EVALUATION OF AUTOANTIBODIES AND HEPATITIS VIRAL MARKERS IN NIGERIANS WITH LIVER DISEASES

BY

JESSE ABIODUN OLUBANJO OTEGBAYO

MBBS (IBADAN), M. Sc., (IBADAN).

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A THESIS IN THE DEPARTMENT OF CHEMICAL PATHOLOGY, SUBMITTED TO THE FACULTY OF BASIC MEDICAL SCIENCES, COLLEGE OF MEDICINE, IN PARTIAL FULFILLMENT OF REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF THE UNIVERSITY OF IBADAN.

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ABSTRACT

Liver disease is a major public health problem. Several diagnostic and aetiologic agents of liver diseases are known, but autoantibodies to liver antigens in Nigerians with liver diseases have not been extensively studied. The study was therefore aimed at detecting the types of autoantibodies and viral antigens in various liver diseases among Nigerians.

One hundred and twenty six consecutive patients with liver diseases and 82 apparently healthy controls were recruited at UCH Ibadan. Clinical features and history of alcohol consumption were documented. Serum samples were analysed for Antinuclear Antibodies (ANA), Antimitochondrial Antibodies (AMA), perinuclear Antineutrophil Cytoplasmic Antibodies (pANCA), anti-Soluble Liver Antigen/Liver Pancreas (anti-SLA/LP) and anti-Liver-Kidney Microsomal-1 (anti-LKM-1) by enzyme linked immunosorbent assay. Similarly, hepatitis B surface antigen (HBsAg), Hepatitis B c Antigen (HBcAg), antibody to HBcAg (anti-HBc), antibody to hepatitis B core antigen (anti-HBc) and antibody to hepatitis C virus (anti-HCV) were analysed. Hepatitis B virus (HBV) DNA was determined by PCR, and samples positive for HBV s-plasmid DNA by electrophoresis were sequenced for genotypes. Liver function and prothrombin time were determined. Data were analysed using relative frequency, odds ratio, Pearson's Chi square, Fisher's exact test and Students' t-test at 5% level of significance.

About 75% were males with mean age of 47.5±14.4 yrs. Sixty one percent had Hepatocellular Carcinoma (HCC), 25.4% Liver Cirrhosis (LC), 7.9% Chronic Hepatitis (CH), 3.2% Acute Viral Hepatitis (AVH) and 1.6% Primary Biliary Cirrhosis (PBC). Hepatomegaly occurred in 78.6%, ascites in 57.1%, 51.3% consumed significant (≥ 80g/day for 5 years) alcohol. There was no difference in ANA among cases and controls. AMA was detectable in 60.3% of cases compared to 43.9% of controls ($p<0.05$). One case and one

control were positive for anti-LKM-1 while all subjects were negative for anti-SLA/LP and pANCA. Anti-HBc was detected in 93.7% of cases and 73.2% controls. The prevalence of HBsAg, HBeAg anti-HBe and anti-HCV were significantly higher in cases than controls ($p<0.05$). The HBV-DNA was higher among cases (46%) than controls (1.2%) (odds ratio 27.3). AVH patients had the highest HBV-DNA viral load with a range of zero to 14 million copies/μL and a mean of 751.86 copies/ μL. Geometric mean HBV-DNA were 63.6, 43.15 and 7.2 copies/ μL amoog cases with LC, HCC and CH respectively. Proportions of CH (40%) and LC (34.4%) with ANA were not significantly higher compared to controls (39.7%), but was significantly higher in HCC patients 61.9% compared to controls. The AMA was significantly higher in CH and HCC compared with controls. HBsAg was significantly higher in HCC compared to controls and other liver cases. HBeAg, anti-HBe, anti-HBc, anti-HCV and HBV-DNA were significantly higher in CH, LC and HCC compared to controls ($p<0.05$). There were 53 genotype E and two genotype A in cases while only one control had genotype E.

Prevalence of autoantibodies to liver antigens is similar in individuals with or without liver disease and, therefore not reliable in predicting autoimmune liver disease. HBsAg, anti-HBcAg, anti-HCV and HBV-DNA were strongly associated with liver disease. HBV genotype E is predominant in Nigeria.

KEYWORDS: Autoantibodies, Viral markers, Liver disease, Nigeria.

WORD COUNT: 500

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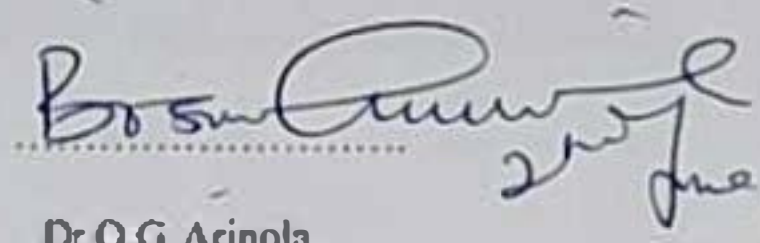
Finally, I give all the glory to my creator for the mental, physical, emotional and spiritual endowment, which he pleased to give me, and for sustaining me all the way.

DEDICATION

I dedicate this Thesis to the Late Professor Lekan Samusa Salimonu, who was called to glory while still on active duty supervising this work, for his fatherly love and thoroughness.

CERTIFICATION

This work was carried out by Jesse Abiodun Otegbayo under my direct supervision in the Department of Chemical Pathology, University of Ibadan, Ibadan.



Dr O.G Arinola

BSc., MSc., PhD.

Department of Chemical Pathology,

University of Ibadan, Ibadan

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CHAPTERONE

INTRODUCTION

1.0 The Liver

The liver is the seat of the major metabolic processes in the body and it performs key roles in carbohydrate, fat, protein, vitamins and mineral metabolism, including drug detoxification and bilirubin excretion. As a result of its key roles in energy homeostasis, it enjoys a dual blood supply from the portal vein and the two hepatic arteries (Figures 1 and 2), which amounts to 20% of the cardiac output, therefore in constant contact with immunological factors and cells from blood circulation. This is in addition to the presence of resident unconventional T lymphocyte subpopulations in the normal adult human liver which may have specialised functions in regional immune responses (Norris *et al.* 1998; Doherty *et al.* 1999) as well as other immune cells which are visible on microscopy (Figure 3).

The biliary system which are the main target organ in some autoimmune liver diseases like primary biliary cirrhosis and primary sclerosing cholangitis (Petrogiannopoulos *et al.* 2004), usually originate as bile canaliculi in the hepatic portal triads and subsequently form bile ductules before finally coalescing into the right and left bile ducts (Figures 1 and 2).

Diseases of the liver and biliary system are major causes of illness and death worldwide and the diseases range from viral hepatitis to gallstones, alcoholic hepatitis, fatty liver disease, inherited and congenital disorders, liver conditions caused by toxins or medications, liver and bile duct cancers as well as autoimmune liver and biliary conditions.

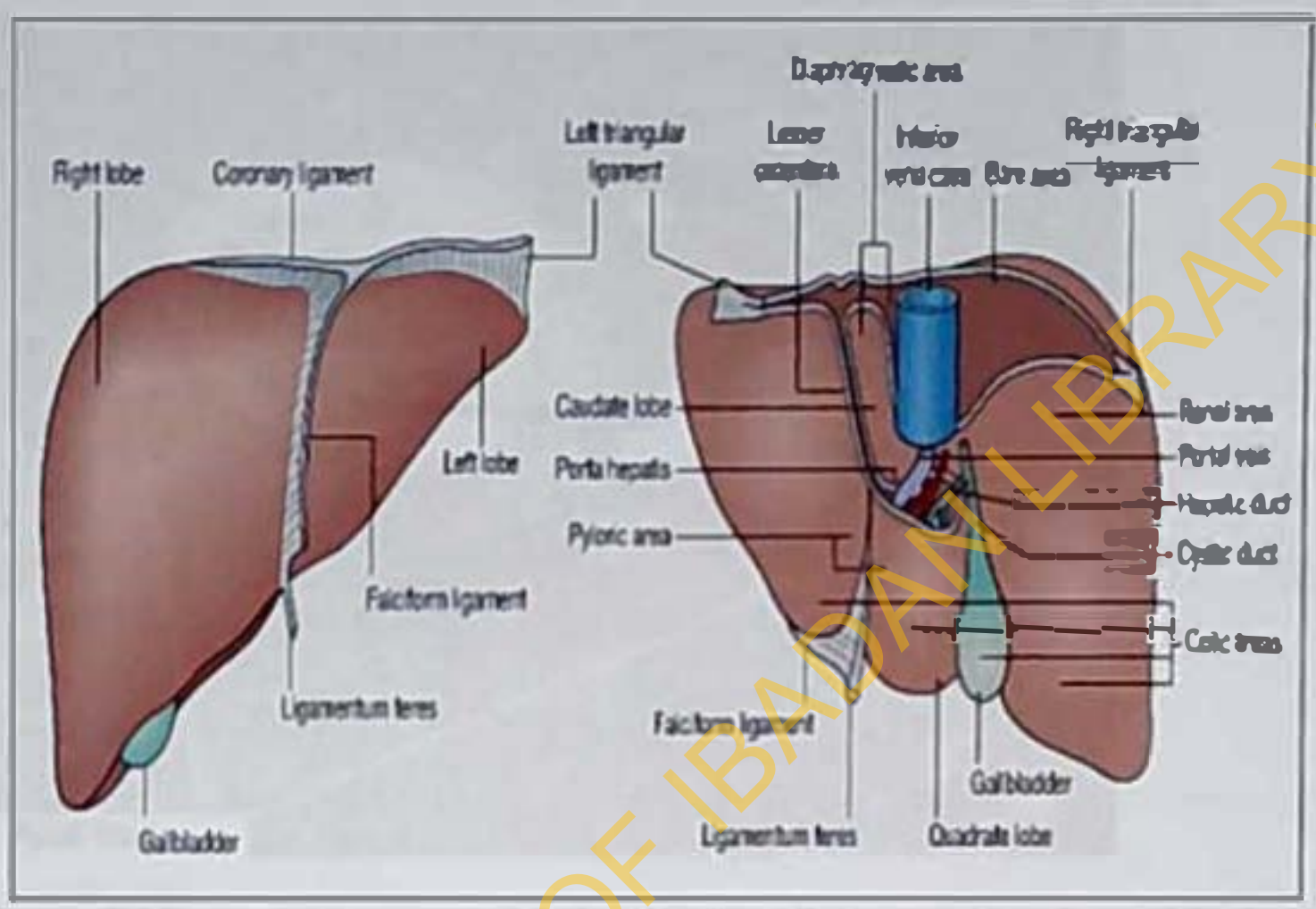


Figure 1. Gross anatomy of the liver showing the vascular, biliary and hepatic features.

Source: Zakim and Boyer Textbook of Hepatology.

