggests that most of the hepatocytes are replicating complete virions during early stage of primary infection.

5.1. HBsAg positive persistent HBV infection

Patients who remain HBsAg positive for 20 weeks or longer after primary infection are very likely to remain positive indefinitely and be designed

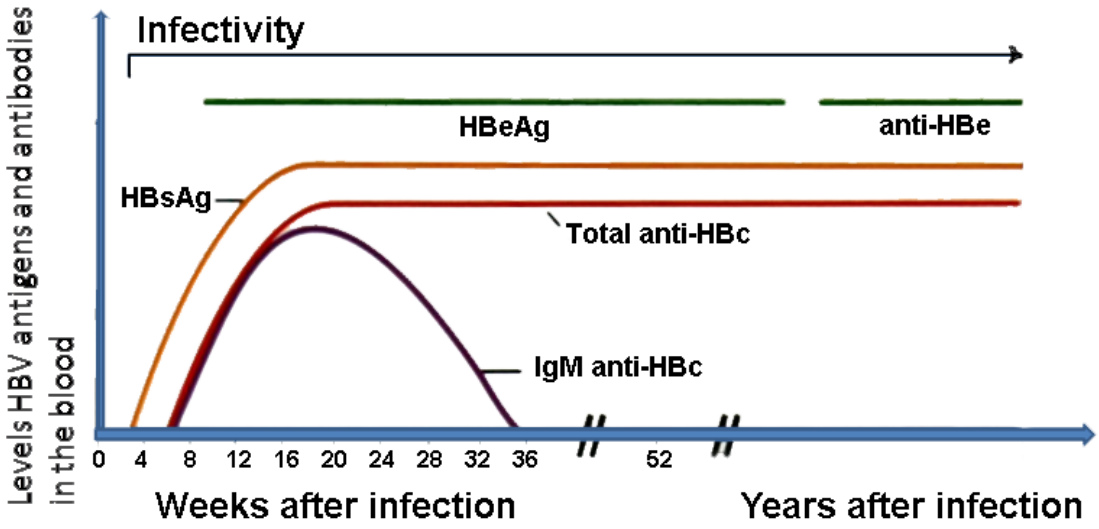


Fig 6. HBV antigen and antibodies detectable in the blood following chronic infection

http://en.wikipedia.org/wiki/Hepatitis

chronic HBsAg carriers. Dane particles, DNA polymerase activity , HBcAg can be detected in the blood of a significant fraction of persistently infected patients positive for HBsAg. All patients with detectable virion DNA polymerase in the blood have HBcAg in the liver biopsies (65) and all patients with HBcAg in the liver have detectable virions in the serum (66). Almost all persistently infected patients have high titre of anti-HBc in the blood. The titre of this antibody are significantly higher during persistent than during most self limited infections or in convalescence. Although most of the antiHBc is undoubtedly in the IgG

fraction , interestingly the IgM anti HBc continues to be made and can be detected indefinitely in the serum of most persistently infected patients (63).Very rarely , HBV carriers fail to produce antibody to HBcAg , which is thought to be on the basis of a selective defect in the immune response.

HBeAg can be detected in the serum of almost all patients early in the primary HBV infection, and anti HBeAg appear in almost all during the resolution of the infection. In persistent infection, on the other hand , HBeAg is present in one fourth to one half of patients , and and antiHBe in almost all the remainder (57). There is a high correlation between the presence of serum HBeAg and virions . There is a wide range of HBsAg titres in persistently infected patients. In general, those with the highest titre of infectious virus and detectable virion DNA polymerase activity and /or HBeAg have the highest HBsAg titre.

Although most HBsAg carriers have high titres of infectious HBV in serum , some carriers appear to have no detectable infectious virus. The highest titres are found in patients with detectable HBeAg (37,38) and /or virion DNA polymerese activity in serum, and those without these markers or with anti-HBe have much lower titres. Patients with detectable viron polymerase and HBeAg in their serum appear to be highly contagious (67,68) which is in agreement with the data on titres of infectious HBV in blood. A few HBsAg carriers with HBeAg and DNA and DNA polymerese-containing virions in the blood have been shown to have free replicating forms of viral DNA probably integrated viral DNA in liver providing biochemical evidence that complete viral replication is proceeding alt least in some cells (43, 69). A variable number of liver cells in such patients appear to contain HBsAg and/or HBcAg . Such patients frequently but not always have chronic persistent or chronic active hepatitis.(70,71).

Investigations of a few HBsAg carriers with no detectable DNA and polymerese containing virions or HBeAg in the blood has revealed evidence of

integrated viral DNA sequences , but no detectable free viral DNA forms HBcAg in liver cells (43, 69). A small fraction of hepatocytes shows the presence of HBsAg. These findings and the infectivity studies indicate that these patients are replicating complete at a very low level or not at all, and that the only viral gene expressed appears to be the HBsAg gene in an integrated state. Many , but not all these patients appear to have little or no liver disease (70) and are considered to be “healthy carriers”.

The long term natural history of persistent HBV infection is not completely defined and prolonged infection appears to be the rule for most chronic carriers , and HBsAg positivity lasting as long as 20 years has been documented (71). The titre of HBsAg and virion DNA polymerese have been shown to be relatively stable over a time period of weeks or a few months (65). However evidence suggest that persistent HBV infection tend to spontaneously wind down over a period of many months to years, with HBsAg titre slowly falling and and virion DNA polymerese and HBe titres falling below the level of detection. Spontaneous disappearance of HBeAg up to 10 percent and of patients per year and up to 45 percent over 2-7 years have been reported (72).

Anti HBe can eventually been detected in most patients after they become HBeAg negative. In HBsAg carriers without virion DNA polymmerese activity or HBeAg in serum , these markers have frequently been observed to reappear spontaneously (73). Although the time after onset of infection when HBeAg and virion level fall below the level of detection appears to vary greatly and these markers may remain detectable in persistently infected patients for years , the duration of infection is clearly and important factor that is strongly correlated with the presence of detectable HBcAg and virions. Spontaneous clearance of all viral markers of active HBV infection , although unusual , can occur at any time. Spontaneous disappearance of HBsAg in persistent infected patients have been reported (70,74). A number of factors that appear to

influence the prevalence of persistent infection such as age, sex, immunological status, and possibly race , may also influence rate of spontaneous remission. Some evidence suggest that persistent infections resolve at a faster rate in females than in males (75).

5.2. HBsAg -Negative persistent HBV infection

There is good evidence that some patients with persistent HBV infection do not have detectable HBsAg in the serum. Also cases of chronic hepatitis with out detectable HBsAg have been ascribed to active HBV infection because of persistent high titres of Anti- HBc (76).

6. Natural history of of chronic hepatitis B

Chronic hepatitis B is a dynamic process The natural history of chronic hepatitis B can be schematically divided into five phases , which can not be necessarily sequential.

- The “immune tolerant phase” is characterized by HBeAg positivity high level of HBV replication (high level of serum HBV DNA levels) , normal or low levels of aminotransferases ,mild or no liver necroinflammation and no or slow progression of fibrosis (47,49). During this phase , the rate of spontaneous HBeAg loss is very low. This first phase is more frequent and more prolonged in subjects infected perinatally or in the first year of life. Because of high level of viremia, these patients are highly contagious

- The “immune reactive phase” is characterized by HBeAg positivity , a lower level of replication (lower serum HBV DNA levels) increasing or fluctuating level of aminotransferases , moderate or severe liver

necroinflammation and more rapid progression of fibrosis compared to the previous phase(47,49). It may last for several weeks to several years. In addition, the rate of spontaneous HBeAg loss is enhanced. This phase may occur after several years of immune tolerance and is more recently reached in subjects infected during adulthood

- The "inactive HBV carrier state" may follow serologic conversion from HBeAg to anti HBe antibodies. It characterized by very low or undetectable serum HBV DNA levels and normal aminotranferases. As a result of immunological control of the infection , this state confers a favourable long term outcome with a very low risk of cirrhosis or HCC in the majority of patients. HBsAg loss and seroconversion to anti HBs antibody may occur spontaneously in 1-3% of cases per year , usually after several years with persistently undetectable HBV DNA (77).

- "HBeAg negative CHB" may follow seroconversion from HBeAg to anti HBeAg antibodies during the immune reactive phase and represents a later phase in the natural history of CHB. It is characterized by reactivation with a pattern of fluctuating levels of HBV DNA and aminotransferases and active hepatitis. These patients are HBeAg negative , and harbour HBV variants with nucleotide substitutions in the pre core and /or the basal core promoter regions unable to express or expressing low level of HBeAg . HBeAg negative CHB is associated with low rates of prolonged spontaneous disease remission. It is important and sometimes difficult to distinguish true inactive HBV carriers from patients with active HBeAg – negative CHB in whom phases of spontaneous remission may occur The former patients have a good prognosis with a very low risk of complications, while the latter patients have active liver disease with a very high risk of progression to advanced hepatic fibrosis , cirrhosis and

subsequent complications such as decompensated cirrhosis and HCC. A careful assessment of the patient is needed and a minimal follow up of one year is need with serum Alanine Aminotransferase (ALT) and HBV DNA level every 3 months usually allows detection of fluctuations of activity in patients with active HBeAg negative CHB (78).

- In the "HBsAg negative phase" after HBsAg loss , low level HBV replication may persists with detectable HBV DNA in the liver. (79ES). Generally, HBV DNA is not detectable in the serum while anti HBc antibodies with or with out anti HBs are detectable. HBsAg loss is associated with improvement of the outcome with reduced risk of cirrhosis, decompensation and HCC. The clinical relevance of occult HBV infection (detectable HBV DNA in the liver with low level -<200IU/ml- HBV DNA in blood) is unclear(79). Immunosopression may lead to reactivation in these patients(80,81).

7. Changing aspects of HBeAg- posive and Negative HBV

The vaccination against HBV , the general improvement in hygiene and socioeconomic status and the availability of effective therapy had led to the rapid changes in the epidemiology of HBV infection that have a significant impact on liver disease. For example, in industrialized countries the proportion HBeAg positive chronic hepatitis B fell during recent years and the age of patients has increased significantly (82). Most of the patients detected to have HBV infection in the developing countries are younger with positive HBeAg (83) where childhood immunisation against HBV is not mandatory and still continues to be a major problem (84). The highest disease burden for HBV infection is in the Asian and Sub Saharan African regions. In these areas , HBV

infection is acquired in the childhood itself through vertical transmission and there is a high rate of chronicity(85).

7.1. HBeAg positive chronic hepatitis

Clinical data indicate that HBV infection acquired in the perinatal period is characterized by a prolonged immunotolerant phase and very low rate of spontaneous HBeAg clearance (86). Most carriers infected at birth or in the first few years of life present with HBeAg positive chronic hepatitis with normal serum aminotransferases and this clinical condition is likely to be maintained up to adulthood by a consistent proportion of patients (87,88). Many of these patients enter the immunoactive phase and develop HBeAg positive chronic hepatitis with elevated ALT levels only after 10 to 30 years of infection (89). In contrast patients who acquire HBV infection in the late childhood, during adolescence or adulthood and become chronic carriers usually present in the immunoactive phase with active liver disease.

The age of adult patients at the time of initial presentation with HBeAg positive chronic hepatitis B ranges between 24 and 36 years (median 31) in several reports and men usually outnumber women, the male to female ratio ranging from 1.5 to 4.9 (90,91). Liver damage ranges from mild (24% to 42%) to moderate or severe chronic hepatitis (44% to 63%) or active cirrhosis (10% to 24%) (91, 92, 93, 94,95). Chronic hepatitis B tends to be milder in children and liver histology reveals minimal to mild chronic hepatitis in the great majority of children (86% to 90%), nevertheless severe liver disease including cirrhosis may occur in a small proportion of patients during childhood (96, 97).

A key event in the natural history of HBeAg positive chronic hepatitis is HBeAg seroconversion. Several longitudinal studies conducted in cohorts of

patients with HBeAg positive chronic hepatitis have shown that seroconversion from HBeAg to anti-HBe with marked reduction of HBV replication is associated with biochemical and histologic regression of inflammatory activity in the majority of patients (90,93,97,98). Histological improvement occurs gradually months to years after HBeAg seroconversion (99). In longitudinal studies the observed probability of clearing HBeAg was about 50% and 70% within 5 and 10 years of diagnosis, respectively (91, 93, 100, 101). Most studies have found that the mean annual rate of spontaneous HBeAg seroconversion ranges from 8% to 15% in children or adults showing biochemical signs of liver disease activity (87, 88, 90, 92, 93, 91, 101,102). On the other hand in Asian children, most with normal ALT, the annual spontaneous HBeAg seroconversion occurs at a very low rate, less than 2% during the first 3 years of age and 4-5% in children older than 3 years .

Some determinants for HBeAg seroconversion have been reported, including sex, age, the degree of biochemical activity and more recently HBV genotypes. Older carriers and female are more likely to clear HBeAg (89, 100, 103); in a study of 532 Alaska Natives with HBeAg positive chronic hepatitis, a multivariate Cox proportional hazards model predicted clearance of HBeAg within 5 years in 33%, 52% and 76% for persons0 to 18 years of age, 19 to 30 years of age, and 31 to 78 years of age, respectively (100). Spontaneous HBeAg seroconversion within 1 year occurs in over 50% of patients with serum ALT levels over5 times the upper limit of normal (ULN) as opposite to less than 10% of those with ALT levels less than 5 times the ULN (104). Frequently acute exacerbation of hepatitis, reflecting immune mediated lysis of HBV infected hepatocytes, with ALT elevations to more than 10 times the upper limit of normal range and more than twice the baseline value and with HBV-DNA levels rising before and falling during the flare, precede HBeAg to anti-HBe seroconversion and usually lasts for 2 to 4 months (105). The probability of

HBeAg seroconversion correlates with the degree of histologic activity during the acute flare of hepatitis. A prospective study in Asian patients has indicated that approximately 70% of ALT flares with histological changes compatible with bridging hepatic necrosis sometimes associated with elevated serum alphafetoprotein level are followed by HBeAg seroconversion compared to only 20% of acute exacerbations without (106). Indeed in some cases these spontaneous flares of hepatitis are not followed by subsequent HBeAg seroconversion and these episodes can be viewed as an abortive attempt at seroconversion. These temporary flares of hepatitis are usually asymptomatic and frequently unrecognized, but some are accompanied by symptoms of acute hepatitis and very rarely, primarily in patients with slightly compensated chronic liver disease, may lead to hepatic decompensation and even death due to massive necrosis (107). HBV can be classified into 8 genotypes A-H and recent studies, all from Asia, have indicated that HBV genotype B is associated with earlier HBeAg seroconversion than genotype C, thus most likely explaining the less progressive disease in patients with genotype B (108,109).