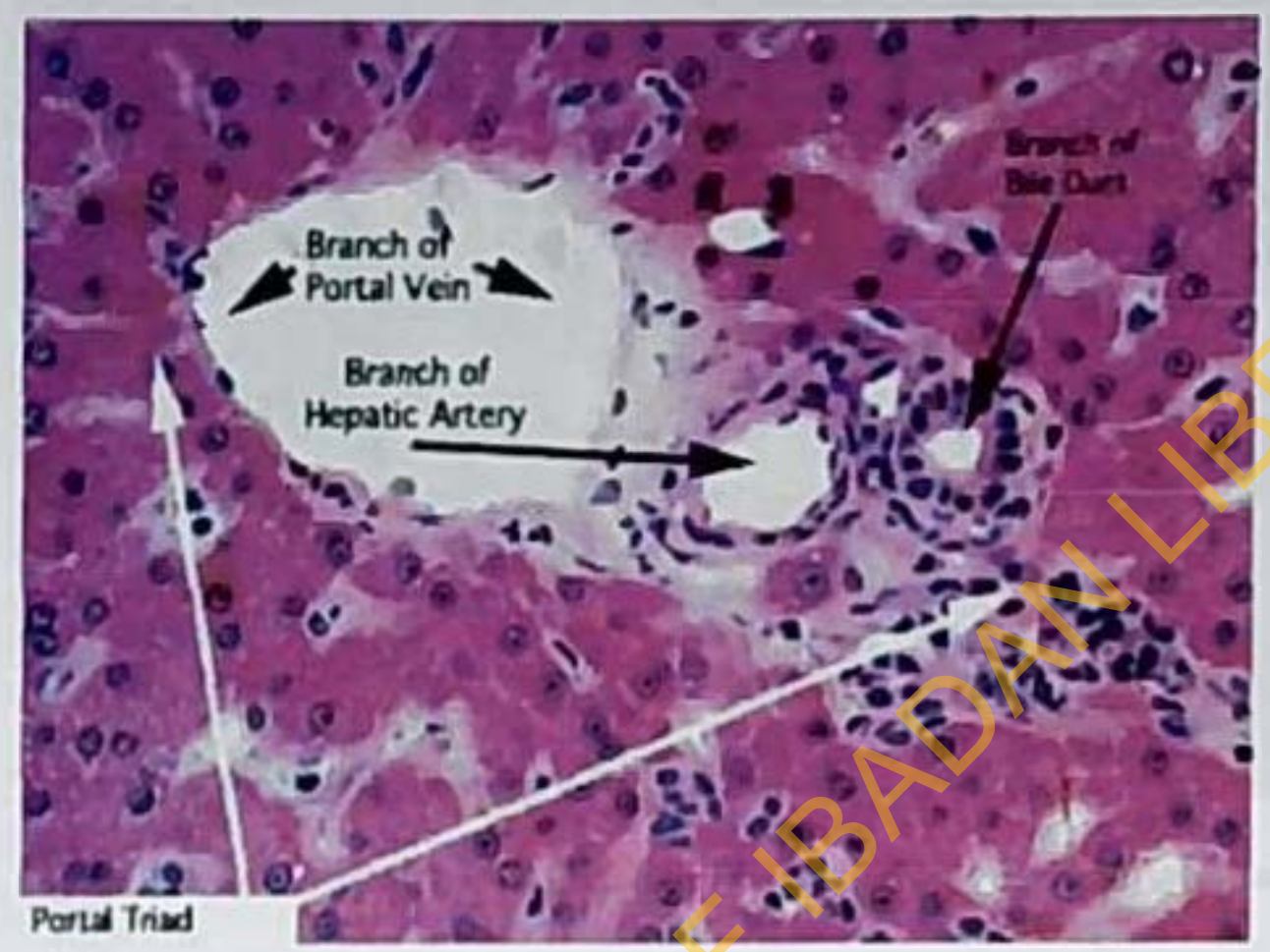
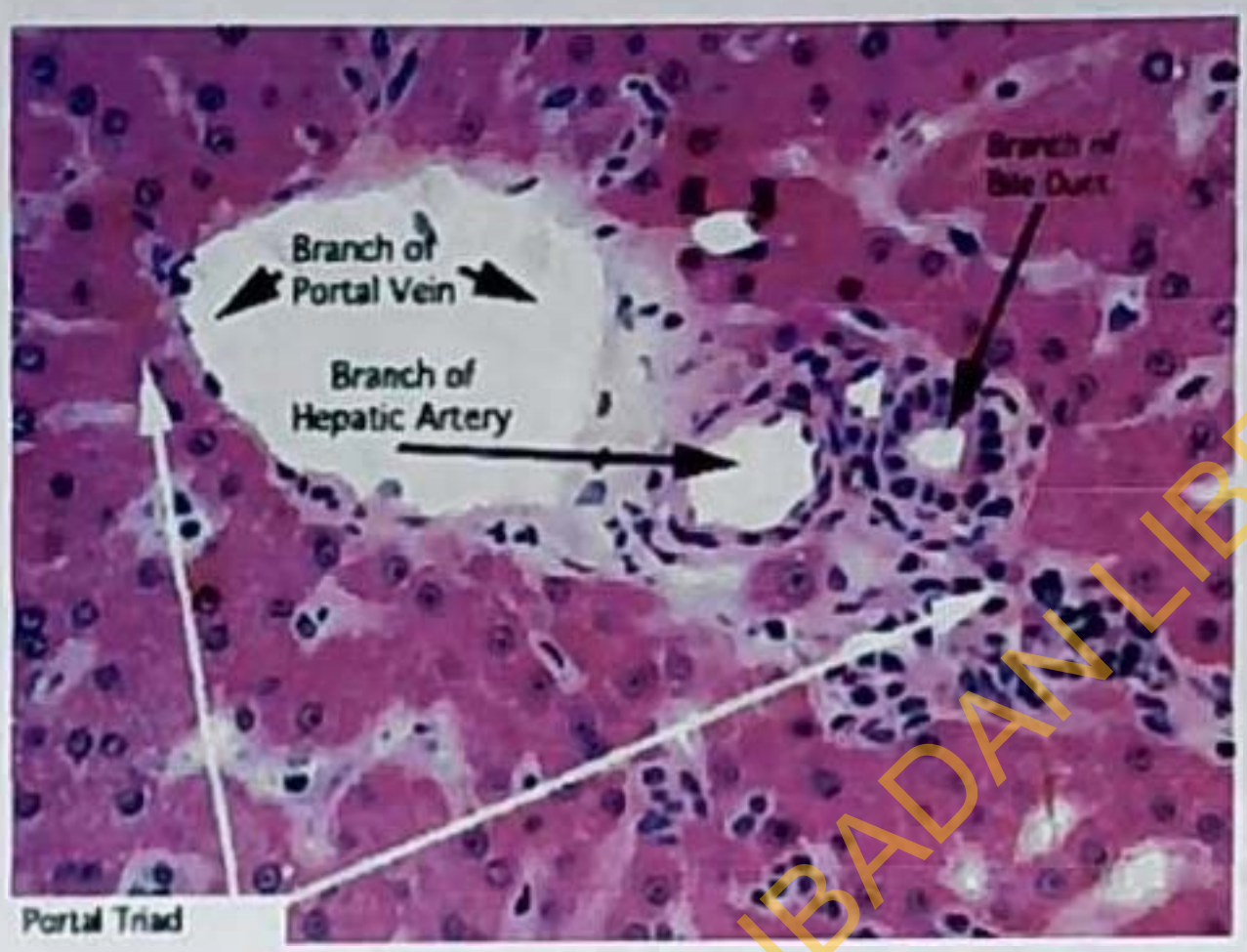


Figure 2. Microscopic anatomy of the liver showing the vascular supply and bile duct

Magnification: X40.

H&E stain

Source: WebPath



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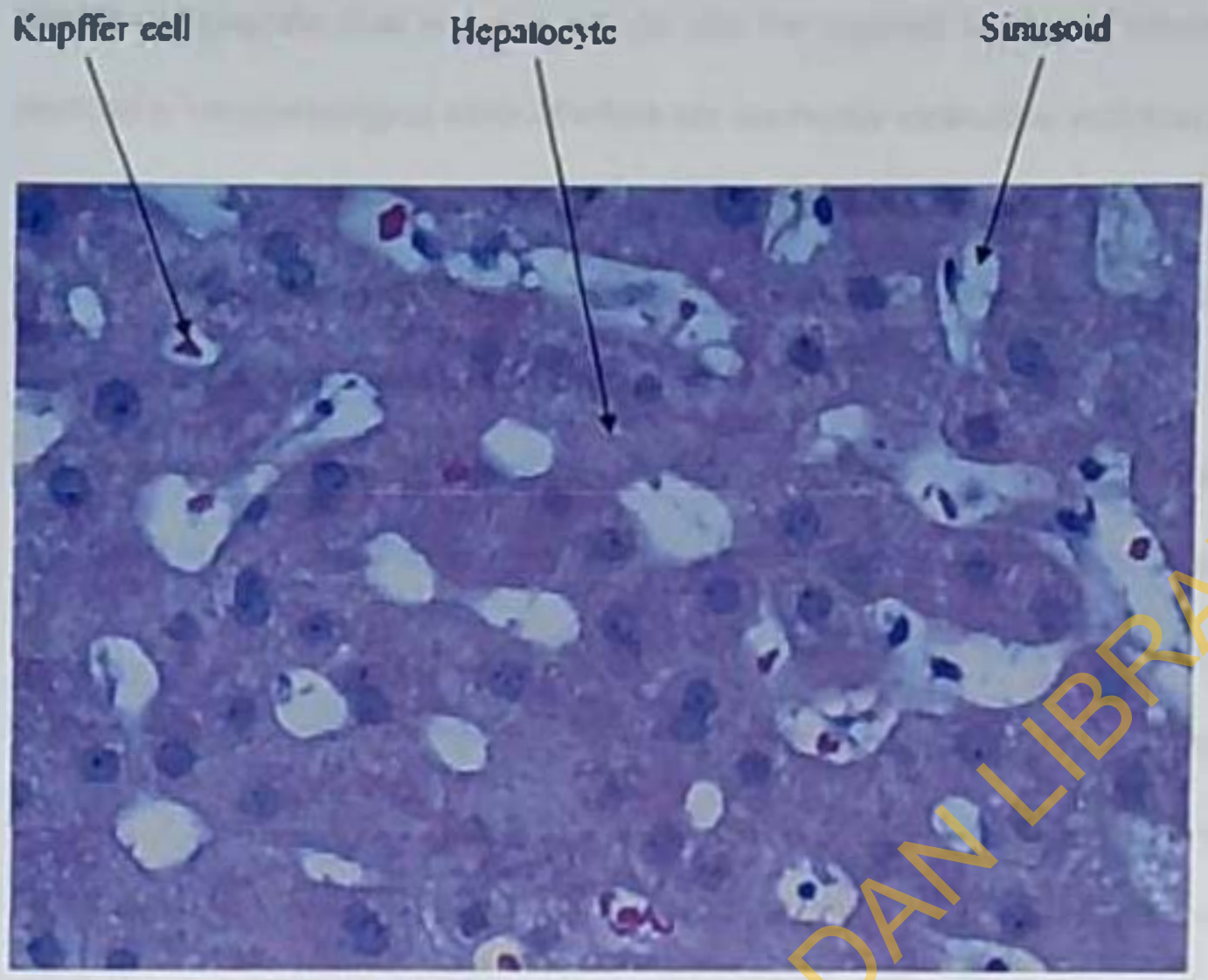

Figure 3. Microscopic anatomy of the liver showing hepatocytes, sinusoids and Kupffer cells

Magnification: X40

H&E stain.

Source: WebPath

The liver being the first major metabolic site for ingested food and substances is exposed to various antigens some of which are xenobiotic molecules, endotoxins and microbial degradation products, which may be injurious to the liver (Lumsden *et al.* 1988). It is not surprising; therefore that the liver participates in immunological activity in the body through specialized cells.

Two cellular elements of the liver are generally recognized: hepatic parenchymal and non-parenchymal cells. Parenchymal cells are hepatocytes that endogenously express MHC Class I molecules, as a result of which they do not elicit strong immune response and are not involved in antibody mediated immune response (Renz and Freise, 2001). On the other hand the non-parenchymal fraction, comprising of bile duct epithelium, dendritic cells, Kupffer cells, Ito cells, interstitum and vascular endothelium, are immunologically active, express both MHC Classes I and II molecules and are also active in antigen presentation (Renz and Freise, 2001).

1.1 Justification for the study

Liver diseases, especially viral hepatitis, are well known to contribute to morbidity and mortality among the Nigerian population (Bojuwoye: 1997). Autoimmune diseases are among the leading causes of death among young and middle-aged women in the United States (Cooper and Strochlo, 2003) and other parts of the world.

No published data are available in developing countries about the prevalence, morbidity and mortality of autoimmune disorders and autoimmune liver disease in particular. There is a need to generate data to fill the gap in knowledge about autoimmune liver diseases and viral hepatitis in Nigeria, and possibly open new vistas in this neglected area of Hepatology in Nigeria.

Indirect immunofluorescence (IIF) has been the standard technique for detecting autoantibodies with the use of substrates such as rat liver kidney or stomach (Kerkar *et al*; 2002). However, the cumbersome nature of the technique, lack of standardization and recent identification of antigenic targets of various autoantibodies has led to the development of ELISA techniques with the advantage of ease, standardization and reproducibility. Moreover, studies have shown agreement between the ELISA and the IIF techniques (Rizzetto & Doniach 1973; Kerkar *et al*, 2002; Vergani and Mieli-Vergani, 2004). This study utilized the ELISA technique in detecting autoantibodies in our study subjects.

1.2 Rationale and objectives

Liver disease is the 5th common cause of death in the UK (Iredale, 2008) and according to data from the Centre for Disease and Control (CDC), the 12th most common cause of death in the USA (Davies and Roberts 2010). In developed countries where data exist on autoimmune liver diseases, it has been shown that autoimmune diseases of the liver or biliary system are significant causes of end stage liver disease (ESLD) which account for approximately 20% of all liver transplantations performed annually in the United States of America (Renz and Freise, 2001), 2.6% of liver transplants at the European Liver Transplant Registry (Milkiewicz et al. 1999) and 5.6% of liver transplantation at the National Institutes of Health (NIH), USA (Wicsner *et al*, 1998).

Low prevalence of autoimmune diseases such as rheumatoid arthritis and vitiligo have been encountered in Nigeria (Adelowo et al. 1998, Talabi *et al* 2003), though, others showed rarity of autoimmune disorders among Nigerians (Famuyiwa and Bella,

1990). Moreover, chronic infections such as Hepatitis B and C, have been found to be accompanied by serological markers of autoimmune liver disease, being more prevalent with HCV than HBV (Clifford *et al.* 1995; Jaeckel and Manns 2005).

There has been no report on autoimmune liver diseases in Nigeria, and only a few reported cases in Africa. There is therefore need to determine the prevalence of autoimmune liver diseases in hospital setting, role of autoimmune factors and viral agents in the pathology of liver disease among Nigerians.

Objectives:

A. Overall objective

The overall objective of this study was to investigate the contribution of autoimmunity to the burden of liver diseases in Nigeria, and correlate with established viral causes of liver diseases such as hepatitis B and C, and alcohol.

B. Specific objectives

The specific objectives of this study were to determine the:

1. prevalence and pattern of specific serum autoantibodies ANA, pANCA, LKM-1, anti-SLA/LP and AMA among patients with liver diseases,
2. prevalence of HBsAg, HBeAg, Anti-HBe, anti-HBc, anti-HCV in patients with liver diseases,
3. frequency of HBV-DNA and viral load among patients with liver diseases in Nigeria, and
4. interactive role of autoimmunity and HBV/HCV markers in the pathogenesis of liver diseases in Nigeria and generate local baseline data on autoimmune liver diseases in Nigeria.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Immunity

Immunity, as it is used today, derives from its earlier usage referring to exemption from military service or from paying taxes. It became a word for protection against infection over a hundred years ago after Pasteur's historic vaccination experiment with attenuated anthrax bacilli in 1881 (Carter, 1988; Stembach, 2003). Immunity is therefore defined as the ability of an organism to protect itself against pathogens. There is therefore an innate ability in organisms to recognize what is self-antigen and what is non-self antigen.

Autoimmunity is an immunological term first introduced by Paul Ehrlich in 1900, in a theory he called "horror autotoxicus", to explain a phenomenon, which is quite different from what the current knowledge is about autoimmunity (Ehrlich and Morgenroth, 1957).

Autoimmunity refers to the ability of the body's immune system to recognize self-antigens. The selective unresponsiveness to self-antigens, termed self-tolerance, is a fundamental feature of the normal immune system. Autoimmune diseases, therefore, develop when there is a breakdown in this self-tolerance (Nossal, 1983; Romagnani, 2006). Autoimmunity, an inherent property of the immune system, leads to autoimmune disease through a pathological process of autoaggression. Other suggested causes of autoimmune diseases are release of normally sequestered antigens, deletion of self antigen recognizing T and B cells and hyperactivity of T-helper cells (Feldmann, 1989).

In spite of advances in Hepatology, a wide gap still exist in knowledge regarding the pathogenetic mechanisms and treatment of liver conditions like hepatic steatosis, liver fibrosis, hepatocarcinogenesis and autoimmune liver diseases, making it imperative for continuation of translational basic and clinical studies that will lessen the burden of liver diseases.

2.2 Mechanisms of Autoimmunity

Autoimmunity is the process by which the host immune system causes disease in the individual and is usually associated with autoantibodies, which are antibodies which have the ability to attack self antigens. The pathogenic role of the autoantibody, however should meet rigorous criteria, such as: it must be detectable in patients with relevant disease in a significantly higher concentration or significantly higher frequency than in the normal population; the autoantibody must be directed against a physiologically or pathogenically relevant antigen; and the disease is reproduced upon injection or induction of the autoantibody in experimental animals (Peter and Shen, 2006). These criteria are however, not always met.

Autoimmunity is an inherent property of the normal immune system as all T and B lymphocytes start out with capacity of autoimmune cells before undergoing some developmental changes and becoming tolerant of self antigens. Clonal deletion and anergy which occur in early life are recognized fundamental mechanisms responsible for self tolerance (Cohen and Young, 1991).

A successful immune response to potentially harmful microorganisms usually depends on the specific recognition of foreign antigens by T and/or B-lymphocytes. Recognition by T-cells involve the T-cell receptor for antigen (TCR), and antigens are

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presented by MHC II molecules on antigen presenting cells (APC). B-cells bind antigen through surface immunoglobulin (Ig), which functions as the specific B-cell receptor for antigen (Kotzin, 2001). The TCR and B-cell receptor (BCR) have the capability to respond to and recognize unlimited number of foreign antigens, but do not normally respond to self-antigens (tolerance). The failure of this selective recognition of self- and non-self results in autoimmune reaction and forms the basis of autoimmune disorders.

Autoimmune disease could broadly be divided into multisystemic and organ-specific in terms of presentation. Virtually all organs in the human body can have organ specific autoimmune disease. Division of autoimmune diseases could also be based on whether the pathology of the disease is mediated by autoantibodies or by autoreactive T-cells. The mechanisms that trigger autoimmune disorders are diverse and complex, and involve interaction of genetic and environmental factors. A conceptual framework for the pathogenesis of autoimmune diseases has been suggested as shown in Figure 4. (Kotzin, 2001). There is a considerable body of knowledge to suggest a strong genetic basis for the development of autoimmune disease. Czaja et al (2002) in a review concluded similarly.

Susceptibility to autoimmunisation is known to depend on multiple genes and not on individual gene, environmental factors or disturbance of the immune system. It is fairly well established that environmental factors, Major Histocompatibility Complex (MHC) genes and "non-MHC genes" interact to promote autoimmunisation.

Well recognized autoimmune diseases are Type-I diabetes mellitus, rheumatoid arthritis, scleroderma, systemic lupus erythematosus, dermatomyositis, myasthenia gravis, Grave's disease and autoimmune liver diseases such as chronic active

hepatitis, primary biliary cirrhosis and primary sclerosing cholangitis. Other examples of autoimmune diseases are listed in Table I.

2.3 Autoantibodies

Autoantibodies are immunoglobulins that react with normal host proteins and may be physiologic or pathologic (Czaja, 1995). The physiologic autoantibodies, also known as polyreactive antibodies, do not fix complements and are produced by the normal humans and animals. They are found in low concentrations in the serum of normal humans of all ages, though commoner in women than in men (Hooper *et al*, 1972). Their origin has not been clearly defined but two hypothesis that explained the origin of autoantibodies suggested that B cells that evaded clonal deletion early in life and are permitted by the suppressor mechanism to produce minute quantities of autoantibodies. The second postulate is that autoantibody formation occurs as a result of cross reaction between foreign and self-determinants. It is suggested that the part of the B cell population which gives rise to autoantibodies carries a polyspecific receptor. Fixation of a foreign antigen to this receptor induces the B cell to undergo a series of divisions and mutations, which under the selective pressure of the antigen leads to production of a highly specific antibody.

Thus, physiologic or natural autoantibodies may constitute the antibodies secreted by B cells prior to encountering foreign antigens (Tomer and Shoenfeld, 1988). They are usually low affinity IgM isotype, though IgA and IgG isotypes are also found. CD5+ cells, which represent 10-25% of circulating B lymphocytes have been found to produce natural autoantibodies (George and Shoenfeld, 1996). 40-50% of fetal B cells are CD5+ whereas only 2-3% from adult lymph nodes are CD5+.

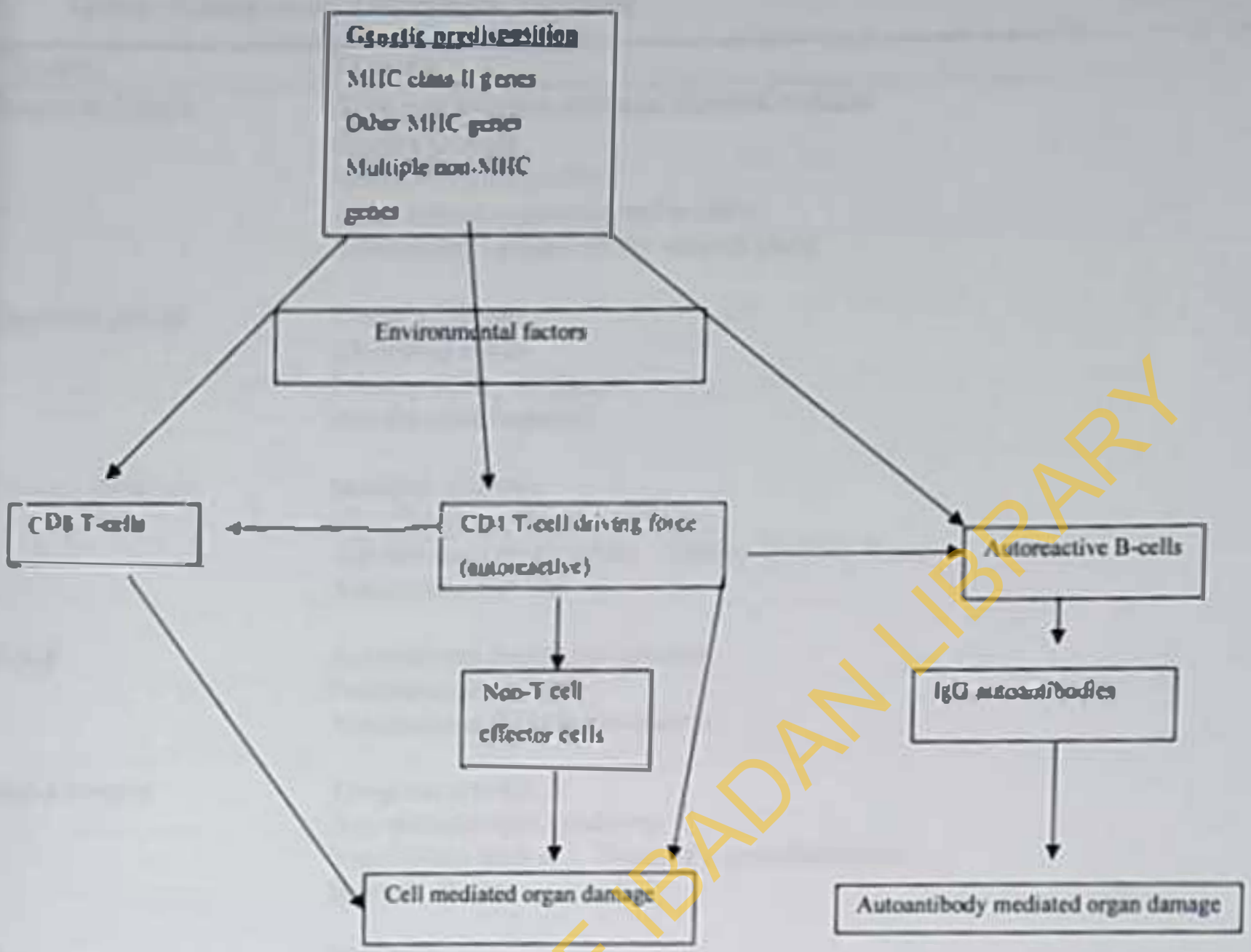


Figure 4. Steps involved in the pathogenesis of autoimmune disease
Source: Clinical Immunology, Principles and Practice

Table1: Examples of Autoimmune Diseases

System	Diseases
Endocrine Glands	Type 1 or immune mediated diabetes mellitus Grave's Disease Hashimoto's thyroiditis Autoimmune oophoritis and orchitis Autoimmune disease of the adrenal gland
Digestive system	Crohn's Disease Ulcerative colitis Primary biliary cirrhosis Autoimmune hepatitis
Nervous Systems	Multiple Sclerosis Myasthenia gravis Autoimmune neuropathies such as Guillain-Barré Autoimmune uveitis
Blood	Autoimmune hemolytic anaemia Pernicious anaemia Autoimmune thrombocytopaenia
Blood Vessels	Temporal arteritis Anti-phospholipid syndrome Vasculitides such as Wagener's granulomatosus Behcet's disease
Skin	Psoriasis Dermatitis herpetiformis Vitiligo Pemphigus vulgaris
Musculoskeletal Systems	Rheumatoid Arthritis Systemic Lupus Erythematosus Scleroderma Polymyositis, Dermatomyositis Spondyloarthropathies such as ankylosing spondylitis. Sjogren's syndrome

It has also been found that CD5+ cells are greatly increased in some autoimmune diseases in rats and man. Natural autoantibodies are known to be common in the first degree relatives of individuals with autoimmune diseases and in the elderly, they could also be found in patients with bacterial, viral or parasitic infections when they may exert a protective effect (George and Shoenfeld, 1996). Natural autoantibodies, although sometimes reactive with the same antigen, differ from autoantibodies produced by CD5-B cells, which are usually monoreactive and have high affinity and are typically detectable only in autoimmune individuals (Cassali and Notkins, 1989; Nossal, 1989; Hentati *et al*, 1991).