HBeAg seroconversion associated with liver disease remission marks the transition from chronic hepatitis B to the inactive HBsAg carrier state, however a small percentage of patients (approximately 5%) may continue to show biochemical activity and high levels of serum HBV-DNA at the time of HBeAg seroconversion (93, 97, 98). These patients as well those undergoing reactivation of hepatitis B after HBeAg seroconversion may generate the group of patients with HBeAg negative chronic hepatitis B.

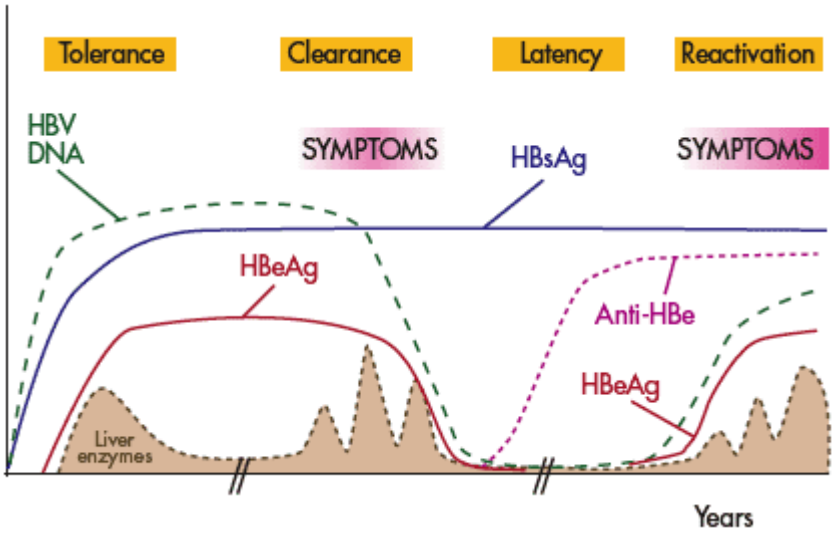


Fig 7 .DNA and Liver Enzymes in HBeAg positive and HBeAg Negative patients.

7.2. HBeAg negative chronic hepatitis B

The diagnosis of HBeAg negative chronic hepatitis B is based on chronic HBsAg carriership, HBeAg negativity with anti-HBe positivity, detectable serum HBV-DNA by molecular hybridization techniques or by quantitative PCR assays, with HBV-DNA usually exceeding 105 and 106 copies per ml, increased ALT levels, liver necroinflammation at histology and exclusion of other concomitant or superimposed causes of liver disease, such as super infection with other hepatitis viruses, alcohol abuse, hepatotoxic drug use, autoimmune or metabolic liver disease (87, 88,). The atypical serological profile is sustained by HBV variants which are unable to express HBeAg. The most frequent pre core mutation is a G to A change at nucleotide1896 (G1896A), which creates a stop codon in the pre core region of HBV genome and completely abolishes the production of HBeAg . Other patients may harbour other changes in the pre core region or mutations in the core promoter region, which reduces pre-core mRNA synthesis and HBeAg production; the most

common core promoter mutation involve a 2 nucleotide substitution, A to T at nucleotide 1762 and G to A at nucleotide 1764 (TA) (110).

HBeAg negative chronic hepatitis has been reported to prevail in certain part of the world such as in the Mediterranean basin, the middle East and Asia (88). Recent data suggest that HBeAg negative chronic hepatitis is more common than previously suspected and that is present worldwide (111). The geographical variations in the prevalence of HBeAg negative chronic hepatitis and associated pre core and core promoter variants is related to the predominant HBV genotype in each area. Indeed the most common pre core mutant (G1896A) can be selected only in patients infected with HBV genotypes B, C, D,E harbouring thymidine (T) at pre core position 1858. On the other hand in HBV genotype A the nucleotide 1858 is a cytocine (C),precluding the selection of the G1896A mutation. This explains why HBeAg negative chronic hepatitis associated with pre core stop codon variant is prevalent in the Mediterranean area where non-A genotypes, particularly D, predominate and is infrequent in North America, Northern Europe and parts of Africa where genotype A predominates . In Asian countries, where both A and non-A genotypes exist, HBeAg negative chronic hepatitis is associated with mutation in the core promoter region in addition to precore mutants (110).Clinical experience suggests that the prevalence of HBeAg negative chronic hepatitis is increasing during the last decade particularly in the Mediterranean area and in Asia, but this issue is supported only by few studies (110, 112).

Patients with HBeAg negative chronic hepatitis are usually older than patients with HBeAg positive chronic hepatitis and the age ranges between 36 and 45 years (median 40); males largely predominates and the reported male/female ratio ranges from 3.9 to 17 (95, 113,114). Current available data indicate that the clinical profile of HBeAg negative chronic hepatitis differs from that seen in patients with HBeAg positive chronic hepatitis. Indeed minimal or

mild chronic hepatitis at histology is infrequent and severe necroinflammation is seen in more than 50% of HBeAg negative cases at diagnosis (113, 114, 115). In large series of patients from the Mediterranean area from 29% to 38% had cirrhosis already developed at the time of their first presentation(114, 115). The older age and the high rate of advanced liver damage at presentation of HBeAg negative as compared to HBeAg positive patients suggest that HBeAg negative chronic hepatitis represents a late phase in the natural history of chronic HBV infection. To further support this concept a recent long term study has reported that HBeAg negative chronic hepatitis accumulated over time after HBeAg seroconversion with a cumulative incidence of approximately 25% at 16 years of follow-up (98). Thus the increasing prevalence of HBeAg negative chronic hepatitis may be only the reflect of the increased duration of infection in chronic HBV carriers and long term monitoring of patients.

Moreover the clinical course of HBeAg negative chronic hepatitis differs from the HBeAg positive forms in relation to large fluctuations over time of viremia and transaminases, with relapses of hepatitis alternate to periods of biochemical remission in most patients and a much lower rate of sustained spontaneous remission. During follow-up three main biochemical profiles are observed, namely recurrent flares of ALT with(pattern 1) or without (pattern 2) spontaneous ALT normalization between flares, and persistently increased ALT without tendency for spontaneous remission (pattern 3). In a recent longitudinal study approximately two thirds of patients showed recurrent ALT flares and the frequency of patterns 1,2 and 3 was reported to be 45%, 20% and 35%, respectively (114). Flares of hepatitis may be severe and disease exacerbations correlate with progression to end stage complications of cirrhosis (114). Periods of remission with normal ALT and low serum HBV-DNA levels (< 105 copies per ml) may be long lasting, but usually the disease recurs and sustained spontaneous remission of disease activity is uncommon . The incidence of

delayed spontaneous HBsAg clearance has been estimated to occur at a low rate of 0.5% per year (98, 115).

8.Role of liver Histology in the assessment of chronic hepatitis B

8. 1. Liver biopsy

Liver diseases in patients with hepatitis B infection represent an interaction between viral replication and the host's immune response that attempts to eradicate the virus. The degree and nature of the injury in any individual patient are determined by the balance between these two factors. Since hepatitis B is not directly cytopathic, it is the immune response that causes tissue damage in the live, which over time may lead to scarring and eventually to cirrhosis and its complications. These processes are reflected in changes in liver histology that can be readily assessed in a liver biopsy (Fig 8). In the recent EASL guidelines, "a liver biopsy is recommended for determining the degree of necroinflammation and fibrosis in patients with either increased ALT or HBV DNA levels > 2000IU/ml or both, since hepatic morphology can assist the decision to start treatment" and , this recommendation is based on high quality evidence(116). Presently liver biopsy remain the gold standard for grading necroinflammation and staging fibrosis in patients with liver disease(117). This requires an adequate size biopsy, 2.5 cm or longer with a 16-guage or wider needle (fig 9) ,and a simple , reproducible staging and grading system.

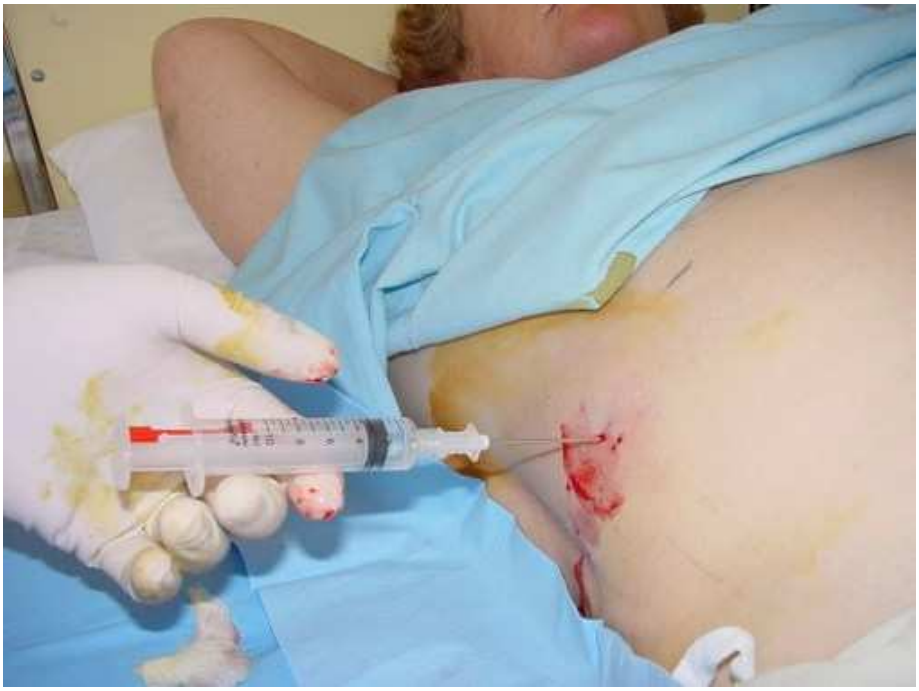


Fig.8. Liver biopsy: Normally liver biopsy is performed by percutaneous method**.** A specimen of at least 1.5 cm in length and 1.2- 2 mm in diameter and contains at least 6 to 8 portal triad is considered adequate.

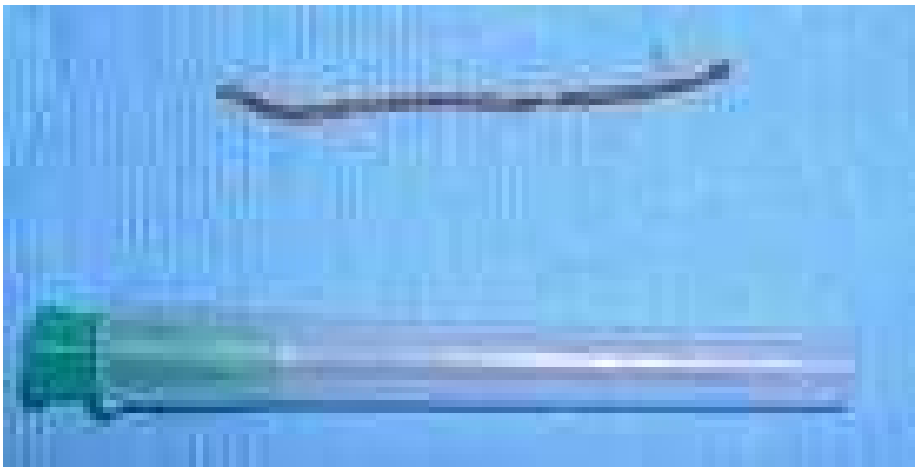


Fig. 9. Biopsy specimen : Specimens are fixed in 10%formalin and four micron sections are stained for histo-pathological examination. Immunohistochemistry is done on deparaffinised sections.

7.3. Grading and staging of liver biopsy specimens

Liver biopsies are usually performed for grading and staging of liver

disease . The stage of any disease is a measure of how far it has progressed its

Periportal±Bridging Necrosis	score	Intra lobular degeneration and focal necrosis	score	Portal inflammation	score	Fibrosis	score
None	**0**	none	**0**	No portal inflammation	**0**	No fibrosis	**0**
Mild piecemeal necrosis	**1**	Mild	**1**	Mild	**1**	Fibrous portal expansion	**1**
Moderate piece meal necrosis	**3**	Moderate	**3**	Moderate	**3**	Bridging fibrosis	**3**
Marked piecemeal necrosis	**4**	Marked	**4**	Marked	**4**	Marked	**4**
Moderate piecemeal necrosis plus bridging necrosis	**5**	-	-	-	-	-	-
Marked piecemeal necrosis plus bridging necrosis	**6**	-	-	-	-	-	-
Multi nodular necrosis	**10**	-	-	-	-	-	-

Table 2.KNODEL Histological Activity Index (HAI) [Desmet V Journal of Hepatology 2003]

Stage	METAVIR	ISHAK
0	**No fibrosis**	**No fibrosis**
1	**Periportal fibrosis**	**Expansion of fibrosis to poral area with or with out septal fibrosis**
2	**Porto-portal septum (> 1 septum)**	**Expansion of fibrosis to more portal areas with or with out septal fibrosis**
3	**Porto-portal septems**	**Expansion of fibrosis to more portal areas with occasional porto-portal bridging**
4	**Cirrhosis**	**Expansion of fibrosis to more portal areas with marked porto-portal bridging**
5		**Marked porto-portal bridging with occasional nodules (incomplete cirrhosis)**
6		**Cirrhosis**

Table 3.Stage of fibrosis in chronic Hepatitis B.F0: No fibrosis F1 : Portal fibrosis without septum F2: : Portal fibrosis with few septumF3: Portal fibrosis with numerous septums without cirrhosis F4 : Cirrhosis.**[Strader et al. Hepatology 2004]**