One other major difference between pathogenic and non-pathogenic autoantibodies is that the former use a restricted number of VH gene segments while the latter uses an assortment of VH gene segments (Coutinho *et al*, 1995).

Natural autoantibodies may however have a potential role in the pathogenesis of autoimmune disease, as they have been found, like pathogenic antibodies, to increase in disease (Hentati *et al*, 1991; George and Shoenfeld, 1996). In addition, in experimental animal models, immunization with natural autoantibodies has been shown to cause end-organ damage (George and Shoenfeld, 1996). Grabar in 1975, however hypothesized that natural autoantibodies are part of a physiologic mechanism for cleansing the organism of self and non-self products in which classical antibodies serve to clear the body of foreign invading agents, while natural autoantibodies rid the organism of its own catabolic product. It has also been suggested that natural autoantibodies function in removing senescent or altered molecules, cells and tumours (Lacroix-Desmazes et al, 1998). The beneficial effect of this scavenger role is the prevention of emergence of autoreactive immunocytes and has a protective function

(Tomer and Shoenfeld, 1988; Coutinho *et al* 1995; Dighiero, 1997; Casali and Schettino, 1999).

The mechanism by which autoantibodies damage their target organs have not been well studied, but are known to be several. The mechanisms include immune complex formation, opsonisation and receptor inhibition or stimulation among others. (Schwartz, 1993).

2.4 Structure and function of the Liver

Parenchymal cells constitute about 80% of the liver volume (Figures 1 and 2), mainly made up of hepatocytes, while the non-parenchymal cells constitute only 6.5% of the liver, but 40% of the cells. The walls of hepatic sinusoid are lined by three different cell types: sinusoidal endothelial cells (SEC), Kupffer cells (KC), and hepatic stellate cells. Liver sinusoidal endothelial cells constitutively express all molecules necessary for antigen presentation (CD54, CD80, CD86, MHC class I and class II and CD40) and can function as antigen-presenting cells for CD4+ and CD8+ T cells. Thus, these cells are thought to contribute to hepatic immune surveillance by activation of effector T cells (Knolle and Gerken, 2000). They are also active in the secretion of cytokines, eicosanoids (prostanoids and leukotrienes), endothelin-1, nitric oxide, and some extracellular matrix components (Kmiec, 2001).

2.5. TYPES OF LIVER DISEASES

2.5.1 Autoimmune hepatitis

Autoimmune hepatitis is the most common of the autoimmune liver diseases affecting women more than men (Czaja, 2003) . It is characterized by chronic progressive

inflammation of the liver due to an autoimmune process which, if left untreated, eventually leads to cirrhosis (Wies. 2006). Cirrhosis (Figures 5a and 5b), being the final common pathway for most chronic liver diseases is a premalignant condition which could culminate in hepatocellular carcinoma (Figure 6). Acute and fulminant hepatitis, though uncommon have been described with AIH, with high fatality.

The primary event that triggers AIH is not well known. It is however suggested that there is a genetic predisposition to autoimmunity, which interact with certain environmental factors such as viruses, drugs and toxins, to induce AIH.

Autoimmune hepatitis are of two major types: Type I and Type II, based on the distribution or frequency of the autoantibodies in them. Type I AIH patients are positive for anti-nuclear antibodies (ANA), anti-smooth muscle antibodies (SMA), anti-soluble liver antigen/Liver pancreas autoantibodies (SLA/LP), perineutrophil cytoplasmic antibodies (pANCA), anti-asialoglycoprotein antibodies (Anti-ASGRP), while Type II are positive for anti-liver kidney microsomal enzyme-1 (LKM-1), anti-liver kidney microsomal enzyme-3 (LKM-3), anti-liver cytosol -1 antibodies (LC-1), Anti-ASGRP (Wies 2006).

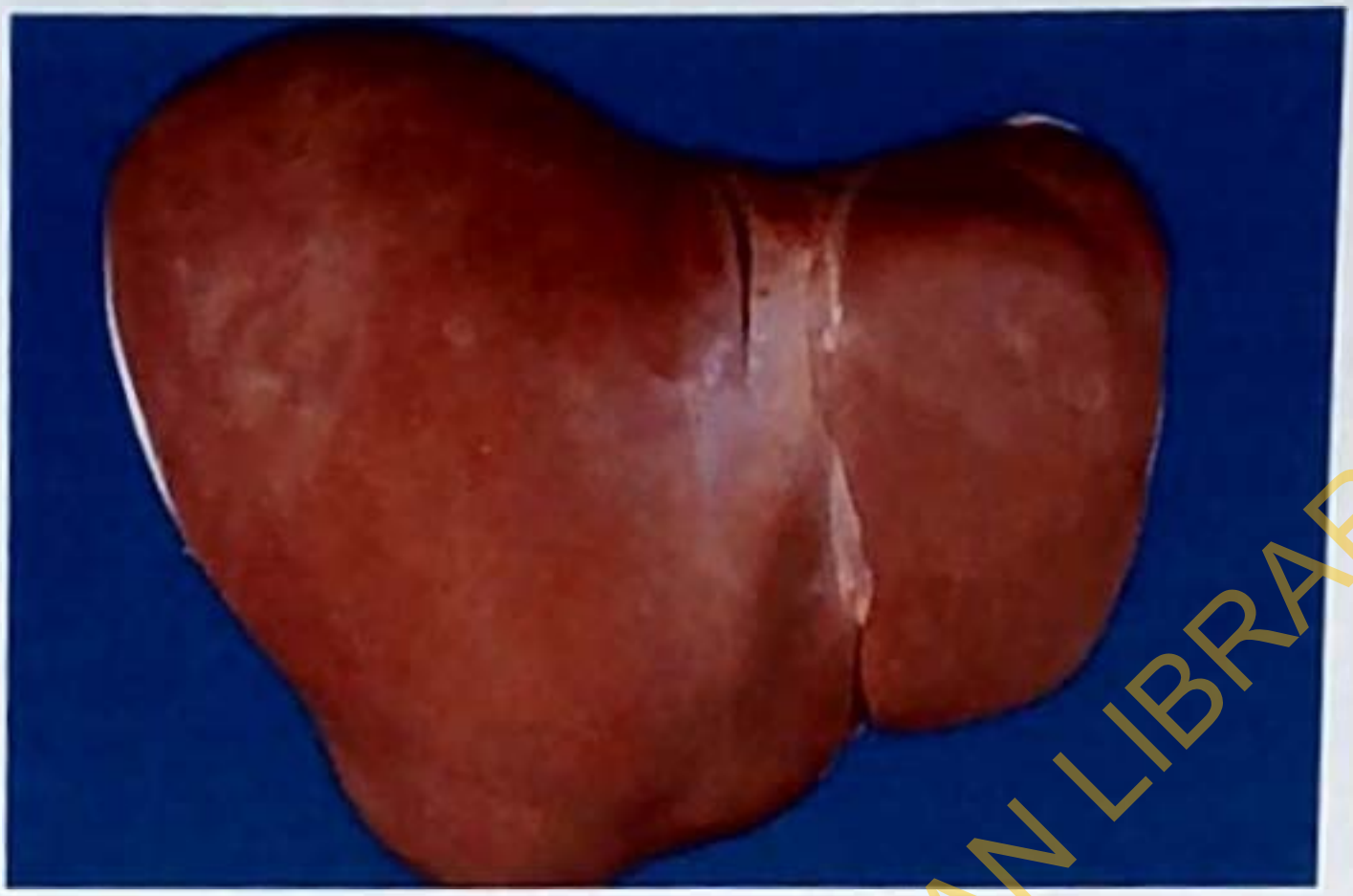
The incidence and characteristics of AIH differ in various geographic regions. Based on limited epidemiological studies, the incidence of type I AIH among Caucasoid populations of Europe and North America ranges from 0.1 to 1.9/100,000/year. The estimated prevalence of AIH in Northern Europe is approximately 160-170 patients/106 inhabitants; 70% of those affected are women. The clinical findings are diverse. The most frequent symptoms being fatigue, jaundice, itching, enlarged liver, abdominal discomfort and arthralgia. There is good response to immunosuppressive treatment, but has a poor prognosis if left untreated. Immunosuppressive drugs lead to

remission (resolution of symptoms, normalisation of transaminase levels and reduction of liver inflammation) in the majority of the patients, but most patients relapse when the treatment is stopped.

Liver transplantation is required in fulminant hepatic failure as well as after progression to cirrhosis. The relative proportion of AIH among cases with chronic hepatitis is low in regions with a high prevalence of viral hepatitis (Boberg. 2002). Areas of the world known for a high prevalence of viral hepatitis are Asia and sub-Sahara Africa. In spite of this, the few studies carried out in these areas have shown appreciable level of AIH.

In India, Asia, Gupta et al (2001) found a prevalence of 3.4% for autoimmune liver disease in a 7 year study and concluded that autoimmune liver disease is not uncommon in India. However, in Africa, data on autoimmune liver diseases are very scanty with a few studies emanating from East and South Africa. In Cameroon, Central Africa, a study by Skalsky et al (1995) concluded that autoantibodies were frequently found in patients with chronic liver disease though this did not seem to correlate with autoimmune liver disease in their study population. In Uganda East Africa, Sadikali and Doniach (1975) studied autoimmune factors and HBV in African cirrhosis and found that 24% were positive for anti-SMA while 5.1% was positive for ANA. They concluded that the study did not favour a role for HBV in causing chronic liver disease by triggering off an autoimmune reaction.

A.



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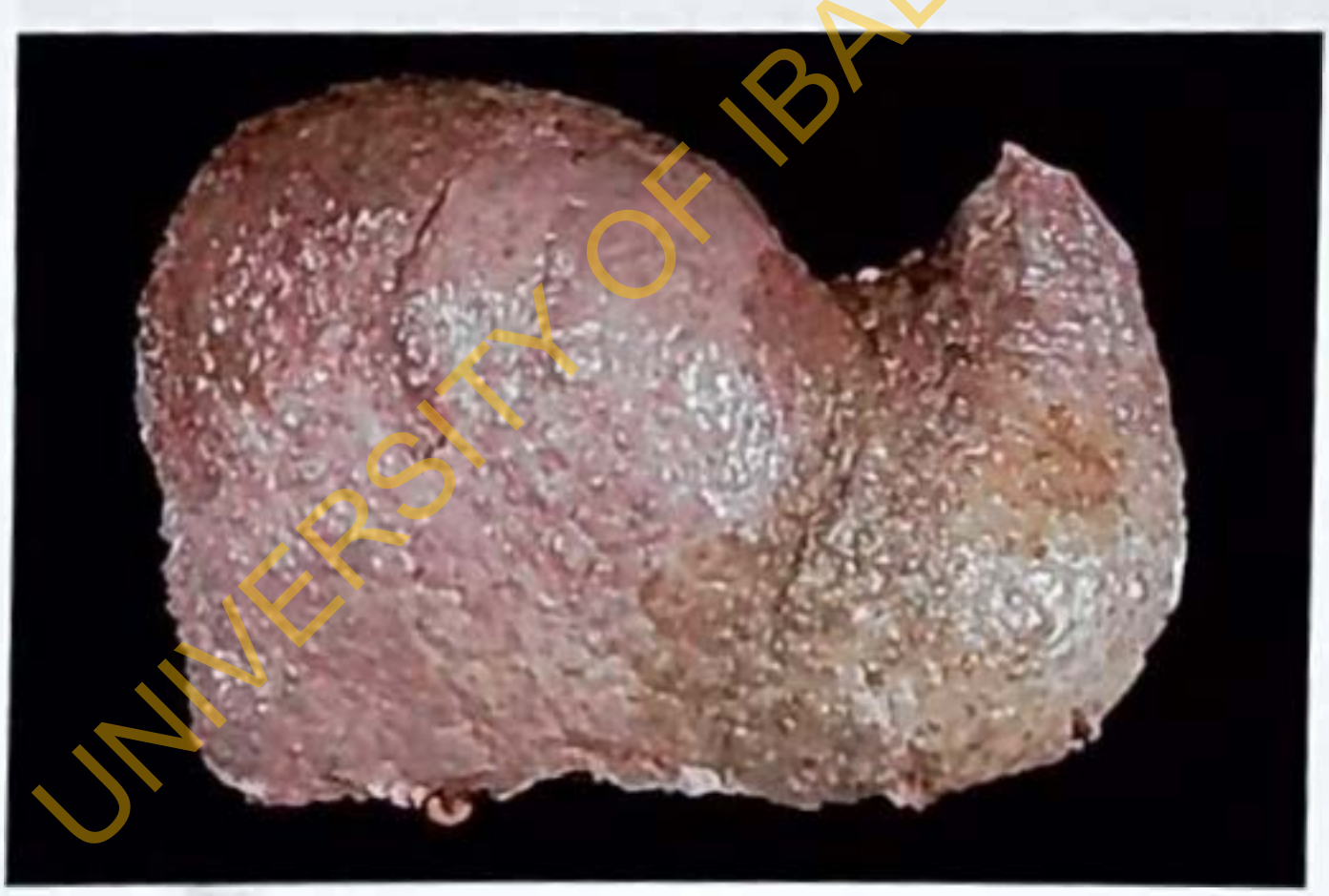

Figures 5 A and B. Gross anatomy of: A - normal liver with smooth appearance; and B - cirrhotic liver showing surface macroscopic nodules.