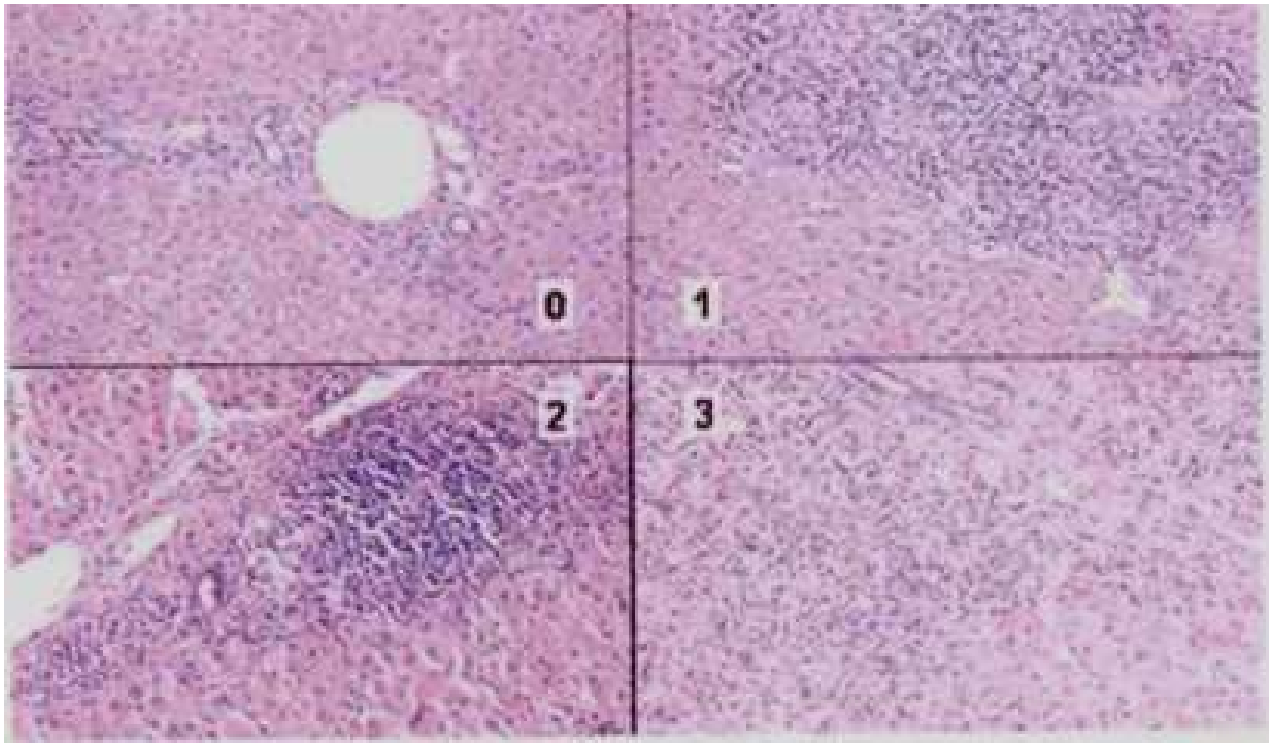


Fig 10.Grade of inflammatory activity in chronic hepatitis B
A0 : No activity A1 : Mild activity A2 : Moderate activity A3 : Severe activity

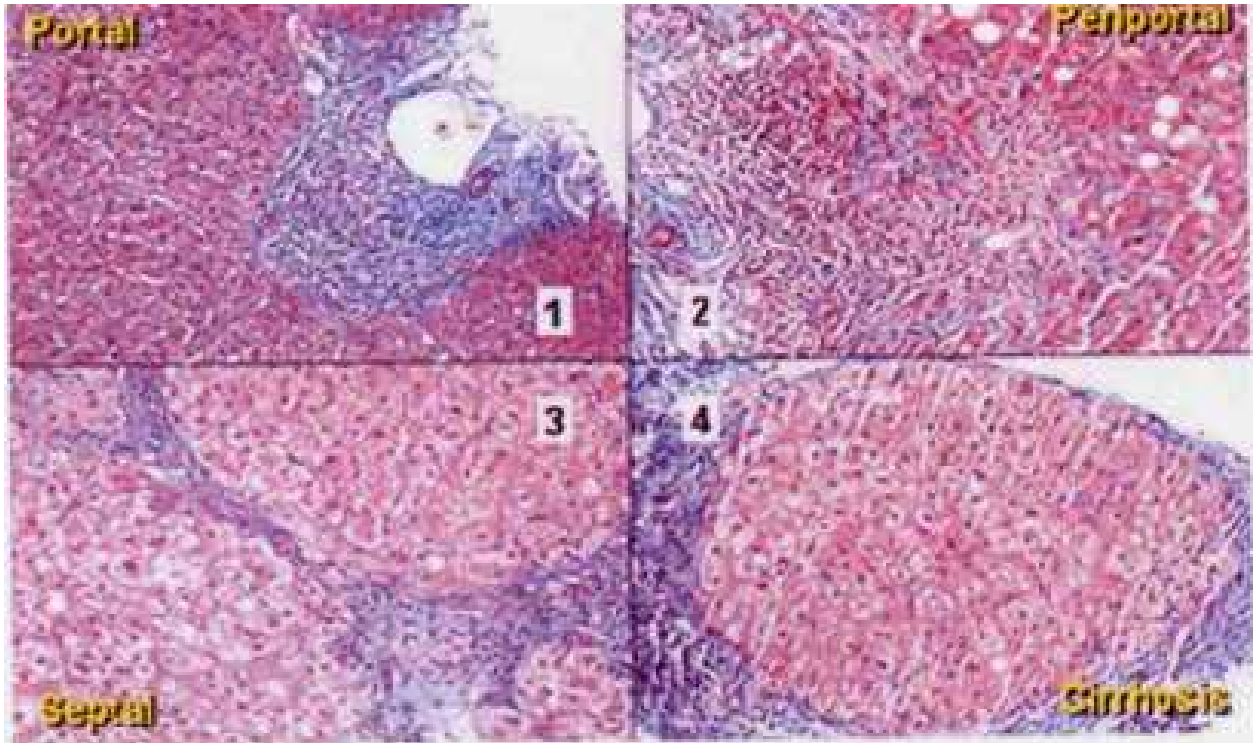


Fig 11.Stage of fibrosis in chronic Hepatitis BF0: No fibrosis F1 : Portal fibrosis without septum F2: : Portal fibrosis with few septumF3: Portal fibrosis with numerous septums without cirrhosis F4 : Cirrhosis
[Photo taken from America Gastroenterological Association Institute (Dr. Tom Smyrk)]

natural history, with the end stage resulting in death of the patient or failure of the organ; and so in chronic hepatitis B the end stage is cirrhosis with clinical decompensation, whereas earlier stages have lesser degree of fibrosis or cirrhosis. The grade of a disease is meant to reflect how quickly the disease will progress to the end stage. In chronic hepatitis, it is thought that hepatocellular injury and inflammation especially interface hepatitis, causes progression of fibrosis. Therefore grading is the assessment of these features (Fig 10&11).

There are two major reasons for histological staging and grading in chronic hepatitis B. First, and the most often, histological grading and staging are used in the management of individual patients, for prognosis, and as a guide to therapy. Secondly, the improvement in liver histology can be used as an end point in clinical trials for new forms of therapy. These two functions of liver histology have different goals and therefore have different requirement and should use different methods for grading and staging(Tables 2&3). Some widely used systems include:

1. Conventional verbal diagnoses- As revised by a special panel under the International Association for the Study of Liver (IASL), the severity of chronic hepatitis can be graded as minimal, mild, moderate or marked, and the degree and location of fibrosis noted precisely.
2. METAVIR- Activity has 3 grades and fibrosis has four stages (118, 119).
3. Batts-Ludwig – Activity has 4 grades and fibrosis has four stages.
4. Scheuer – Scores added to give an activity of 0 to 8 and stage 0 to 4 (120,121,122).
5. Knodell – scores added to give an activity of 0 to 18 and stage 0, 1,3 or 4(123).

6.Ishak – score added to give an activity of 0 to 18 and stage of 0 to 6 (124).

For clinical trials employing large cohorts of patients, a wide range of values are better for statistical analysis. For activity, both Knodell and Ishak scores have a theoretical range of 0 to 18. The Ishak fibrosis score, with a range of 0 to6 and no missing numbers, is better for assessing fibrosis. The date can be analysed in many ways, although it is important to remember that the numbers correspond to categories rather than equidistant units.

8. Aim of the study

The correlation between the expression of viral antigens in the hepatocytes of patients with chronic hepatitis B viral(HBV)infection , the liver histology and the stage of viral replication remains less explored. The aim of this study is to asses the liver histology by using the Histological Activity Index (HAI) and correlate it with the expression of HBV core antigen (HBcAg) and surface antigens (HBsAg) in the hepatocytes of patients with chronic HBV infection of various phase of viral infection.

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9. Materials and methods

The study was conducted between January 2008 and April 2009 in the Department of Gastroenterology and Hepatology , Medical College Calicut of the University of Kerala, in South India, with the collaboration of our Department. The liver histology and immunohistological analysis was performed by the Department of Pathology, “Bambin Gesu Hospital”, Rome, Italy. Patients attending the Liver clinic of the Institution with documented chronic HBV infection at six months apart were recruited for study. All patients gave informed written consent for liver biopsy and the study protocol was approved by the institutional ethics committee. They underwent thorough evaluation by detailed history, physical examination, routine haematology and biochemical tests including liver functions tests and blood coagulation profile. HBsAg, HBeAg , Anti HBe, Anti HCV,(Abbot) HIV antibodies and HBV DNA were done in all patients. Every patient had routine abdominal ultrasonogram and liver biopsy through the inter-costal route using Bard liver

biopsy gun. The exclusion criteria were; cirrhosis of the liver, pregnancy, coagulation disorder, HCV and HIV infection, BMI >25 , alcohol intake of > 40 gm /day, those already on antiviral therapy and those unwilling for liver biopsy .

All liver biopsy specimens were fixed in 10% formalin and four micron sections were stained for routine histopathology examination using haematoxylin and eosin and orcein. The disease activity was assessed using the HAI scoring index(124). Immunohistochemistry was done on deparaffinised sections for cytoplasmic and nuclear HBcAg and cytoplasmic HBsAg using polyclonal anti HBc and anti HBS using standard immunoperoxidase method (125). The pathologist was blinded to the clinical and serological profile of the patients. The patients were broadly divided in to HBeAg positive and negative and the HAI and pattern of core antigen and surface antigen expression in liver were compared later. The results were analysed using non parametric tests like chi square test, Wilcoxon test and Mann-Whitney test and the statistical software used was SPSS.

10. Results:

The study was conducted between January 2008 and April 2009. 19 patients who satisfied the inclusion criteria underwent liver biopsy during this period. The baseline characteristics of 19 patients are given in Tables 4&5. 12(63.15%) patients were HBeAg positive and were younger with a mean age of 26.1 ± 7.3 while HBeAg negative patients were older with a mean age of 40 ± 10.4(p 0.003). In this study the major number of patients were male representing 68.43%. The HBeAg positive patients presented with a higher value of HBV DNA, **$2.35x10^7$ (**range**$1.07x10^7$-10^8) vs $1.25x10^4$ (**range **$8.06x10^3$-$1.15x10^5$)** ($p<0.05$) in HBeAg negative patients . No much difference was noted in the other parameters: serum bilirubin, 1.2± 0.6 vs 1.2 ± 0.4 (p

0.65); prothrombin time, 15.6± 1.4 vs 15.3± 1.5 (p 0.65); platelet count 2.6± 0.6 vs 2.8± 0.7 (p 0.34); AST 53.2± 37.4 vs 43± 16.6 (p 0.84) and ALT 66.7± 67.5 vs 46.3± 28.8 (p 0.77), in the respective groups. There was history of intake of indigenous drugs in 7 out of 19 patients. Most of them took self made extracts of the stem and leaves of the plant Phyllantus amarus ssp. niruri, for varying periods ranging from 2-24 months. This plant is widely claimed to be useful for treatment of liver diseases among practitioners of indigenous medicine in India. None of the patients had clinical evidence of cirrhosis of the liver.

Considering the above parameters, the mean age of the patients and the HBV DNA values were statistically significant.

10.1. Liver histology and immunohistology

For HBeAg positive patients, the mean grade for inflammatory activity was 5 (range.2-12) and staging for fibrosis was 0 (range 0-2), the minimum grade of 2 was represented in 1 out of 12 (8.33%)patients and the maximum grade of 12 was presented only in 1 out of 12 (8.33%) while majority , 8 out of 12 (66.66%) had a fibrosis stage of 0. For HBeAg negative patients, the mean grade for inflammatory activity was 6 (range2-9) and staging for fibrosis was 1 (range0-5), the minimum grade of 2 was noted in 2 out of 7 (28.5%) patients and the maximum grade of 9 was noted only in 1 out of 7 (14.48%) and 3 out of 7 (42.85%) had a fibrosis staging of 0 and the maximum staging of 5 was noted only in 1 patient out of 7 (14.48%) . Diffuse ground glass appearance (Fig15) was seen in 16cases (82.2 %). Orcein staining confirmed the presence of Shikata cells bearing HBsAg in 17 cases. (Figure 1) . By immunohisotology, there were three patterns of staining for HBcAg in the hepatocytes namely predominantly nuclear, predominantly cytoplasmic and a mixed pattern. (Figure 12) The pattern of staining for HBsAg was membranous, submembranous and diffuse type.

(Table 12, Fig 13) The cells expressing the antigens were found either in clusters or were diffusely scattered. The details of liver histology as per HAI and the pattern of staining by immunohisotology for the two groups are given in Table 7. Two cases (10.5%) had evidence of macrovescicular steatosis (Fig16) predominantly affecting the centrizonal regions, one patient had evidence of nodule formation in the liver and one patient had evidence of lymphoid follicle.

There was strong correlation between the presence of nuclear HBcAg , low disease activity index by HAI and positive HBeAg in the serum. Similarly cytoplasmic HBcAg was strongly correlated with significant liver disease as per HAI and negative HBeAg status (chi square test 4.08). Membranous expression of HBsAg was associated with inflammatory activity. Discrete expression of HBsAg in the cytoplasm was inversely related with disease activity (p=0.03) (Tables 7).

11. Discussion

The prevalence of Hepatitis B in the normal population of Calicut in the northern part of Kerala is considered as low with only 0.52%, compared to other parts of India, where the seroprevalence of Hepatitis B is intermediate between 2-7% (126). Hepatitis B continues to be a major problem in the state of Kerala , as in other developing countries, where childhood immunization is not mandatory (84). In these areas HBV infection is acquired in the childhood itself through vertical transmission and there is a high rate of chronicity (85), eventhough most of these young persons with HBV infection are asymptomatic, the infection is usually detected during routine medical check-up for unrelated conditions (126). Nearly two third of our patients in our study group are HBeAg positive .Most of the patients detected to have HBV infection in the

Asian regions are younger with positive HBe (83). It is contrary to the present situation in industrialized countries where the population of HBeAg positive chronic hepatitis B shows a steep decline and the age of the patient has increased significantly (51,82). In Italy , nation wide surveys of patients with hepatitis B seen in clinical centres documented a fall in HBeAg positive cases from 60% in the years 1975- 1984 to approximately 10% in 1997 and 2001 (82,127). Mass vaccination of newborns and 12 year subjects since 1991 has contributed to this change.

Recently a multi centric study conducted in Italy by Stroffolini et al has demonstrated the presence of up to 89% patients with HBeAg negative chronic hepatitis B(128). Interestingly, the balance between HBeAg positive and negative cases has reached a steady state in Italy during the last 10 years. After a dramatic fall in the prevalence of HBeAg by the end of the 1980s , since the year 1997, it has ranged between 10-15% in different surveys performed with similar selection criteria.

Nearly two thirds of our patients (63 %) with chronic HBV infection were HBeAg positive. The mean age of these patients were 26.1(19-42) as against the mean age of 40.9 (28-55) for HBeAg negative patients. Various reports from Asian countries have shown a relative preponderance of younger patients with HBV infection as against elderly patients in the developed world (83). It may be noted that in Asian countries, the mode of infection is most likely vertical in younger patients where as it is most likely horizontal in elderly patients. Conversion from HBeAg positive to negative status is usually synonymous with immunoclerance . However HBV DNA is detectable in the serum of these person albeit in lower titres, suggesting that there is low level of viral replication probably with mutation at pre core or core promoter regions of the HBV viral genome (129).

During the phase of immunotolerance, the serum HBV DNA titres are high, but the disease activity in the liver is very low and often the patients have normal transaminase values. In this study group almost all the patients with positive HBeAg status presented with a comparatively high viral load, **$2.35x10^7$ (**range**$1.07x10^7$-10^8)** (Table 5). There are some reports in the literature that persons with higher serum DNA levels are at a greater risk of development of severe liver disease including hepatocellular carcinoma (130). Persons with HBV infection in the immune tolerance phase are not eligible for any form of antiviral therapy, however they have the risk of development of liver inflammation and hepaotocellular carcinoma in future (131).

The exact mechanism of the induction of immunotolerace is not yet fully established . During HBV infection , the host immune response , particularly the cellular immune response, mediate clearance of HBV infection (132). Unfortunately , the patient often exhibits impairment of HBV specific T cell activity during chronic HBV infection (133). Circulating CD4+ CD25+ regulatory T cells (T regs) have been demonstrated to maintain immunotolerance and suppress the antigen specific or antigen non specific T cell response(134/ Immun. 2008). In this study conducted by Guoping Peng et al , it is proved that there is a marked increase in circulating CD4+ CD25+ Tregs in CHB patients. T regs play a negative role not only in modulating the effectors of immune response by inhibiting IFN gamma secretion and cellular proliferation upon HBV antigen stimulation , but also in influencing the viral load and disease persistence. It seems to have a positive correlation between T reg frequency and serum HBV DNA load , suggesting an up- regulation of T regs associated with an increase in HBV replication (134). In fact, in this study group almost all the patients with HBeAg positive status, demonstrated a comparatively high DNA level compared to the patients with HBeAg negative status ($p<0.05$), supporting the above findings.

However, both HBeAg and HBcAg appear to play a role in viral persistence. It has been suggested that the HBeAg may promote HBV chronicity by functioning as an immunoregulatory protein via the induction of tolerance and Th1/Th2 cross-regulation. HBeAg appears more efficient at eliciting T cell tolerance than the HBcAg , and this split immune tolerance may have significance implications (135). In natural HBV infection , a dichotomy exists between the apparent tolerogenic and immunogenic functions of the HBeAg (136). High concentrations of serum HBeAg in vivo severely compromise the ability of the T cells to produce cytokines or proliferate to recall antigens on vitro and to provide T-cell help for in vivo anti HBe antibody production. In the so called immunotolerace phase of CHB infection , the majority of HBe specific T cell clones would be expected to be tolerant , although the mechanism of tolerance may vary. Mainly three mechanisms are elicited in this context , i.e., deletion , anergy or ignorance. The status quo of the various clonal tolerance phenotypes would likely be maintained as long as the HBeAg concentration and/ or the non inflammatory hepatic environment remains unchanged. However , a non specific increase in hepatic inflammation or a decrease in HBeAg serum concentration, perhaps due to the emergence of core promoter region (137)or precore region(138) mutants, may allow activation of low avidity ignorant HBeAg specific T cells and/or reverse the antigenic state of others, respectively. Such a shift from HBeAg –specific T cell tolerance to T cell activation may precipitate the so called clearance phase of CHB infection. High avidity HBeAg specific T cell clones are likely to be physically or functionally deleted and not available to participate in the antiviral clearance mechanisms after a chronic infection has become established. Intermediate or low avidity HBeAg specific T cell clones that are not physically deleted at least have the potential to be activated either through the reversal of adaptive tolerance (anergy)or the primary activation of ignorant HBeAg specific T cells (139).

The clonal heterogeneity of the HBeAg specific T cell tolerance may also explain how a primarily tolerogenic protein can exert pressure on the immune response to select an HBeAg negative mutant. For example, high avidity HBeAg specific T cells clones may be tolerized and simultaneously lower avidity T cells clones may be activated and involved in selecting HBeAg negative mutant in the same patient (139).

In the natural history of HBV infection, the most important event is HBeAg seroconversion characterized by loss of HBeAg and development of antibody to HBeAg (Anti HBe)(140). This generally occurs years after replicative phase and indicates transition to a low/non replicative phase with potential for resolution of infection and improvement of necro-inflammation in the liver. Age of acquisition of the virus, immune competence of the host and the strength of immune response to the viral antigens are some of the determinants of timing and efficiency of seroconversion. The prognosis of chronic HBV infection is dependent upon the amount of inflammation, necrosis and fibrosis in the liver at this point of seroconversion. If significant liver damage is already present at this point, then the prognosis after seroconversion, spontaneous or treatment related is unlikely to be good, despite suppression of viral replication. On the other hand, if the seroconversion had occurred early and is maintained, then the long-term prognosis is excellent. It has been shown that the probability of development of hepatocellular carcinoma is many fold higher in persons who are HBeAg positive, than who are only HBsAg positive and HBeAg negative. In a subset of persons, this relationship between seroconversion and suppression of viral replication does not hold true (141). In them, despite anti-HBe positivity, active viral replication persists due to emergence of mutants in the pre core and basal core promoter regions of HBV. This state, characterized by continuing

viral replication despite anti HBe positivity has been termed as HBe Ag negative hepatitis (142,143,144). It has been increasingly recognized that HBeAg negative hepatitis is progressively increasing in prevalence globally , so also in India . The outcome of HBeAg negative hepatitis is different from that of the HBeAg positive phenotype. Fluctuating disease activity with periodic ALT flares accompany viral replication that progresses indolently to chronic liver disease. Response to anti viral therapy in HBeAg negative hepatitis is also different from the HBeAg positive disease (87,145). It would therefore be important to delineate the molecular character, viral load in association with immunohistolology to evaluate the stage of chronic HBV infection.

An important facet of global HBV epidemiology is the emergence and increasing significance of HBeAg negative infections as well as the distribution and significance of HBV mutants, particularly those in the pre core (PC) and basal core promoter (BCP) regions of the HBV genome. The prevalence of this e negative chronic hepatitis B and its molecular basis varies geographically. Thus, in the Mediterranean countries, nearly 90% of the HBeAg negative infections are associated with the precore mutants, while this is 50% in the Far East and 25% in the USA(145).

Very little information on the prevalence and molecular character of HBeAg negative hepatitis has emerged from India. In North India, the prevalence of precore mutants has been reported to be 15% amongst chronic liver disease patients (146). In the state of West Bengal (North India), amongst asymptomatic HBV infected, HBeAg negative, Anti HBeAg positive subjects, the prevalence of pre core mutants was 9% and that of the basal core promoter mutants (BCP) was 4%. Rest (87%) of the HBeAg negative infections in the community, mostly inactive biochemically were associated with the wild type virus (147). Different genotypes of hepatitis B have its own significance. Based

on the nucleotide sequence homology and divergence amongst HBV isolates globally, eight genotypes of HBV are described (A to H). The distribution of HBV genotypes varies geographically. HBV genotypes have been correlated with disease progression (genotype C progresses to chronic hepatitis faster than B, and D faster than A), timing of e antigen seroconversion (genotype B earlier than genotype C), and poorer response to antiviral therapy (Genotype C). Moreover, HBV genotypes have also been correlated with human population migration in Japan (148). In India, genotype D had been the predominant genotype both in North and Eastern India. However genotype C too had been described from other parts of the country (147,149).

In our series , the mean grade for inflammatory activity was 5 for NBeAg positive patients and 6 for HBeAg negative patients (Table 7). The overall inflammatory activity score for our patients was 11 (Table 7). The low score shows that the inflammatory activity in the liver is minimal and it reflects the immune tolerance phase of the patients. The expression of HBV core antigen was predominantly nuclear in 42% of cases suggesting active replication in them.

The expression pattern of HBcAg in hepatocytes was found to be related to the activity of liver disease in chronic HBV infection (150) especially when HBcAg was located in the cytoplasm of the hepatocyte (151). It also was found that nuclear but not cytoplasmic expression of HBcAg is associated with high HBV replication and low activity of liver disease in the chronic B viral infection (152). In this study, all these factors were assessed in comparatively younger population which in fact made it easy to compare the effects of these factors on the degree of HBcAg expression. The results suggested that only the extent of nuclear HBcAg expression correlated with HBV replication and also the extent of cytoplasmic HBcAg expression correlated with histological activity of liver disease in chronic HBV infection. The limitation of this study was that the HBV

DNA levels were only measured at a single time point and most of patients had mild or moderate histological activities of liver disease.

The interesting and significant finding was that there was a positive correlation between the histological activity of liver disease and the degrees of expression of HBcAg in the hepatocyte cytoplasm in both HBeAg-positive and negative patients. In the HBeAg-positive patients, there was an inverse correlation between the degree of expression of HBcAg in the hepatocyte nucleus and the histological activity of liver disease. This finding supported the importance of hepatocyte injury in determination of HBcAg expression pattern. The degree of expression of HBcAg in the hepatocyte cytoplasm as assayed by immunohistochemical techniques is helpful for estimating histological activity of liver disease in the young patients with chronic HBV infection. This inverse relation could suggest that the lysis of HBV infected hepatocytes was followed not only with the decreasing of serum viral load but also the degree of expression of HBcAg in the hepatocyte nucleus.

In 1987, Hsu et al (153) showed that HBcAg was expressed at a relatively higher level on the nucleus than on the cytoplasm during the immune tolerance phase, in which there was little or no inflammatory activity in the liver, whereas expression of HBcAg in the nuclei decreased with a concomitant increase in the expression of HBcAg in cytoplasm during the immune clearance phase In 1987, Hsu et al (153) showed that HBcAg was expressed at a, in which there is active and ongoing hepatitis. These studies postulated that expression of HBcAg on the cell membrane is the important event that triggers cytotoxic T cells with HBcAg receptors, resulting in lysis of HBV infected hepatocytes.

In the present study, the interesting other finding was that there was a highly significant positive correlation between the levels of HBV DNA in serum and the degrees of expression of HBcAg in the hepatocyte nucleus, but there was no correlation between the degree of expression of HBcAg in the hepatocyte

cytoplasm and the level of viral replication in the HBeAg-positive patients. Our result confirmed the relationship between nuclear HBcAg expression and an enhanced viral replication detected by high serum value of DNA. It has been shown that HBcAg was localized in the nucleus in quiescent cells but diminished in proliferating cells, and the replication of HBV was enhanced in quiescent cells but diminished in the proliferating cells (151). Expression of HBcAg in the hepatocyte nucleus was likely to be important in viral replication.

Though HBeAg seroconversion was commonly taken as a therapeutic endpoint in the past, increasing evidence showed that disease progression can continue in a significant portion of patients after HBeAg seroconversion, especially in the Asia and Mediterranean population (154,155). Lack of HBeAg is usually connected with biochemically and histologically inactive disease as well as with the significant reduction of HBV replication , but up to 9% of such patients show active inflammatory process despite anti-HBe seroconversion (156,157).

It demonstrated that there was correlation between HBV DNA levels with lobular activity of liver disease in the HBeAg-negative patients (158,159). This finding confirms that active viral replication is still present in a certain proportion of HBeAg-negative patients, and increased HBV DNA might be used as a marker to identify HBeAg-negative patients who have a higher risk of active liver disease. Some HBeAg-negative patients who have the inflammatory response and viral clearance might have pre core mutation or/and core promoter mutations to escape immune clearance (160,161,162,163). In contrast, one study conducted by Wu PC et al reported that core promoter mutations were not associated with the enhanced viral replication (164). These mutations and unknown other factors may be related to the localization of HBcAg in the hepatocytes, therefore the virus might gradually replicate and develop

inflammatory response in the liver. More studies are required to document whether core promotor mutations have any effects on the chronic hepatitis B disease.