Source: WebPath

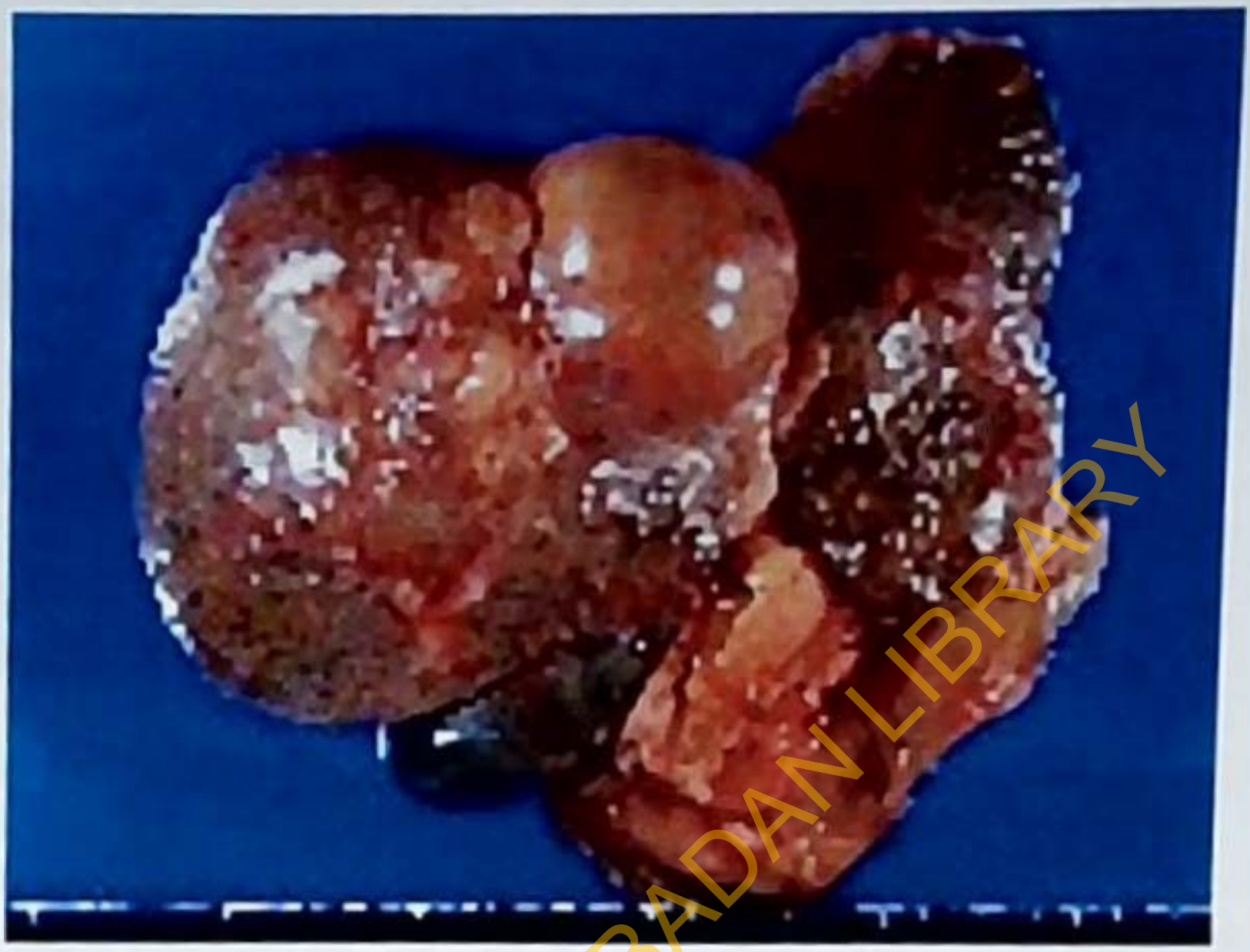

Figure 6. Gross anatomy of hepatocellular carcinoma showing distorted architecture.

Source: WebPath

Table 2. Pathogenetic mechanisms of autoantibodies.

Mechanism	Example
Complement-dependent lysis	Paroxysmal cold haemoglobinuria
Opsonisation	Immune thrombocytopaenia
Immune complexes	Systemic lupus erythematosus
Receptor inhibition	Myasthenia gravis
Receptor stimulation	Thyrotoxicosis
Inhibition of physiological peptide	Pernicious anaemia

Source: Clinical Immunology: Principles and Practice

Pathogenesis of AIH

The role of autoantibodies in the pathogenesis of AIH is not yet known. Given that AIH is a liver specific disease, non-liver specific autoantibodies are unlikely to be involved in inflammation of and damage to the liver (Wies. 2006). The ANA/SMA autoantibody status is neither predictive of AIH-1 nor correlates with its course. ANA/SMA appear to be uninvolved in the pathogenesis itself; they may instead be elevated due to the disregulation of the immune system. It is generally believed that their assay is more useful for diagnosis of AIH than for prognosis (Wies. 2006). A possible pathogenetic role was suggested for SLA/LP autoantibodies as these are the only antibodies which are 100% specific for AIH (Wies et al 2000). Interestingly, SLA/LP show a dominant immune reactivity to a special epitope of the autoantigen, indicating a specific autoantigen-driven induction and maturation of B cells but so far the actual mechanism is still to be elucidated. It has been suggested that LKM-1 autoantibodies are involved in liver tissue damage since it has been shown that the target antigen CYP2D6 is expressed on the surface of hepatocytes (Strassburg and Manns 2002). Possible mechanisms are either direct binding of LKM-1 to hepatocytes leading to liver cell lysis, or the activation of liver-infiltrating T lymphocytes via a combination of B and T cell activity. Moreover, cross reactivity of LKM-1 autoantibodies with viral epitopes (HCV, HSV, CMV) indicates that the autoimmune response may be triggered by viral antigens which mimic the body's own proteins molecular mimicry (Invernizzi and Mackay 2008). Further research is needed to clarify the mechanisms of pathogenesis of AIH in order to develop a specific, and reliable therapy without side effects.

Diagnosis of AIH

The characteristics of AIH aiding in diagnosis are:

(1) increased levels of plasma transaminases (ALT/AST) while alkaline phosphatase (AP) and gamma-glutamyltranspeptidase (GGT) levels remain normal or only marginally elevated;

(2) selectively increased IgG levels;

(3) histological pattern showing inflammation of the liver parenchyma, piecemeal necrosis and. in the final stages, cirrhosis; and

(4) high titres of specific serum autoantibodies.

2.5.2 *Primary Biliary Cirrhosis*

Primary biliary cirrhosis (PBC) is a chronic and progressive cholestatic disease of the liver. It is a non-suppurative destructive cholangitis which occurs worldwide, but commoner in women (9:1) and does not occur in children (Floreani et al 2010). It predominantly affects middle-aged women (30-65years old) and the incidence is rising. In the UK incidence has risen from 23 per million in 1987 to 32 per million in 1994 (James *et al* 1999). Life expectancy in the untreated is about 12 years.

Presumed to be autoimmune in nature and caused by granulomatous destruction of the interlobular bile ducts, PBC is characterized by a T-lymphocyte-mediated attack on small intralobular bile ducts (Figure 7). A continuous assault on the bile duct epithelial cells leads to their gradual destruction and eventual disappearance. The sustained loss of intralobular bile ducts causes the signs and symptoms of cholestasis, and eventually results in cirrhosis and liver failure (Joshita et al 2010).

The precise cause of this attack is unknown but appears to be related to both genetic and environmental factors such as *Escherichia coli*, *Mycobacterium gordonae*, and viruses (Agmon-Levin et al 2009). In addition to the T-lymphocyte mediated destruction of small bile ducts, secondary damage to hepatocytes may occur from the accumulation in the liver of increased concentrations of potentially toxic substances, such as bile acids, which are normally secreted into bile. The naturally occurring bile acids – cholic acid, chenodeoxycholic acid, and deoxycholic acid – are all detergents and can dissolve cell membranes if present in a sufficiently high concentration; such toxic concentrations are reached in states of cholestasis. Cholestasis per se causes increased expression of HLA class I antigens on hepatocytes, thereby rendering them better targets for activated T-lymphocytes (Agarwal *et al* 1999; Charatcharoenwitthaya and Lindor, 2005).

Genetic susceptibility is also suspected as the prevalence of PBC in families with one affected member is estimated to be 1000 times greater than that in the general population. However, the disorder is not inherited in any simple recessive or dominant pattern. Familial occurrences of the disease have included sisters, brothers, brothers and sisters, and parent and child. In addition, unaffected family members are more likely than controls to have impaired T-cell regulation and increased numbers of circulating autoantibodies. There is, however, no significant increase in AMA, the serologic marker of PBC in healthy family members (Hayase *et al* 2005; Joshita *et al* 2010).

The mechanism underlying the genetic susceptibility in some patients with PBC is not known. There is a weak association between PBC and haplotype HLA-DR8 and the HLA-DPB1 gene in some populations. Accumulated data suggest that there may be an

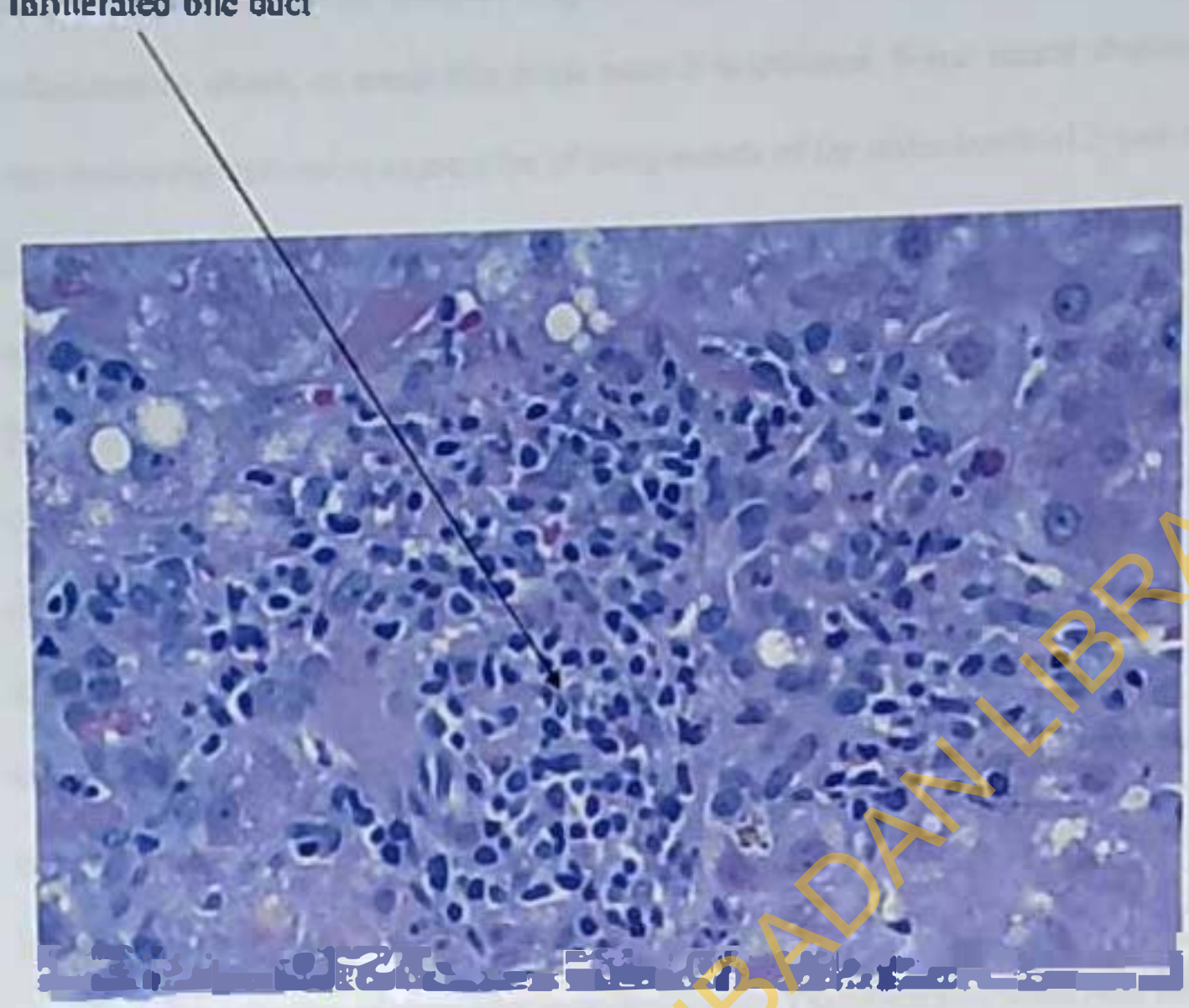

Figure 7 Histology of primary biliary cirrhosis showing a bile duct being infilterated by lymphocytes.

H&E stain

Magnification X40

Source: WebPath

inherited abnormality of immune regulation, perhaps an inability to suppress an inflammatory attack on small bile ducts once it is initiated. Some recent studies have also shown aberrations in expression of components of the mitochondrial 2-oxo acid dehydrogenase complex. Overlap syndromes do occur with PBC especially in association with other autoimmune disorders.

Diagnostic work-up for PBC includes serum alkaline phosphatase, which is almost always markedly elevated. Gamma glutamyl transferase and 5'nucleotidase levels parallel those of alkaline phosphatase. Aminotransferases are normal or mildly elevated (<5-fold), while bilirubin is normal in the early phase but increases in about 60% with progression of the disease. Unlike the aminotransferases, increased bilirubin is a poor prognostic sign (Dickson *et al* 1989; Angulo and Lindor 1999).

Increased blood eosinophils is demonstrated especially in the early phase. This is also seen in the liver and may suggest a pathogenic role. Thrombocytopaenia may be auto-antibody-induced or due to hypersplenism of portal hypertension (Panzer *et al* 1990; Feistauer *et al* 1997).

Antimitochondrial antibodies are the serologic hallmark of PBC (in almost 100%). There is also increased serum immunoglobulin M. Cholesterol levels are elevated in >50% of patients and may be severely high, (eg > 1000mg/dl) especially in the presence of xanthomas (Kanda *et al* 2004). Remarkably, in spite of the striking hypercholesterolaemia, PBC patients are not at risk of death from atherosclerosis. This is due to the striking elevations in HDL relative to LDL and VLDL. Another protective factor is the low level of lipoprotein(a), an atherogenic factor. Striking elevations in LDL levels, decrease HDL and Lipoprotein-X is only seen in advanced disease (Sorokin *et al* 2007).

Other notable biochemical abnormalities are increased serum caeruloplasmin, bile acids and hyaluronate. Raised hyaluronate levels correlate with serum bilirubin with histologic worsening.

2.5.3 *Primary Sclerosing Cholangitis*

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease characterized by progressive destruction of the intra-hepatic and /or extrahepatic bile ducts (Figure 8). Actiology is poorly understood but it involves an uncontrolled inflammatory response in the bile ducts with fibrosis and ultimately biliary cirrhosis. There is a strong association with inflammatory bowel disease (IBD) especially ulcerative colitis (UC) (~90%). About 5% of UC patients also have PSC (Sano *et al* 2010). Continued destruction of bile ducts in PSC leads to end stage liver disease (ESLD) and portal hypertension. Though suspected to be autoimmune disease, no typical immune markers are found in the serum but different types of immune-competent cells can be seen in the liver tissue (Portincasa *et al* 2005). The activated lymphocytes are able to destroy the biliary tree and the liver lobules. Complications include cholestasis associated problems, biliary stricture, cholangitis/cholelithiasis, cholangiocarcinoma and colon cancer.

Primary sclerosing cholangitis is seen mainly in men and seldom in women. It is sometimes seen in children and adolescents. Majority are asymptomatic at the time of diagnosis even in advanced disease. Possibility of PSC should be considered in ulcerative colitis patients with unexplained abnormal liver biochemistry especially elevated alkaline phosphatase.

The earliest symptoms are fatigue and pruritus. In addition jaundice, fever, chills, night sweats and right upper quadrant pain occur in about 10-15% at presentation.

This may be due to episodic bacterial cholangitis from biliary obstruction rather than advancement of disease. Peripheral features of chronic cholestasis are present as disease advances. Florid features of liver cirrhosis are seen in the cirrhotic stage of disease.

Liver function tests (LFT) usually show cholestatic features with raised alkaline phosphate predominating. Aminotransferases are usually below 300iu/l. Albumin is normal in early disease but hypoalbuminaemia may be seen in UC. Hypergammaglobulinaemia is found in about 30% with increased serum IgM in 40-45% (Rust and Beuers 2008). Perinuclear antineutrophil cytoplasmic antibodies (p-ANCA) are found in 65-80%. ANA/ASMA when present are non-specific, but AMA is usually absent. Serum and hepatic copper is usually increased, while serum caeruloplasmin is reduced in most cases (Gross et al 1985).

2.5.4 *Viral Infections and the Liver*

Viruses are obligate intracellular parasites that require the host to replicate and to effect their spread. Most human viruses replicate only in certain target tissues as a consequence of viral receptor distribution (Rouse and Ahmed, 2001). Several protective mechanisms prevent viruses from getting to their target tissues and this may be innate and or adaptive. The skin and the mucosal surfaces serve as effective barrier to viruses. Adaptive immune response is usually evoked after the virus has gained entry into the body (Rouse and Ahmed 2001). Failure of all the protective mechanisms leads to overt disease.

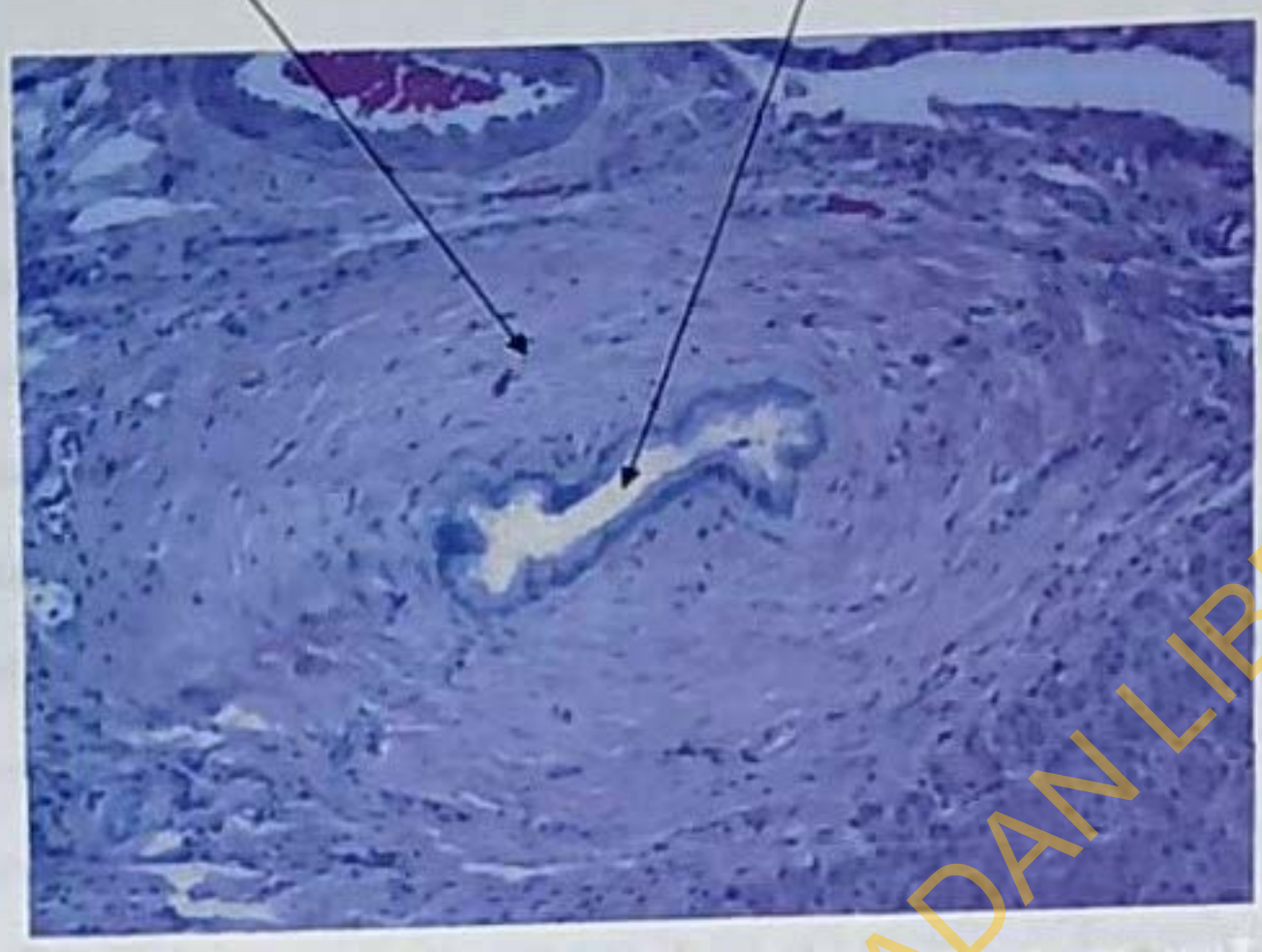

Figure 8. Histology of primary sclerosing cholangitis showing intense fibrosis around a bile duct

Magnification x40

H&E stain

Source: WebPath

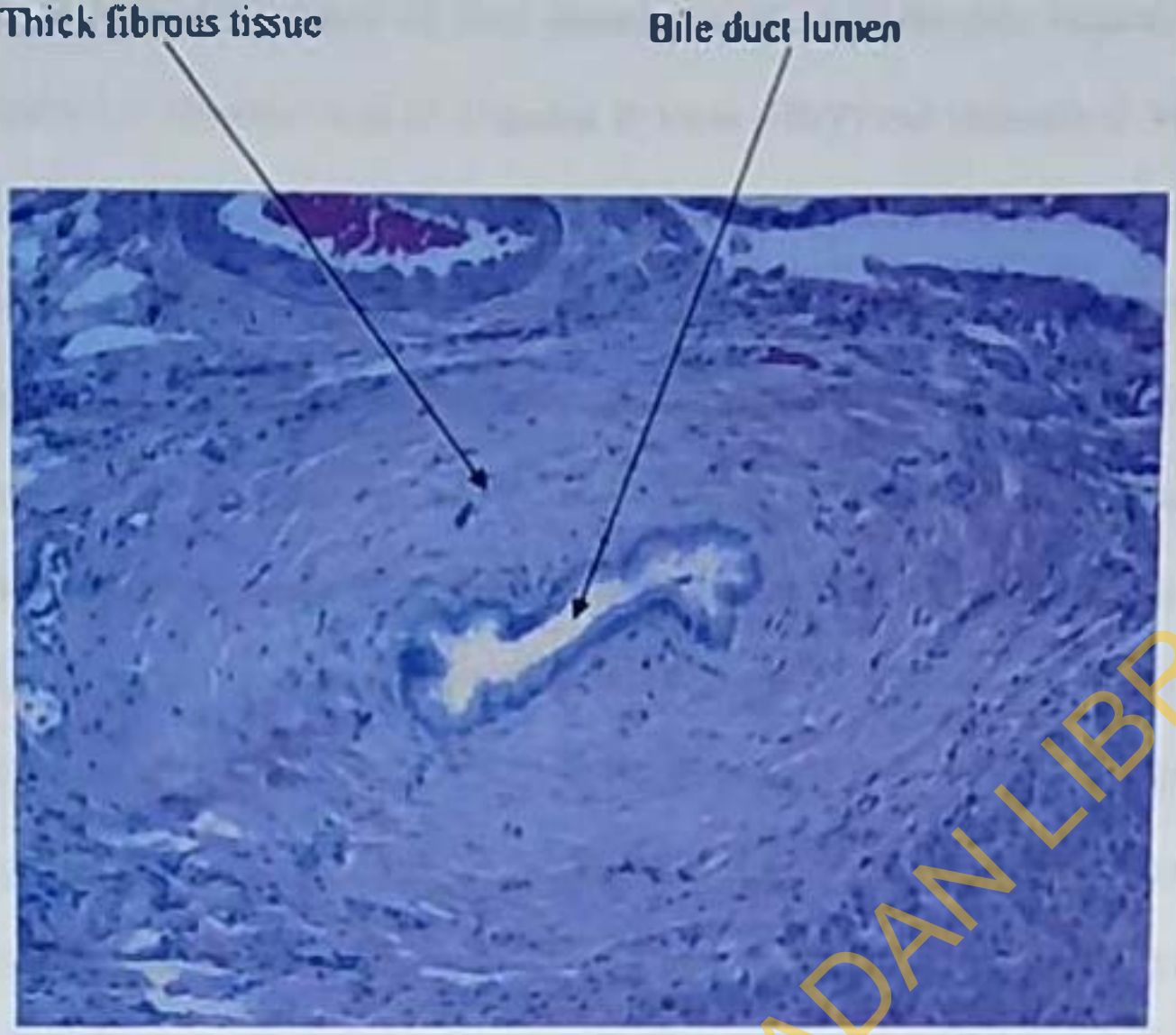

Figure 8. Histology of primary sclerosing cholangitis showing intense fibrosis around a bile duct