On correlating the HBeAg status with HAI and immunohisotology ,it was found that nuclear expression of HBV core antigen was the predominant observation. (Table .7) On the other hand, cytoplasmic expression of HBV core antigen is reported to be a feature of immune clearance phase. This phase is associated with higher inflammatory activity score in the liver. As all our patients who were negative for HBeAg had a positive HBV DNA in the serum also, it could mean that these sub group of patients were harbouring HBV virus with mutation at the core or the pre core regions. It is possible that the cytoplasmic expression of HBV core antigen is a feature of mutation at core/ pre core region of the virus and this speculation requires further study. Those patients who had a mixed pattern with both nuclear and cytoplasmic HBV core antigen could be attempting to clear the virus or it may be expression of simultaneous presence of wild infection which is probably being replaced by mutant infection. The mean fibrosis score for HBeAg positive patients were 0 and for HBeAg negative patients were 1. There was no correlation with fibrosis and various types of staining for HBV core antigen

HBV surface antigen staining was positive in the liver of 15 out of 19 patients (79 %). Of these, 89% cases had a ground glass type of appearance of the cytoplasm. There are three patterns for expression of HBsAg in the hepatocytes : the membranous, sub membranous and the cytoplasmic types (165). In this study 7 (36.8%) had cytoplasmic expression of surface antigen and 6 (31.5%) had membranous pattern of expression, while the rest had mixed pattern. Different studies associate membranous pattern of HBsAg with active viral replication and disease activity (166,167,168). However there are contradictory report by Wee et al and Ramakrisna et al (165,169) which stated

that there is no such correlation between different patterns of expression of surface antigen and disease activity. In our study , membranous distribution of surface antigen was associated with high disease activity and cytoplasmic distribution of surface antigen was associated with low disease activity. Those with high viral replication showed discrete cellular staining whereas those with low viral load showed a diffuse pattern of expression of surface antigen (170). This pattern may appear paradoxical. However subjects with low viral replication may have integrated surface antigen gene and large quantities of HBsAg may be produced due to the transcription from the hepatocytes genome.

In conclusion, the viral antigen expression in the hepatocytes of persons affected by chronic HBV infection in our study conforms to previous reports on this subject. About two thirds of our patients were young and they were positive for HBeAg in the serum, suggestive of immune tolerance phase of chronic infection. The disease activity as per HAI was low suggesting that there is only low level inflammation in them. The nuclear expression of HBcAg and discrete distributed cytoplasmic pattern of HBsAg was associated with immune tolerance phase whereas the cytoplasmic expression of HBcAg and diffuse pattern of HBsAg expression was more often associated with immune clearance phase.

12. Tables and Figures

Sex		Number of patients	Percentage
Female		06/19	31.57%
Male		13/19	68.43%
	Age		
HBeAg +	26.1 yrs		
AntiHBeAg	40.9 yrs		
HBeAg +		12/19	63.15%
Female		05/12	41.66%
Male		07/12	58.34%
Anti HBeAg		07/19	36.85%
Female		01/07	14.28%
Male		06/07	85.72%

Table 4. Characteristics of patients studied.

	HBeAg positive	HBeAg negative	p
No of patients	12	7	
Age	26.1 ± 7.3 (19-42)	40.9 ± 10.4 (28-55)	0.003
Bilirubin(Total)	1.2 ± 0.6 (0.5-2.4)	1.2 ± 0.4 (0.5-1.8)	0.65
Prothrombin time (sec)	15.6 ± 1.4 (14-19)	15.3 ± 1.5 (14-18)	0.65
Platelets (lakhs)	2.6 ± 0.6 (1.5-3.8)	2.8 ± 0.7 (1.9-3.8)	0.34
AST IU/L	53.2 ± 37.4 (18-134)	43 ± 16.6 (22-68)	0.84
ALT IU/L	66.7 ± 67.5 (18-252)	46.3 ± 28.8 (22-106)	0.77
HBV DNA IU/ml	$2.35x10^7$ ($1.07x10^7$-10^8)	$1.25x10^4$ ($8.06x10^3$-$1.15x10^5$)	<0.05

Table 5.Clinical and laboratory dates of patients studied.

Distribution pattern of intrhepatic HBcAg	HBeAg Positive	Percentage	HBeAg Negative	Percentage
Nuclear pattern	7	58.33 %	1	14.28 %
Cytoplasmic pattern	1	8.33 %	4	57.14 %
Mixed pattern	4	33.33 %	2	28.57 %

Table 6. Distribution pattern of HBcAg in hepatocytes according to HBeAg status

	HAI		Immunohistology of hepatocytes					
			HBV Core Antigen			HBV Surface Antigen		
	Staging	Grading	Nuclear	cytoplasmic	both	membranous	sub-membranous	cytoplasmic
HBeAg positive	**0**	**5**	**7**	**1**	**4**	**2**	**3**	**7**
HBeAg negative	**1**	**6**	**1**	**4**	**2**	**4**	**3**	**-**

Table7. Distribution of HBV core and surface antigens in the hepatocytes and the HAI.

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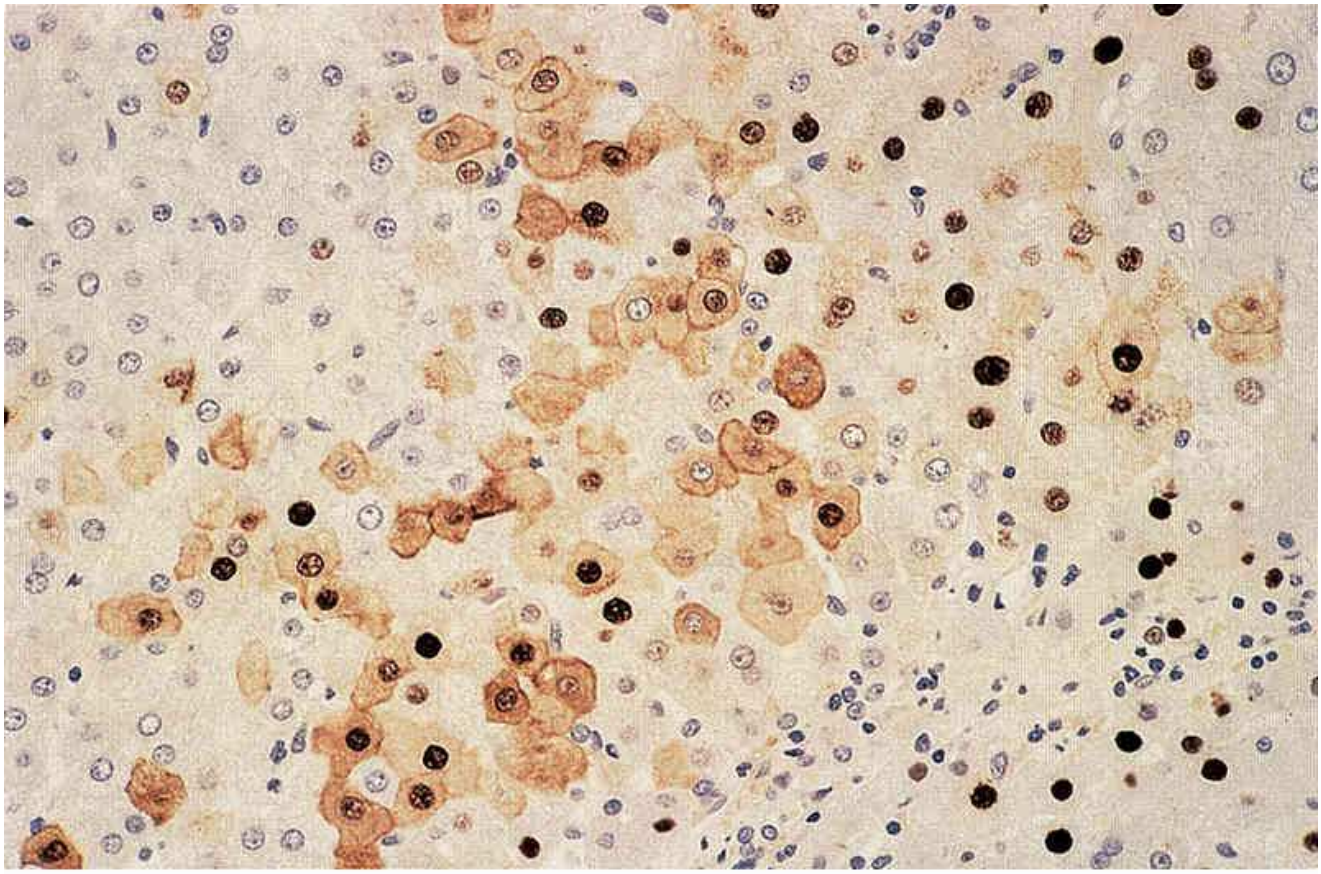

Fig 12. HBcAg :Chronic HBV- Viral replicative phase. Hepatitis B core antigen (HBcAg) is localized in hepatocellular nuclei and, in several hepatocytes; also in the cytoplasm and cell membrane. (Immunoperoxidase stain for HBcAg)

IMMUNOHISTOLOGY

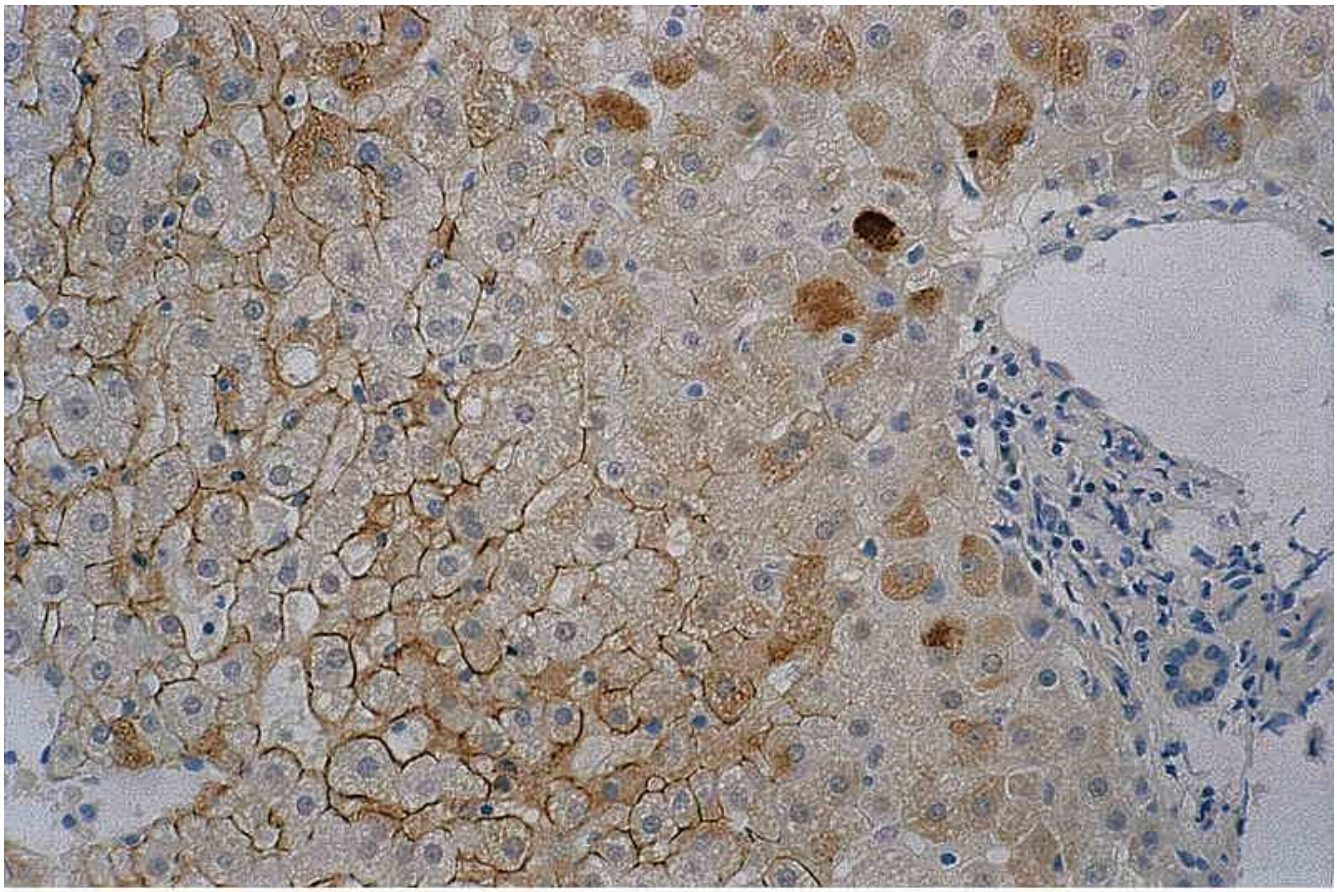

Fig. 13: Chronic viral hepatitis B: viral replicative phase. Hepatitis B surface antigen(HBsAg) is localized in variable quantities in the cytoplasm and in the cell membrane of several hepatocytes. Note only mild lymphocytic infiltrate in portal tract and lobule. (Immunoperoxidase stain for HBsAg)

IMMUNOHISTOLOGY

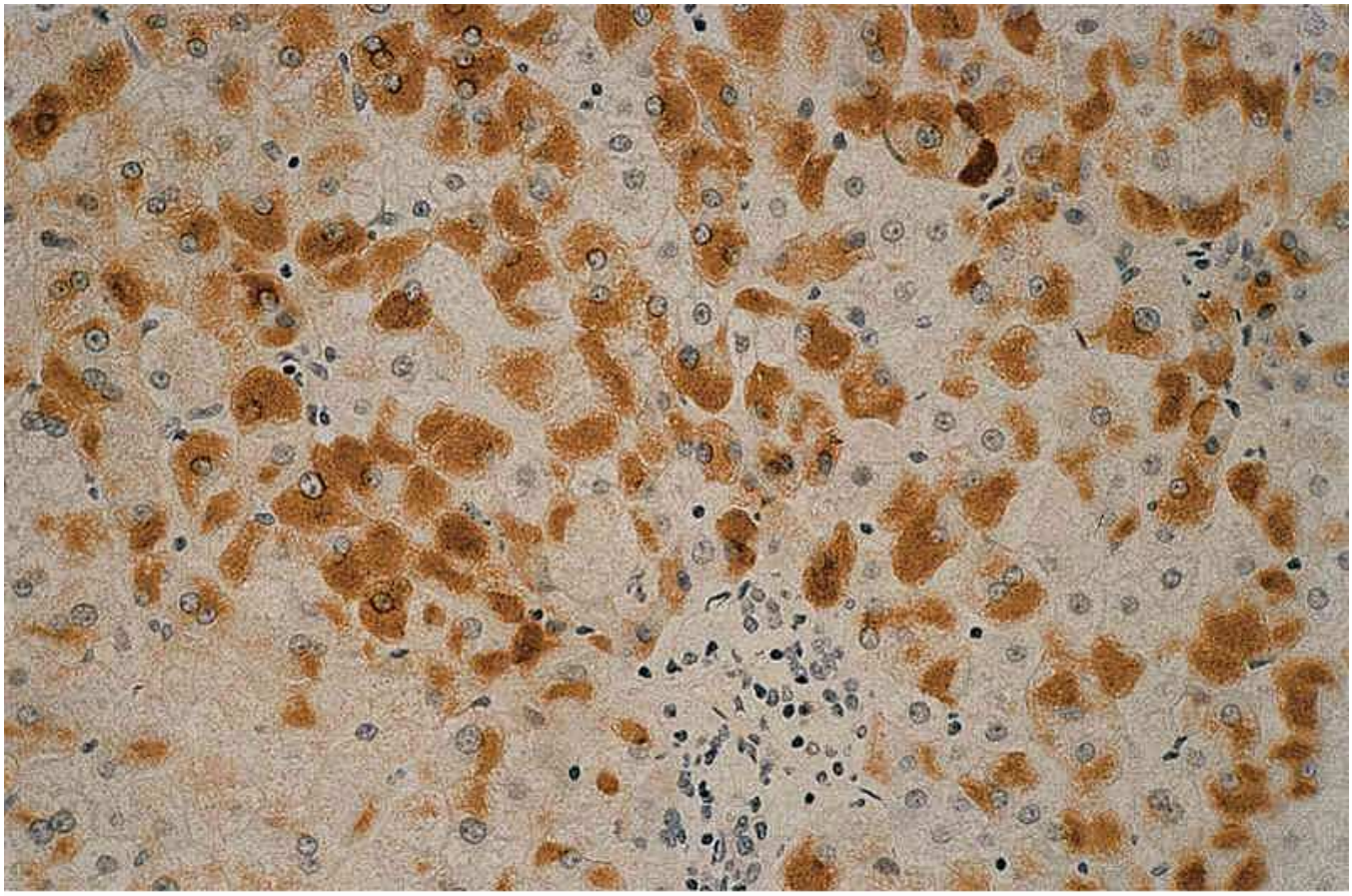

Fig. 14 : Chronic viral hepatitis B: viral nonreplicative (integration) phase. Hepatitis B surface antigen is localized in considerable quantity in the cytoplasm of a contiguous group (‘clone’) of hepatocytes. Note relatively mild lymphocytic infiltrate in portal tract and lobule. The more intensely staining cells appear as ‘ground glass hepatocytes’ on H&E staining. (Immunoperoxidase stain for HBsAg)

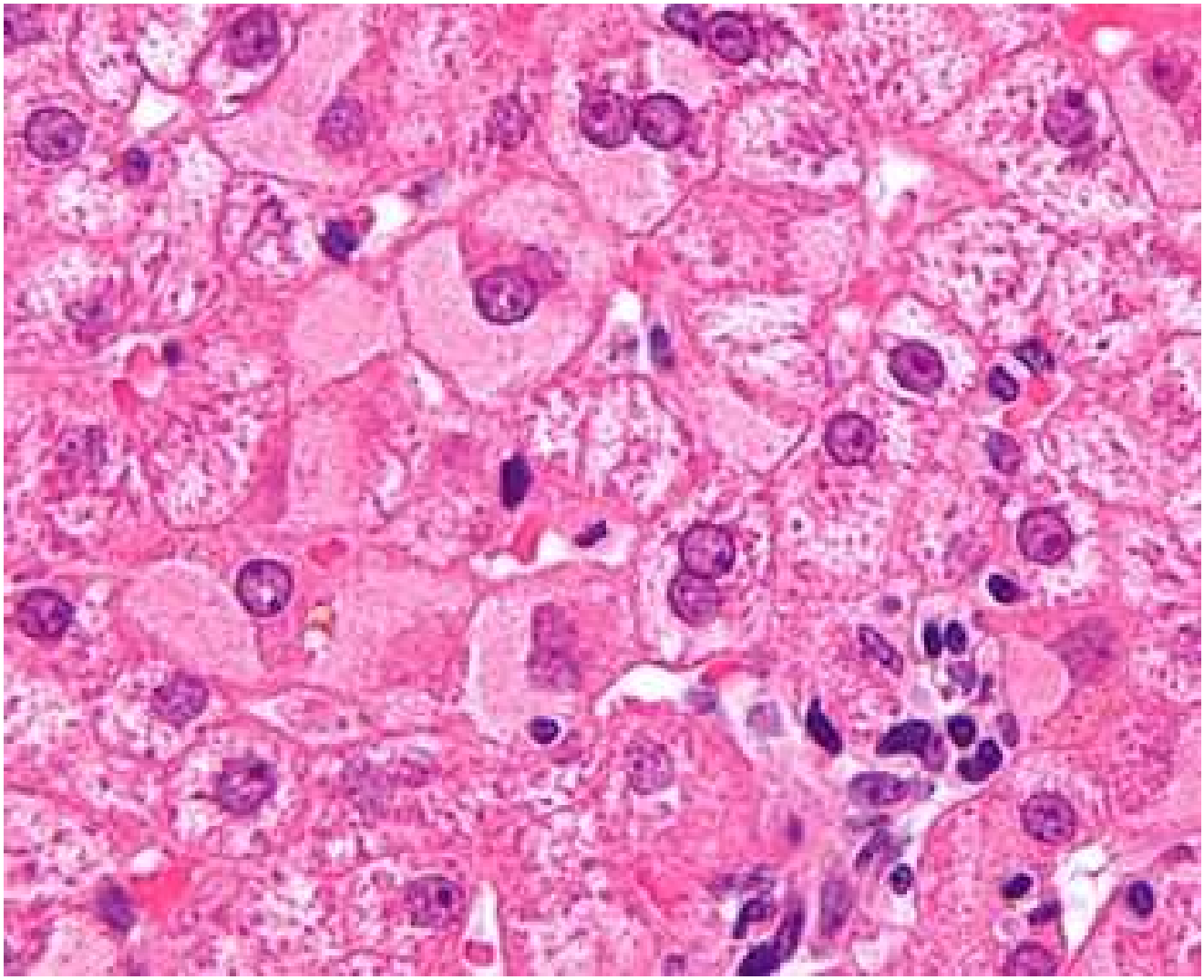

Fig. 15: Chronic viral hepatitis B, high magnification. Ground glass hepatocytes, characterized by more pale, eosinophilic, and homogeneous cytoplasm than surrounding normal (more granular) hepatocytes. Note (artefactual) cleft between 'ground glass' cytoplasm and hepatocellular cell membrane.

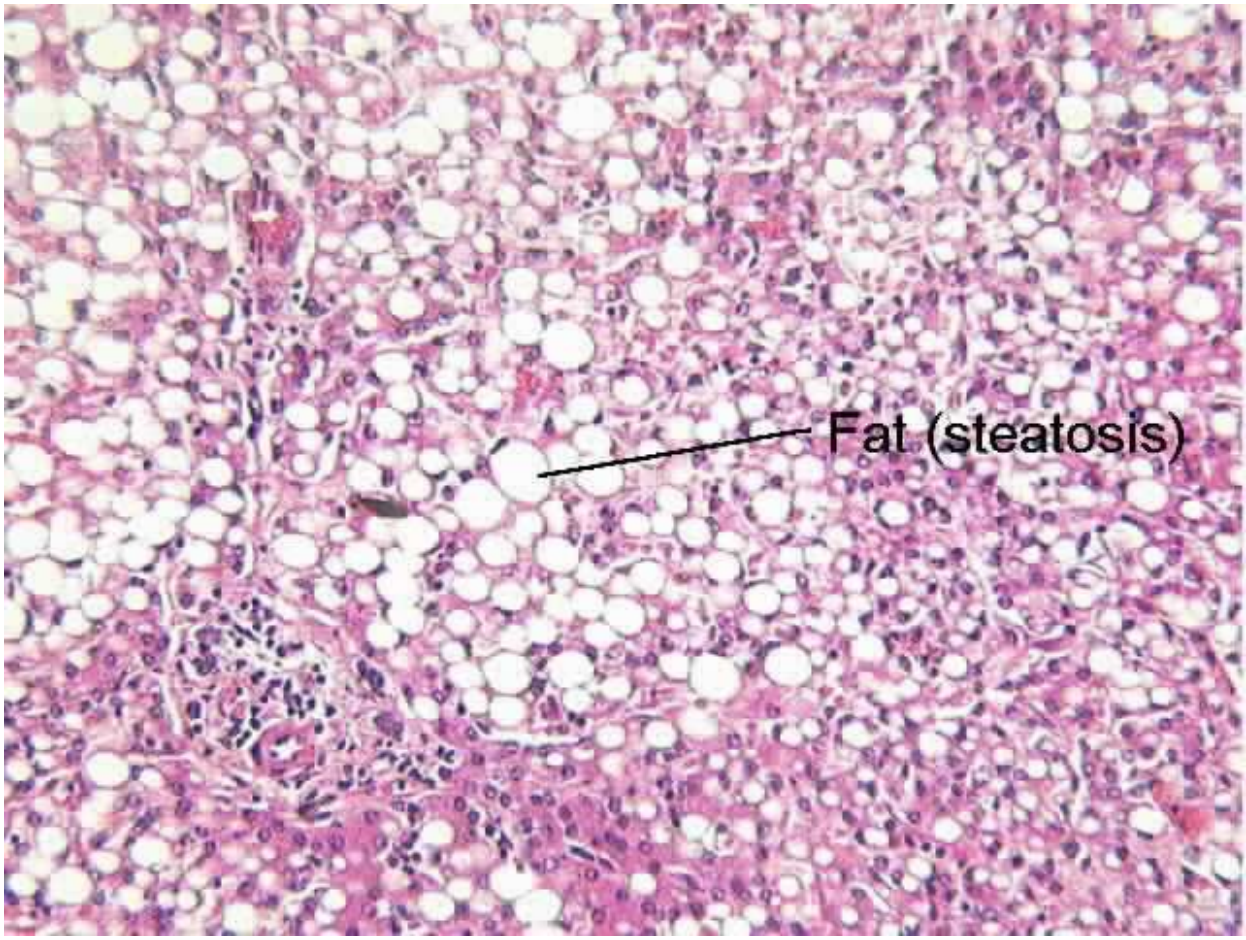

Fig 16: Statosis : biopsy specimen of the liver of a patient who denied use of alcohol (non alcoholic steatosis) Note fat granules of different sizes.

13. References

1.Lurman A. Eine icterus epidemic. Berl Klin Wochenschr1855;22:20.

2.Flaum A, Malmros et al. Eine nosocomiale icterus epidemic.Act Med Scand Suppl 1926;16:544.

3.Bigger J.W, Dubi S.D. Jaundice in syphilitics under treatment. Lancet 1943;1:457.

4.MacCallum F.O. Transmission of arsenotherapy jaundice by blood: failure with face and nasopharyngeal washings. Lancet 1945;1:342.

5.MacNatty A.S. Great Britain Ministry Health report Of Chief Medical officer Annual Report .London 1937.

6.Blumberg BS, Alter SJ, Visnich S. A "new" antigen in leukaemia sera. JMA 1965;191:541.

7.Alter S.J, Blumberg B.S. Further studies on a "new" human isoprecipitin system (Australian antigen). Blood 1966;27:297.

8.Blumberg G.S,Gerstley J.S, Hungerford D.A et al. A serum antigen (Australia antigen)in Down's syndrome leukaemia and hepatitis . Ann Intern Med. 1967;66:924.

9.Okachi K, Murakami S. Observation of Australian antigen in Japanese. Vox Sang 1968;15:374.

10.Prince A.M . An antigen detected in the blood during the incubation period of serum hepatitis. Proc Natl Acad Sci USA. 1968;60:814.

11.SzmunessW. Hepatocellular carcinoma and the hepatitis B virus: evidence for a casual association. Prog Med Virol 1978;24:40.

12. zukerman AJ .Hepatitis virus.In:Baron's Medical Microbiology,(4th edition)Univer of texas Medical Branch.ISBN 1996; 0-9631172-1-1.

13.Dane D.S, Cameron C.H, Briggs M. Virus-like particles in serum of patients with Australian antigen associated hepatitis. Lancet 1970;2:695-8.

14.Landers T.A , Grrenberg H.B Robinson W.S. Structyre of hepatitis B Dane particle DNA and nature of endogenous DNA polymerase reaction J Virol.1977;23:368-76.

15.Hruska JF, ClytonD.Aet al. Structure of hepatitis B Dane particle DNA before and after the Dane particle DNA polymerase reaction. J Virol1977;21:666-72.

16.Tiollais P, Pourcel C, Dejean A . The hepatitis B virus Nature 1985;317:489-95.

17.Takahashi K, Akahane Yet al. Demonstratipn of hepatitis e antigen in the core of Dane particles. J Immunol 1979;122:275-9.

18.RalmondoG, Stemler M et al . Latency and reactivation of a precore mutant hepatitis B virus in a chronically infected patient. J Hepatol1990;11:374-80.

19.Wakita T, Kakumu S et al. Detection of pre C and core region antigen mutants of hepatitis B virus in chronic hepatitis B virus carriers. J Clin Invest 1991;88: 1793-801.

20.Heerman K.H , Goldman U et al. Large surface protein of hepatitis B virus coating the pre-s sequence. J Virol 1984;52:396-402.

21.Stibbe W, Gerlich W.H. Structural relationships between minor and major proteins of hepatitis B surface antigen. J.Virol 1983; 46:626-8.

22.Toh H, Hyashida H et al. Sequence homology between retroviral reverse transcriptase and putative polymerese of hepatitis B virus and cauliflower mosaic virus. Nature 1983;305:827-9.

23.Miller R.H, Robinson W.H. Common evolutionary origin of hepatitis B virus and retroviruses. Proc Natl Acad Sci USA. 1986;83:2531-5.