Magnification x40

H&E stain

Source: WebPath

Major actiologic agents of liver disease are the hepatotropic viruses, which are labelled as Hepatitis A to G. Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) are however the most devastating agents causing both acute and chronic liver diseases with long term sequelae of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Marcellin 2009). Serological markers for the diagnosis of HBV and HCV are currently available, but reports have shown that in advanced liver disease some of the antigens especially the hepatitis B surface antigen (HBsAg), may not be expressed and therefore become undetectable in the serum, a phenomenon referred to as occult HBV infection (Thedja *et al* 2010). At this stage, only molecular markers of the virus such as the DNA could confirm the presence of the virus. Similarly some antibodies may cross react and yield a positive result to antibody to HCV (anti-HCV) testing, in effect RNA analysis of the virus may be the only modality of diagnosis of the virus (Castillo *et al* 2010; Pham *et al* 2010).

Importantly also, acute and chronic viral infections have been implicated in the pathogenesis of various autoimmune diseases (Jaeckel and Manns, 2005). No study is yet to address the interplay of hepatotrotropic viruses and autoimmunisation in the pathogenesis of liver diseases in Nigeria.

2.5.4.1 *Hepatitis B Virus and the Liver*

In Nigeria HBV infection and anti-HCV antibodies are the most studied actiological agents of liver diseases; (Nasidi *et al* (1986), Ojo *et al* (1995), Ola *et al* (2002)). The prevalence of hepatitis B virus is high in Nigeria and varies depending on study and population group being addressed. Odaibo *et al* (2003) found a prevalence of 18.3% among patients undergoing dental extraction, while Olubuyide *et al* (1993) found a

prevalence of 47-49% in non-hospitalised rural and urban dwellers in south-west Nigeria. On average, the prevalence of HBV in Nigeria is about 20%. Many risk factors are known for HBV infections, ranging from blood and blood product transfusion, indiscriminate injections, tattoos, use of sharp instruments, surgical procedures with unsterilized equipment, sex, especially among homosexuals, and intravenous drug abuse among others (Forbi *et al* 2009; Ola *et al* 2008 and Mackenzie *et al* 2003). Healthworkers are also exposed. In Nigeria, body scarification and indiscriminate injections were the commonest risk factor found among blood donors (Otegbayo *et al* 2003).

Hepatitis B, however is the most studied of the hepatotropic viruses because its diagnosis has been made easy by the historic discovery of the Australian antigen now called Hepatitis B surface antigen (HBsAg), by Blumberg et al (1965). Hepatitis B Virus (HBV) has a global distribution with about 350 million carriers. The WHO estimates that by year 2000, the carrier rate of HBV infection would be about 400 million.

HBV causes about 2 million deaths annually with 500,000 from fulminant hepatitis. About 2 billion people have markers of disease worldwide. There is a striking geographical variation in the prevalence of HBV. It is estimated that 77.9% of carriers are in Asia and 12.3% in Africa (Oon 1995). In sub-Saharan Africa prevalence rate varies between 5% to 20% while in the USA and Northern Europe the prevalence in volunteer blood donors is 0.1% (Figure 9). There is a notable increase in the prevalence of HBV infection, recently, even in developed countries (Steinke et al, 2002 and Barclay et al 2010).

The WHO estimated that 40% of infected people will die of chronic liver disease (WHO 2002). The most effective means of transmission is blood and blood products, however the virus is found virtually in all body secretions with the highest concentrations found in serum, semen and saliva. Hepatitis B virus (HBV) is about a 100 times as infectious as the human immunodeficiency virus (HIV). The virus can also be transmitted non-parenterally or perinatally (vertical transmission)

The complete infectious virion (Dane particle) is a 42nm spherical particle containing the:

a). HBsAg: The outer lipoprotein surface envelope that includes the Pre-S proteins that may mediate the attachment of HBV to the hepatocytes. Its presence indicates acute or chronic infection with HBV and potential infectivity. It is the first viral marker to appear.

b). HBcAg: The core protein which surrounds the viral genome or DNA. It is not detected in circulation, but might be demonstrated by special stains in the liver. Antibody against it indicate a recent infection if IgM is present in high titres. IgG anti-core antibody on the other hand suggests chronic infection or immune state if HBsAg is negative.

c). HBeAg A sub-unit of HBsAg bearing particle, which is antigenically complex with at least 5 antigenic specificities (el. e2, e3, etc). Its presence connotes infectivity. Antibodies to e antigen suggest convalescence or a low infectivity state. The precore mutant strain may present with absent HBeAg and high anti-HBe in the presence of HBsAg

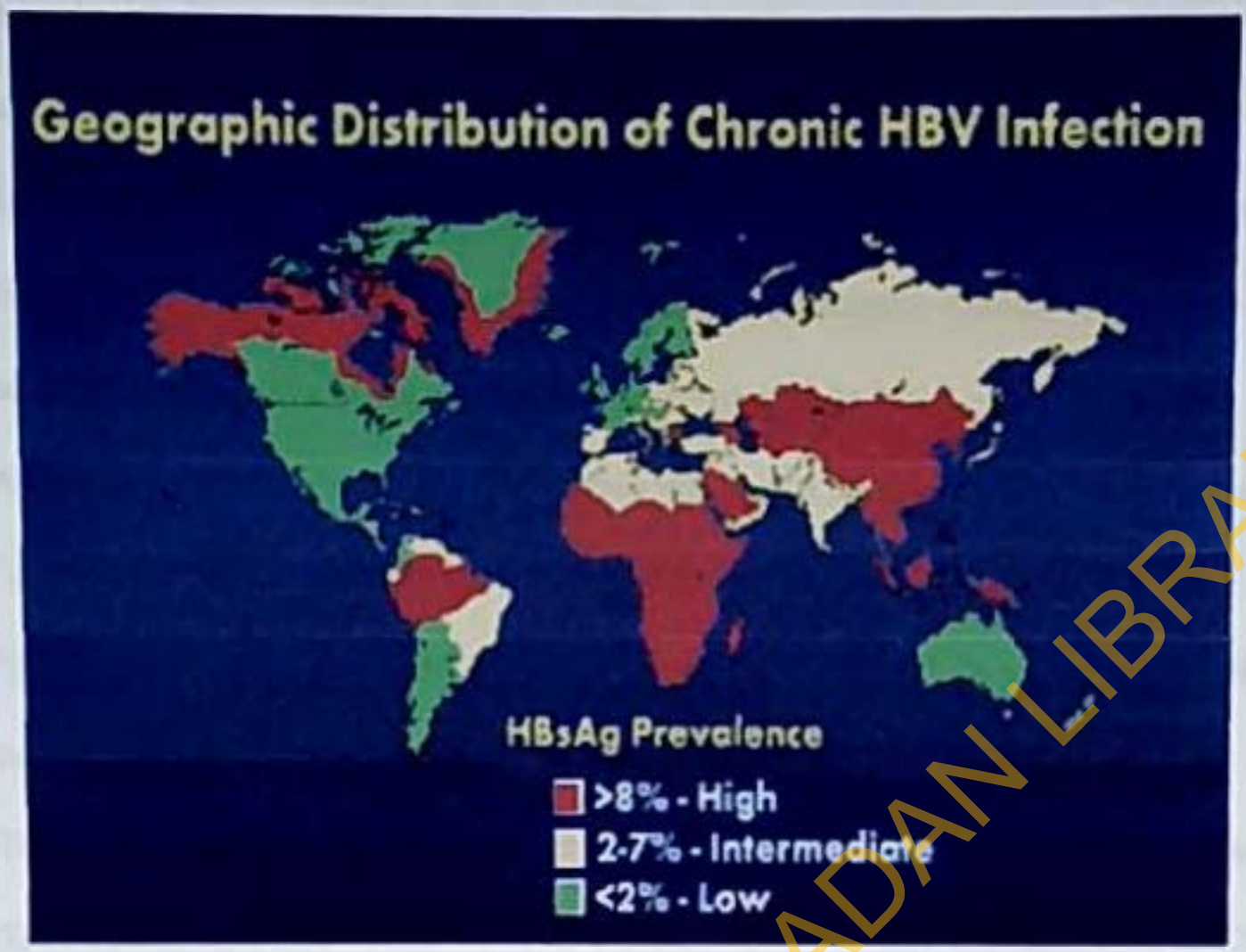

Figure 9. World map of prevalence of hepatitis B virus infection

Source: World Health Organisation, Geneva.

d). DNA and DNA polymerase: The presence of DNA and the DNA-polymerase indicate continuous viral replication, and may be present even when HBsAg is undetectable in the serum.

All HBsAg subtypes share a group determinant "a". There are two pairs of subtypes determinant; these are d, y and w, r which are mutually exclusive and therefore behave as alleles. There are now 8 HBV genotypes (A to H).

Antigenic heterogeneity of the "w" determinants and additional determinants such as "q", "s" and "g" have also been demonstrated.

Eight HBsAg subtypes, viz. ay. w, ay; w2, ayw3, ayw4, ay; ra, adw, adw2, and adr have also been identified, these are mainly of epidemiological significance

The HBV genome is an incomplete double stranded DNA molecule of approximately 3200 bases, HBV replicates by reverse transcription of RNA intermediate to DNA. The DNA polymerase synthesizes the second positive DNA strand. Open reading frame encode the viral envelope, neclecocapsid (HBcAg and HBeAg) and the viral polymerase and X protein (Zakim and Boyer 1990). (Figure 10).

HBV may progress to a chronic carrier state (>6 months) in 5-10% of patients and 10% of these will develop chronic liver disease (chronic hepatitis, liver cirrhosis, hepatocellular carcinoma)

Immunopathogenesis of Hepatitis B virus

HBV is not cytopathic, the basis of liver damage is immunological response by the immunocompetent host to viral attack (Dudley *et al* 1972, Chang and Lewin 2007). The more vigorous the host response, the worse the extent of liver damage and hence the clinical presentation. The immune response leads to cell damage by the CD 8+

cytotoxic T lymphocyte. On the other hand low immune response or immunotolerance leads to chronicity of infection (Visvanathan and Lewin, 2006).

Immune complex mediated tissue damage also play a role in the extrahepatic manifestations of HBV infection. Deposition of soluble immune complexes in tissues lead to glomerulonephritis, polyarteritis nodosa, and Gianotti crosti lesion among others (Pyrsopoulos and Reddy, 2001; Tan *et al* 2008).

Serum Markers of Hepatitis B Virus

Laboratory diagnosis of HBV include screening for HBsAg, anti-HBs, anti-HBc (IgM, IgG). Several techniques have been used in the past, but enzyme linked immunoabsorbent assay (ELISA) and recombinant immunoblot assay (RIBA) are the most current (Leon et al. 1998). Polymerase chain reaction (PCR) is useful for amplifying and quantifying serum DNA.