24.Bosh V, Bartenschlager R et al. The duck hepatitis B virus P- gene codes for protein strongly associated with the 5'-end of the viral DNA minus strand. Virology 1988.;166:475-85.

25.Colgrove R, simon G et al. Transcription activation of homologous and heterologous genes by the hepatitis B virus x gene product in cells permissive for viral replication. J Virol 1989; 63:4019-26.

26.Spandau D F, Lee C.H .Trans-activation of viral enhancers by the hepatitis B virus X protein. V Hepatol 1988; 62:427-34.

27.Bancroft W.H, Mundo F.K et al. Detection of additional determinants of hepatitis B antigens. J Immunol1972;109:842-8.

28.Kidd-Ljunggen K et al.Genetic variability in hepatitis B virus.J Gen Virol 2002;83:1267-1280.

29.Arauz-Ruiz P, Norder H et al. Genotype H: A new new median genotype of hepatitis b virus revealed in Central America. J Gen Virol 2002;83:2059-73.

30.Kao JH, Chen PJ et al .Genotypes And clinical phenotypes of hepatitis b virus in patients with chronic hepatitis B virus infection. Clin Microbiol 2002; 40:1207-09.

31.McMahon BJ, Holck P et al. Serologic and clinical outcome of 1536 Alaska natives chronically infected with hepatitis B virus. Ann Intern Med 2001;135:759-68.

32.Sumi H, Yohisuka O et al. Influence of hepatitis B virus genotypes on the progression of chronic hepatitis B liver disease. Hepatology 2003;37:19-26.

33.Chu CJ, Lok ASF. Clinical significance of hepatitis B virus genotypes. Hepatology 2002;35:1274-76.

34.Chu CJ, KeeffeeEB et al. Prevalence of HBV pre core /core promoter variants in the united States. Hepatology 2003; 38:619-628.

35.Pujoll FH, Novas MC et al. "World wide genetic diversity HBV genotypes and risk of hepatocellular carcinoma". Cancer Lett 2009, Aug 13.

36.Perrillo RP. Acute flares in chronic hepatitis B: The natural and unnatural history of an immunologically mediated liver disease. Gastroenterology 2001;120:1009-1022.

37.Scullard G, Greenberg HB et al. Antiviral treatment of chronic hepatitis B viral infection: Infectious virus can not be detected in patient serum after permanent response to treatment. Hepatology 1982;2:39.

38.Shikata T, KarasawaT et al. Hepatitis e antigen and infectivity of hepatitis B virus. J Infect Dis 1977;136:571.

39.Summers J, Manson W.S .Replication of the genome of a hepatitis B like virus by reverse transcription of an RNA intermediate . Cell 1982;29:403-15.

40Barker L.F, Chisari F et al. Transmission of type B viral hepatitis to chimpanzee. J infect Dis 1973;127:648.

41.Gudat F, Bianchi O et al. Pattern of core and surface expression in liver tissue reflects state of specific immune response in hepatitis B. Lab Invest 1975;32:1.

42. Ray M.B, Desmet V.I et al. Distribution pattern pf hepatitis B surface antigen (HBsAg in liver of hepatitis patients) Gastroenterology 1976;71:462.

43.Huang S.A. Hepatitis associated antigen hepatitis: An electronmicroscopic study of virus like particles in liver cells. Am J Pathol 1971;64

44.Redd, J.; Baumbach, J.; Kohn, W.; Nainan, O.; Khristova, M.; Williams, I.. Patient-to-patient transmission of hepatitis B virus associated with oral surgery (PDF). The Journal of infectious diseases 2007;195 (9): 1311–1314.

45.Alter, M. . Epidemiology and prevention of hepatitis B. Seminars in liver disease 2003;23 (1): 39–46.

46.Ganem D, Prince AM. Hepatitis B virus infection-natural history and clinical consequences. N Engl Med 2004;350:1118-1129.

47.Hoofnagle JH, Doo E et al. Management of hepatitis B:summary of a clinical research workshop. Hepatology 2007;45:1056-1075.

48.Liaw YF. Prevention and surveillance of hepatitis B viral related hepatocellular carcinoma. Semin Liver Dis 2005;25:40-47.

49.Lok AS, McMahon BJ. Chronic hepatitis B. Hepatology 2007;45:507-539.

50.Rizzetto M, Ciancio A. A chronic hepatitis B related liver disease. Mol Aspects Med 2008;29:72-84.

51.Zarski JP, Marcellin P et al. Characteristics of patients with chronic hepatitis B in france: predominant frequency of HBe antigen negative cases. J Hepatol 2006;45:355-360.

52.Chu CM, Liaw YF . Hepatitis B virus related cirrhosis: natural history and treatment. Semin Liver Dis 2006;26:142-152.

53.Fattovich G, Stroffolini T et al. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology 2004;127: S35-S50.

54. EASL Clinical Practice Guidelines: Management of chronic hepatitis B.2009;50:227-42.

55.Krugman S, Overby LR et al. Viral hepatitis type B: studies on natural history and prevention re examined. N Eng J Med 1979;300:101-6.

56.Hoofnagle JH, Seeff LB et al. Serologic responses in hepatitis B . In: Vyas GN, Schmid R,eds .Viral hepatitis : A contemporary Assessment of Epidemiology, Pathogenesis and Prevention. Philadelphia: Franklin Institute Press;1978:219-42.

57.Takahashi K, Fukuda M et al. Determination of e antigen and antibody to e by means of passive haemagglutination method. J Immunol 1979;119:1556-9.

58.Ling c, Mushahwar IK et al.Hepatitis B e antigen and its correlation with other serologic markers in chimpanzees. Infect Immunol 1979;24:352-6.

59.Aldershvile J, Fosner GG et al .Hepatitis B e antigen and antibody radioimmunoassay in acute hepatitis B surface antigen-positive hepatitis. J Infect Dis 1980;141:293.

60.Kaplan PM, Gerin JL et al. Hepatitis B specific-DNA polymerase activity during post transfusion hepatitis. Nature 1974;249:762.

61.Krugman S, Hoofnagle JH et al. Viral hepatits type B: DNA polymerase activity and antibody to hepatitis B core antigen. N Eng J Med 1974;290:1331-5.

62.Hansson BG: Age and sex related distribution of antibodies to hepatitis B surface and core antigens in Sweedish population. Acta Pathol Microbiol Scand, Sect B 1976;84:342.

63.Gerlich WH, Leur W et al. Diagnosis of acute and in apparent hepatitis B virus infection by measurement of IgM antibody to hepatitis B core antigen. J Infect Dis 1980;142:95-101.

64.Seef LB, Wrigt EC et al. Type B hepatitis after needle stick exposure :prevention with hepatitis B immune globulin. A final report of the Veterans Administration Cooperative study. Ann Intern Med 1978;88:285.

65.Scullard GH, Pollard RB et al. Antiviral treatment of chronic hepatitis B viral infection.Changes in viral markers with interferon combined with adenine arabiniside.J Infect Dis . 1981;143:772.

66.Bonino F, Hoyer B et al. Hepatitis B virus DNA in the sera of HBs Ag carriers: A marker of active HBV replication in the liver. Gastroenterology. 1980;79:1009

67.Okada K, Kainiyama I et al. E antigen and anti e antigen in the serum of asymtomatic carrior mothers as indicators of positive and negative transmission of hepatitis B virus in their infants. N Engl J Med 1976;294:746.

68.Perrillo RP,Gelb L et al. Hepatitis B e antigen, DNA polymerase activity , and infection of household contacts with hepatitis B virus .Gastroenterology 1979;76:1319-25.

69.Koshy R, Maupas P et al. Detection of hepatitis B virus- specific DNA in the genome of human hepatocellular carcinoma and liver cirrhosis tissue. J Gen Virol 1981;57:91.

70.Norkrans G, Nordenfeldt E et al. Long term follow up of chronic hepatitis with HBsAg, HBeAg and Dane particles associated DNA polymerase. Scand J Infect Dis 1980;12:159.

71.Zukerman AJ , Tylor PE . Persistence of serum hepatitis (SH-Australia)antigen for many years. Nature 1969;223:89.

72.Realdi G, Alberti A et al. Seroconvertion from hepatitis B e antigen to anti HBeAg in chronic hepatitis B virus infection. Gaseroenterology 1980;79:195.

73.Pirrillo RP, Campbell CR et al. Spontaneous clearance and reactivation of HBV infection among male homosexuals with chronic type B hepatitis. Ann Intern Med 1984; 100:43.

74.Feinman SV, Cooter N et al. Clinical and epidemiological significance of the HBsAg (Australian antigen): Carrier state. Gastroenterology 1975;68:113.

75.Szmuness W, Harley EJ et al. Socio demographic aspects of the epidemiology of hepatitis B.In:Vyas GN, Cohen SN. Viral hepatitis . Philadelphia: Franklin Institute press; 1978:297.

76.Bories P, Coursaget P et al. Antibody to hepatitis B core antigen in chronic active hepatitis. Br Med J 1978; 1:396-7.

77.Martinot-Peignoux M, Boyer N et al. Serum hepatitis B virus DNA levels and liver histology in inactive HBsAg carriers. J Hepatol 2002; 36:542-46.

78.Hadziyannis SJ, Vassilopoulos D. Hepatitis B e antigen negative chronic hepatitis B. Hepatology 2001;34:617-624.

79.Raimondo G, Allain JP et al. Statements from the Taormina expert meeting on occult hepatitis B infection .J Hepatol 2008;49:652-57.

80.Knoll A, Pietrzyk M et al. Solid organ transplantation in HBsAg –negative patients with antibodies to HBV core antigen : low risk of HBV reactivation. Transplantation2005;79:1631-1633.

81.Marcellin P, Giostra A et al. Redevelopment of hepatitis B surface antigen after renal transplantation. Gastroenterology 1991;100:1432-1434.

82.Stroffolini. The changing pattern of hepatitis B virus over the three past decades in Italy. Dig Liver Dis 2005;37:622-27.

83.Dixit VK, Panda K et al . Asymtomatic chronic hepatitis B virus infection in Northern India. Indian J Gasroenterol 2007;26:159-161.

84.Lavanchy D. Hepatitis B virus epidemiology , disease burden, treatment, and current and emerging prevention and control measures. J Viral Hepatol 2004;11: 97-107.

85.World Health Organization. Hepatitis B. World Health Organization Fact Sheet O204(Revised October 2000) WHO web site 2000.http:/who.int/inf-fs/en/fact204.html..last accesed on 27 May 2009.

86. Chang MH. Natural history of hepatitis B infection in children. J Gastroenterol Hepatol 2000; 15 (Suppl): E11-19.

87. Lok AS, Heathcote EJ, Hoofnagle JH. Management of hepatitis B: 2000-Summary of a workshop. Gastroenterology 2001; 120:1828-1853.

88. Lok ASF, McMahon BJ. Chronic hepatitis B. Hepatology 2001; 34: 1225-1241

89. Lok ASF, Lai CL, Wu PC, Leung EKY, Lam TS. Spontaneous hepatits B e antigen to antibody seroconversion and reversion in Chinese patients with chronic hepatitis B virus infection. Gastroenterology 1987; 92: 1839-1843.

90. Realdi G, Alberti A, Rugge M, Bortolotti F, Rigoli AM, Tremolada F, Ruol A. Seroconversion from hepatitis B e antigen to anti-HBe in chronic hepatitis B virus infection. Gastroenterology 1980; 79: 195-199.

91. Yuen MF, Hui CK, Cheng CC, Wu CH, Lai YP, Lai CL. Long-term follow-up of interferon alfa treatment in Chinese patients with chronic hepatitis B infection: the effect on hepatitis B e antigen seroconversion and the development of cirrhosis-relarted complications. Hepatology 2001; 34: 139-145

92. Hoofnagle JH, Dusheiko GM, Seef LB, Jones EA, Waggoner JG, Bales ZB. Seroconversion from hepatitis B e antigen to antibody in chronic type B hepatitis. Ann Intern Med 1981; 94: 744-748.

93. Fattovich G, Rugge M, Brollo L, Pontisso P, Noventa F, Guido M, Alberti A, Realdi G.Clinical, virologic and histologic outcome following seroconversion from HBeAg to anti-HBe in chronic hepatitis type B. Hepatology 1986; 6: 167-172.

94. Moreno-Otero R, Garcia-Monzòn C, Garcia-Sànchez A, Buey LG, Pajares JM, Di Bisceglie AM. Development of cirrhosis after chronic type B hepatitis: a clinicopathologic and follow-up study of 46 HBeAg-positive asymptomatic patients. Am J Gastroenterol 1991; 86: 560-564.

95. Di Marco V, Lo Iacono O, Cammà C, Vaccaro A, Giunta M, Martorana G, Fuschi P, Almasio PL, Craxì A. The long-term course of chronic hepatitis B. Hepatology 1999: 30: 257-264.

96. Chang MH, Hsu HY, Hsu HC, Ni YH, Chen JS, Chen DS. The significance of spontaneous hepatitis B e antigen seroconversion in childhood: with special emphasis on the clearance of hepatitis B e antigen before 3 years of age. Hepatology 1995; 22:1387-1392.

97. Bortolotti F, Jara P, Crivellaro C, Hierro L, Cadrobbi P, Frauca E, Camarena C, De La Vega A, Diaz C, De Moliner L, Noventa F. Outcome of chronic hepatitis B in Caucasian children during a 20-year observation period. J Hepatol 1998; 29: 184-190.

98. Hsu YS, Chien RN, Yeh CT, Sheen IS, Chiou HY, Chu CM, Liaw YF. Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. Hepatology 2002; 35: 1522- 1527.

99. Fong TL, Di Bisceglie AM, Gerber MA, Waggoner JG, Hoofnagle JH. Persistence of hepatitis B virus DNA in the liver after loss of HBsAg in chronic hepatitis B. Hepatology 1993; 18: 1313-1318.

100. McMahon BJ, Holck P, Bulkow L, Snowball M. Serologic and clinical outcomes of 1536 Alaska natives chronically infected with hepatitis B virus. Ann Intern Med 2001; 135: 759-768.

101. Bortolotti F, Cadrobbi P, Crivellaro C, Guido M, Rugge M, Noventa F, et al. Long-term outcome of chronic type B hepatitis in patients who acquire hepatitis B virus infection in childhood. Gastroenterology 1990; 99: 805-810

102. Vajro P, Migliaro F, Fontanella A, Orso G. Interferon: a meta-analysis of published studies in pediatric chronic hepatitis B. Acta Gastroenterol Belg 1998; 61: 219-223.

103. Alward WLM, McMahon BJ, Hall DB, Heyward WL, Francis DP, Bender TR. The long-term serological course of asymptomatic hepatitis B virus carriers and the development of primary hepatocellular carcinoma. J Infect Dis 1985; 151: 604-609.

104. Liaw YF. Current trends in therapy for chronic viral hepatitis. J Gastroenterol Hepatol 1997; 12: S346-S353.

105. Perillo RP. Acute flares in chronic hepatitis B: the natural and unnatural history of animmunologically mediated liver disease. Gastroenterology 2001; 120: 1009-1022.

106. Liaw YF, Tsai SL. Pathogenesis and clinical significance of spontaneous exacerbations and remissions in chronic HBV infection. Viral Hepatitis Rev 1997; 3: 143-154.

107. Sheen IS, Liaw YF, Tai DI, Chu CM. Hepatic decompensation associated with hepatitis B e antigen clearance in chronic type B hepatitis. Gastroenterology 1985; 89: 732-735.

108. Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, Okanoue T, Yotsuyanagi H, Iino S. A case-control study for clinical and molecular biological diffferences between hepatitis B viruses of genotype B and C. Japan HBV Genotype Research Group. Hepatology 2001; 33: 218-223.

109. Chu CJ, Hussain M, Lok ASF. Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared to hepatitis B virus genotype C. Gastroenterology 2002; 122: 1756-1762.

110. Chan HLY, Leung NWY, Hussain M, Wong ML, Lok ASF. Hepatitis B e antigen negative chronic hepatitis B in Hong Kong. Hepatology 2000; 31: 763-768.

111. Funk ML, Rosenberg DM, Lok ASF. World-wide epidemiology of HBeAg-negative chronic hepatitis B and associated pre core and core promoter variants. J Viral Hepat 2002; 9: 52-61.

112. Rizzetto M, Volpes R, Smedile A. Response of pre-core mutant chronic hepatitis B infection to lamivudine. J Med Virol 2000; 61: 398-402.

113. Fattovich G, Farci P, Rugge M, Brollo L, Mandas A, Pontisso P, Giustina G, Lai E, Belussi F, Buratto G, Balestrieri A, Ruol A, Alberti A. Randomized controlled trial of lymphoblastoid interferon alfa in patients with chronic hepatitis B who lacked hepatitis B e antigen. Hepatology 1992; 15: 584-589.

114. Brunetto MR, Oliveri F, Coco B, Leandro G, Colombatto P, Gorin JM, Bonino F. Outcome of anti- HBe positive chronic hepatitis B in alpha-interferon treated and untreated patients: a long term cohort study. J Hepatol 2002; 36: 263-270.

115. Papatheodoridis GV, Manesis E, Hadziyannis SJ. The long-term outcome of interferon-treated and untreated patients with HBeAg-negative chronic hepatitis B. J Hepatol 2001; 34: 306-313.

116.Europian Association for the Study of the Liver. EASL clinical practice guidelines: management of chronic hepatitis B J Hepatol 2009; 50:227-42.

117. Atif Zaman, Kenneth Ingram et al. Diagnostic Liver Biopsy. E-Medicine: Medscape's Continually Updated Clinical Reference . (Updated Nov 18, 2009)

118.METAVIT TFCSG. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C . Hepatology 1994;20:15-20.

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

120.Scheuer P. Classification of chronic viral hepatitis: a need for a reassessment. J Hepatology 1991;13:372-374.

121.Scheuer PJ, Standish RA et al. Scoring of chronic hepatitis. Clin Liver Dis 2002;6:335-347, V-Vi.

122. Scheuer PJ. Assessment of liver biopsies in chronic hepatitis: how is it best done? J Hepatol 2003;38:240-42.

123.Knodell RG, Ishak KG et al. Formulation and application of a numerical scoring system foe assessing histological activity in asymptomatic chronic active hepatitis. Hepatology 1981;1:431-435.

124.Ishak K, Baptista A et al. Histological staging and grading of chronic hepatitis. J Hepatol 1995;22: 696-699.

125.Callea F , Facchetti Fbonera E et al.Significance of Viral Antigens in Liver tissue.In Viral Hepatitis(Ed) Callea F. Springer-varlag Berlin 1986:41-54

126.Sandesh K, Varghese T, Harikumar R, Beena P, Sasidharan VP, Bindu CS, Tony J, Harish K, Sunilkumar K, Ramachandran TM. Prevalence of Hepatitis B and C in the normal population and high risk groups in north Kerala. Trop Gastroenterol 2006 ;27:8

127.Gaeta GB, Stornaiuolo G, Stroffolini T et al. Epidemiological and clinical burden of chronic hepatitis B virus/ hepatitis C virus infection . a multicenter Italian study. J Hepatol 2003;39:1036-1041.

128.Stroffolini T, Almasio PL et .al evolving clinical landscape of chronic hepatitis B: A multicenter Italian study. J Med Virol 2009; 9999:1-8

129.Wai CT ,Fontana RJ et al. Clinical significance of hepatitis B virus genotypes, variants and mutants . clinical Liv Dis 2004;8:321-352.

130.Chen CJ; Yang HI et al. REVEAL –HBV Study group. Risk of Hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level . JMA 2006;295:65-73.

131.Europian Association for the study of Liver: EASL clinical practice Guidelines: management of chronic hepatitis B.J Hepatol 2009;50:227-242.

132.Maiani MK, Boni C et al. Direct ex vivo analysis of hepatitis B virus specific CD8+ T cell associated with the control of infection. Gastroenterology. 1999; 117:1386-96.[pubmed]

133.Webster GJ, Reignat S et al. Logitudinal analysis of CD8+ T cells specific for structural and non structural hepatitis B viral proteins in patients with chronic hepatitis B. Implication for immunotherapy. J Virol.2004; 78: 5707-19.[pubmed]

134.Guoping Peng, Shuping Li et al. Circulating CD4+ CD25+ regulatory T cells correlate with chronic hepatitis B infection. Immunology 2008 January; 123(1): 57-65.

135.Millich DR, Chen NR et al . The secreted hepatitis B pre core antigen can modulate the immune response to the nucleocapsid: a mechanism for persistence. J Immunol 1998; 160:2013-2021.

136.Millich D, Liang TJ. Exploring the biological basiss of hepatitis B e antigen in hepatitis B viral infection. Hepatology 2003; 38:1075-1081.

137.Carmen WF, Jacyna MR et al . Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection Lancet 1989; ii:588-590.

138.Okomoto H, Tsuda F et al. Hepatitis B virus with mutation in the core promoter for an e antigen negative phenotype in carriers with antibody to e antigen. J Virol 1994;68:8102-8110.

139.Margaret Chen, Matti Sallberg et al. Immune tolerance split between hepatitis B virus pre core and core proteins. J of Virol 2005;79:3016-3027.

140.Lok AS. Natural history and control of perinatally acquired hepatitis B virus infection. Dig Dis 1992;10:46-52. [PUBMED]

141.Chowdhury A. Epidemiology of Hepatitis B virus in India. Hep.B Annual 2004;1:17-24.

142.Hadziyannis SJ, Bramou T, Alexopoulou A, Makris A. Immunopathogenesis and natural course of anti-HBe positive chronic hepatitis with replicating B virus. In: Hollinger FB, Lemon SM, Margolis HS, Eds. Viral Hepatitis and Liver Disease. Baltimore: Williams and Wilkins 1991:673-6.

143.Hunt CM, McGill JM, Allen MI, Condreay LD. Clinical relevance of hepatitis B viral mutations. Hepatology 2000; 31:1037-44

144.Miyakawa Y, Okamoto H, Mayumi M. The molecular basis of hepatitis B e antigen (HBeAg)-negative infections. J Viral Hepatol 1997;4

145.Funk ML, Rosenberg DM, Lok ASF. World wide epidemiology of HBeAg negative chronic hepatitis B and associated precore and core promoter variants. J Viral Hepatol 2002;9:52-61

146.Chowdhury A, Santra A, Chaudhuri S et al. Hepatitis C virus infection in the general population: A community -based study in West Bengal, India. Hepatology 2003;37:802-9.

147.Tandon BN, Acharya SK, Tandon A. Epidemiology of hepatitis B virus infection in India. Gut 1996;389(Suppl 2) S56-S

148.Guptan RC, Thakur V, Sarin SK, Banerjee K, Khandekar P. Frequency and clinical profile of precore and surface hepatitis B mutants in Asian-Indian patients with chronic liver disease. Am J Gastroenterol 1996;91:1312-7.

149.Thakur V, Guptan RC, Kazim SN, Malhotra V, Sarin SK. Profile, spectrum and significance of HBV genotypes in chronic liver disease patients in the Indian subcontinent. J Gastroenterol Hepatol 2002;17:113-5.

150. Mangia A, Chung YH, Hoofnagle JH, Birkenmeyer L, Mushahwar I, Di Bisceglie AM. Pathogenesis of chronic liver disease in patients with chronic hepatitis B virus infection without serum HBeAg. Dig Dis Sci. 1996;41:2447–2452. [PubMed]

151. Chu CM, Yeh CT, Sheen IS, Liaw YF. Subcellular localization of hepatitis B core antigen in relation to hepatocyte regeneration in chronic hepatitis B. Gastroenterology. 1995;109:1926–1932. [PubMed]

152. Chu CM, Yeh CT, Chien RN, Sheen IS, Liaw YF. The degrees of hepatocyte nuclear but not cytoplasmic expression of hepatitis B core antigen reflects the level of viral replication in chronic hepatitis B virus infection. J Clin Microbiol. 1997;35:102–105. [PubMed]

153. Hsu HC, Su IJ, Lai MY, Chen DS, Chang MH, Chang SM, Sung JL. Biologic and prognostic significance of hepatocyte hepatitis B core antigen expressions in the natural course of chronic hepatitis B virus infection. J Hepatol 1987;5:45–50. [

154.. Hsu YS, Chien RN, Yeh CT, Sheen IS, Chiou HY, Chu CM, Liaw YF. Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. Hepatology 2002;35:1522–1527. [PubMed]

155.. Yuen MF, Yuan HJ, Hui CK, Wong DK, Wong WM, Chan AO, Wong BC, Lai CL. A large population study of spontaneous HBeAg seroconversion and acute exacerbation of chronic hepatitis B infection: implications for antiviral therapy. Gut 2003;52:416–419. [PubMed]

156. ter Borg F, ten Kate FJ, Cuypers HT, Leentvaar-Kuijpers A, Oosting J, Wertheim-van Dillen PM, Honkoop P. Relation between laboratory test results and histological hepatitis activity in individuals positive for hepatitis B surface antigen and antibodies to hepatitis B e antigen. Lancet 1998;351:1914–1918. [PubMed]

157. Chan HL, Hui Y, Leung NW, Ching JY, Chan FK, Sung JJ. Risk factors for active liver disease in HBeAg-negative chronic hepatitis B virus-infected patients. Am J Gastroenterol. 2000;95:3547–3551. [PubMed]

158. Lindh M, Horal P, Dhillon AP, Norkrans G. Hepatitis B virus DNA levels, precore mutations, genotypes and histological activity in chronic hepatitis B. J Viral Hepat. 2000;7:258–267. [PubMed]

159. Karayiannis P, Fowler MJ, Lok AS, Greenfield C, Monjardino J, Thomas HC. Detection of serum HBV-DNA by molecular hybridisation. Correlation with HBeAg/anti-HBe status, racial origin, liver histology and hepatocellular carcinoma. J Hepatol 1985;1:99–106. [PubMed]

160.. Lindh M, Gustavson C, Mardberg K, Norkrans G, Dhillon AP, Horal P. Mutation of nucleotide 1762 in the core promoter region during hepatitis B e seroconversion and its relation to liver damage in hepatitis B e antigen carriers. J Med Virol. 1998;55:185–190. [PubMed]

161. Kidd-Ljunggren K, Oberg M, Kidd AH. Hepatitis B virus X gene 1751-1764 mutations: implications for HBeAg status and disease. J Gen Virol 1997;78:1469–1478. [PubMed]

162. Li J, Buckwold VE, Hon MW, Ou JH. Mechanism of suppression of hepatitis B virus precore RNA transcription by a frequent double mutation. J Virol 1999;73:1239–1244. [PubMed]

163. Buckwold VE, Xu Z, Chen M, Yen TS, Ou JH. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J Virol 1996;70:5845–5851. [PubMed]

164. Wu PC, Lau JY, Lau TK, Lau SK, Lai CL. Relationship between intrahepatic expression of hepatitis B viral antigens and histology in Chinese patients with chronic hepatitis B virus infection. Am J Clin Pathol 1993;100:648–653. [PubMed]

165.Wee A, yap I et al. Hepatocyte hepatitis B surface antigen expression in chronic hepatitis B virus carriers in Singapore: correlation with viral replication and liver pathology. Hepatology 1998; 8:749-54.

166.Ray MB, Desmet VJ et al. differential distribution of hepatitis b surface antigen and hepatitis B core antigen in the liver hepatitis B patients. Gastroenterology 1976;71:462-9.

167.Hsu HC, Lai MY et al. Correlation of hepatocyte HBsAg expression with viral replication and liver pathology.Hepatology 1988; 8:749-54.

168.Chu CM, Liaw YF et al .Membrane staining of hepatitis B surface antigen on hepatocytes : a sensitive and specific marker of active viral replication in hepatitis B . J Clin Pathology 1995;48:470-3.

169. Ramakrishna Bhanumathy , Mukhopadhya A, Kurian George . Correlation of hepatocyte expression of hepatitis B viral antigens with histological activity and viral titer in chronic hepatitis B viral infection: An immunohistochemical study. J of Gastrol and Hepatol2008;23:1734-1738.

170.Hay-chi Hsu,Ming-Yang Lai et al. Correlation of hepatocyte hBsAg expression with virus replication and liver pathology.Hepatology 2005;8:749-754.