2.5.4.2 *Hepatitis C virus and the liver*

Hepatitis C virus (HCV) infection was discovered in 1988 when it was cloned by Choo and his colleagues from copy DNA extracted from infected chimpanzee. It has since become the leading cause of chronic liver disease worldwide. About 3% of world population (170 million) is estimated to be chronically infected with HCV (Raggam *et als* 2009). Chronic infection is a major cause of cirrhosis and hepatocellular carcinoma in the developed world. The virus is found worldwide (Figure 11) with relatively high prevalence in Japan, the southern part of the USA, the Mediterranean countries of Europe and the Middle-East where 0.5-1.5% of blood donors are anti-HCV positive (Gaeta and Giusti 1990). Prevalence is very low in Northern Europe and N/America. In Africa epidemiological data is deficient but a

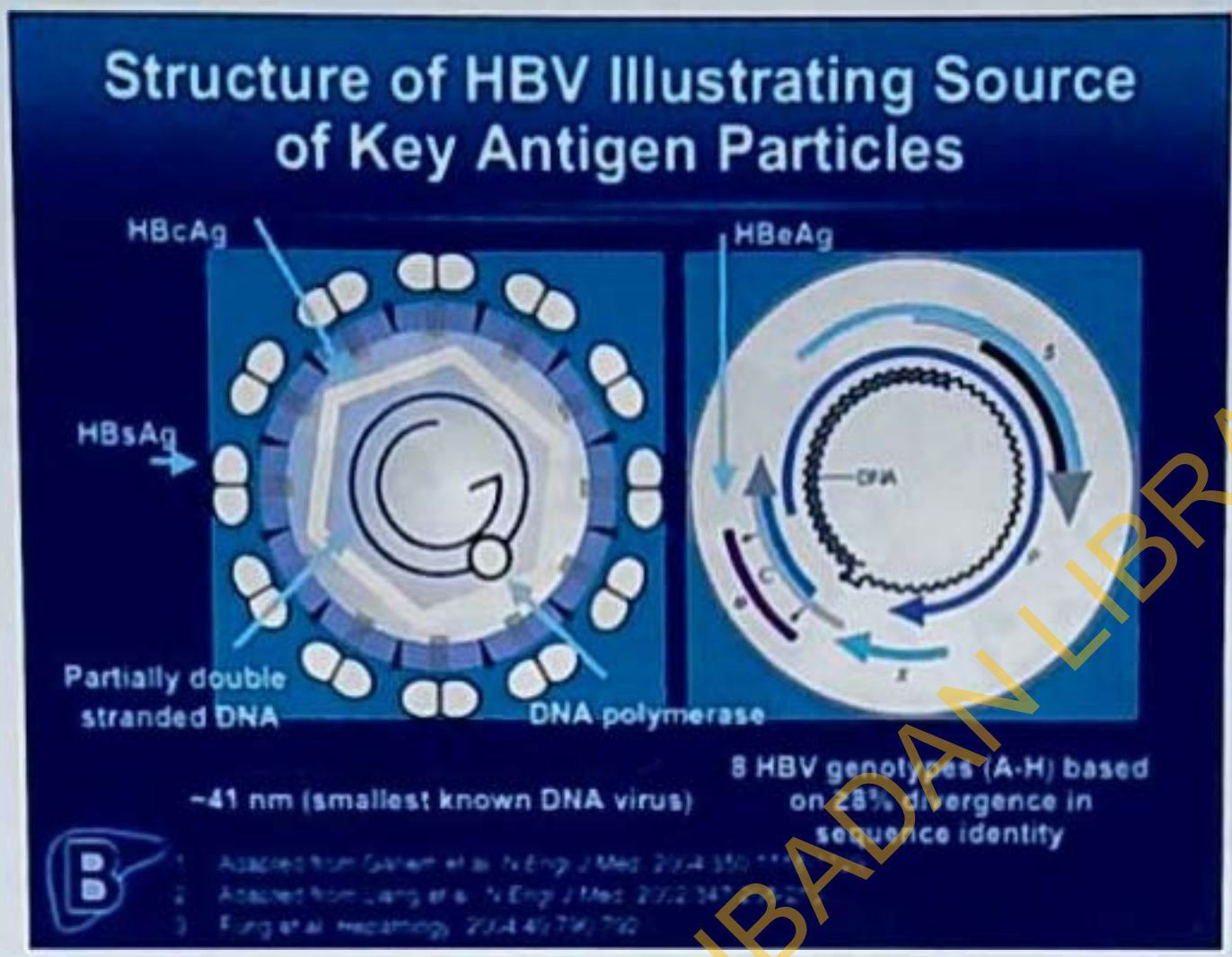

Figure 10. Major antigens of hepatitis B virus

World Health Organization. Hepatitis C: global prevalence. Wkly. 2003. Ford P, et al. Semin Liver Dis. 2000;20:103-108. Wasley A, et al. Semin Liver Dis. 2000;20:1-16.

Figure 11. Global distribution of hepatitis C virus

prevalence of 6% has been documented (Berkes and Cotler 2005). In Nigeria studies have shown a range of 3% to 6% among the groups studied (Opaleye *et al* 2010; Buseri *et al* 2009 and Fasola et al 2008). Most infection (85%) leads to chronicity with only 15% resolving in the acute phase. Transmission is most effective through serum. Unlike HBV, sexual transmission is rare because of the usually low level of viraemia but vertical transmission is possible with marked viraemia. The incubation period on average is nine weeks and infection is usually asymptomatic.

HCV is made up of a heterogeneous group of RNA viruses. It is a small single stranded RNA virus, 30-60mm in diameter with a lipoid envelope and has about 9379-9481 nucleotides. It has a single open reading frame with a 5' and 3' terminals. The 5' terminal has 92% homology among different HCV types. Six major genotypes (1-6) and subtypes a, b, c, etc have been described, with varied geographical distribution and response to treatment. Type 1a is predominant in the USA, while type1b is found mainly in Japan and has been associated with chronic liver disease and poor response to interferon therapy. In Nigeria a pilot survey in healthy adult donors and children of preschool age showed genotypes 1a, 4, and 1d to be the predominant subtypes (Oni and Harrison 1996).

Immunopathology of HCV

An immunopathogenic mechanism similar to that of HBV is suspected as a mechanism of liver injury as the virus has been found not to be cytopathic (Ramirez et al 2008). Chronic carrier state is found in 80% of infected people and 20% of these progress to chronic liver disease, about 10% develop liver cancer in 20 years (Tassopoulos, 1996).

Extrahepatic manifestations of HCV hepatitis include, essential mixed cryoglobulinaemia, membranoproliferative glomerulonephritis, porphyria cutanea tarda, lichen planus, mooren corneal ulcer, autoimmune thyroiditis, idiopathic pulmonary fibrosis and Sjogren's syndrome (Pyrsopoulos and Reddy, 2001). Several autoantibodies have been found in patients with HCV infection. Ten percent of HCV infected patients are positive for serum autoantibodies. Among the antibodies found in HCV infection are ANA, SMA, LKM-1, LC-1, rheumatoid factor, antithyroid antibody, IgG and IgM anticardiolipin, ANCA and anti-gastric parietal cells antibody (Jaeckel and Manns, 2005).

Serum markers of hepatitis C virus

Enzyme-linked immunosorbent assay (ELISA) is used to detect antibody to HCV (anti-HCV), and Recombinant immunoblot assay, is used for confirmation. Serum RNA extraction followed by polymerase chain reaction amplification and subsequent sequencing are used to determine the presence of HCV genome.

Transaminase levels are usually raised, although 60% of patients have normal ALT. A fluctuating pattern of rise has been noticed to be characteristic but not invariable in acute HCV hepatitis (Dienstag, 1983). A recent Italian study suggest a high level of alanine transaminase (ALT) in HCV patients with superimposed HAV, this has raised the question of HAV vaccination in patients with chronic HCV infection (Vento et al, 1998).

2.6 Serum Markers Of Autoimmune Liver Disease

The laboratory markers of autoimmune liver disease are either histological, characterized by infiltration of the liver by activated T-cells, or serological, characterized by the presence of pathogenic autoantibodies.

The first experiment establishing that autoantibodies can cause a human disease was that by William Harrington (Harrington *et al*, 1951). Harrington volunteered to receive an infusion of plasma from a patient with autoimmune thrombocytopaenia, after which his platelets dropped precipitously. This was clearly a presentation of autoimmune thrombolysis.

Autoimmune hepatitis (AIH) is a chronic progressive inflammatory liver disease of unknown origin, which responds well to immunosuppressive therapy, but has a poor prognosis if untreated. Early and accurate diagnosis is therefore of great importance. AIH is characterized by histological features of periportal hepatitis in the absence of viral markers, by hypergammaglobulinaemia, and in the majority of patients, by autoantibodies in the serum. The International Autoimmune Hepatitis Group (IAHG) has developed a comprehensive criteria for the diagnosis of autoimmune hepatitis, based on several parameters such as serum autoantibodies, gamma globulins, ferritin etc into probable and definite autoimmune disease (Alvarez et al, 1999). Table 3 shows the IAIHG diagnostic criteria.

Antinuclear antibodies (ANA), smooth muscle antibody (SMA), mitochondrial antibodies (AMA), soluble liver antigen antibodies (SLA), liver kidney microsomal antibodies (LKM) are recognized autoantibodies used in the diagnosis of AIH. Antinuclear antibodies and anti-smooth muscle antibodies (SMA) also occur in 10-15% (Clifford *et al*, 1995) of patients with viral hepatitis and other autoimmune

disorders. LKM-1 autoantibodies are also associated with hepatitis C. Three types of LKM-1 antibodies can be distinguished according to the target antigen. LKM-1 is directed against cytochrome p450 IID6, a cytoplasmic protein found in hepatocytes and renal tubular proximal cells. LKM2 antibodies are associated with tienynafen (tielinic acid, a uricosuric agent)-induced hepatitis. The target antigen is cytochrome p450 IIC9, a cytochrome p450 enzyme that catalyses metabolic oxidation of the drug. LKM-3 antibodies are associated with hepatitis D virus (HDV). The target antigen is UDP-1 glucoronosyl transferase. The main autoantibodies demonstrable in the sera of patients with autoimmune liver disease are:

2.6.1 Antinuclear antibodies (ANA)

Antinuclear antibodies (ANA) are serologic hallmarks for systemic or organ specific autoimmune disease (Hahn, 1998) and are the most common circulating antibodies in autoimmune hepatitis. They are a group of autoantibodies against various cell nuclear antigens, some of which are considered to be quite useful as disease markers in autoimmune disorders (Tan *et al*, 1988, Tan 1989).

The most likely molecular targets of ANA in AIH are various nuclear antigens without a specific pattern, including the known nuclear antigens dsDNA, tRNA, SS-A, snRNPs, laminins A and C and histones. There are, however, additional, still unknown, nuclear antigens that are also suspected. Liver disease-specific ANAs have, however, not been identified so far.

2.6.2 Antimitochondrial antibodies (AMA)

These are serologic hallmark of primary biliary cirrhosis, and it is found in 95% of patients with primary biliary cirrhosis (PBC). Titres of 1:160 are practically diagnostic of PBC. The antigens against which AMA are targeted have been cloned (Coppel *et al* 1988). The antigens are in the dehydrolipoamide acetyl transferase component (E2 subunit) of a functionally related family of enzymes, the 2-oxo-acid dehydrogenase (M2 subtype of mitochondrial autoantigens). These enzymes include pyruvate dehydrogenase, branched chain keto-acid dehydrogenase, and ketoglutarate dehydrogenase. Each enzyme complex catalyzes the reductive transfer of an acetyl group from its respective substrates to co-enzyme A for oxidation in the Kreb's cycle. The enzymes serve as an antigen for AMA, but antibodies to pyruvate dehydrogenase are most prevalent. Human AMA inhibit the enzymatic activity of these enzymes in vitro. Thus far, all of the mitochondrial autoantigen screened have been targets of only the anti-M2 AMA (Kaplan, 2000).

Antimitochondrial antibodies are usually absent in jaundiced patients with extrahepatic obstruction, drug sensitivity and viral hepatitis.

2.6.3 Anti-smooth muscle antibodies (ASMA)

SMA autoantibodies, initially detected by Johnson et al (1965), were originally named because of their ability to stain smooth muscle of arterial vessel walls and the muscular layer of the stomach during indirect immunofluorescence (IIF) testing. They are the second major class of autoantibodies that have proved to be of value in the diagnosis of AIH-1. They are less prevalent than ANA, but are more specific for AIH-1. When present in AIH, they are predominantly directed against filamentary actin (F-

actin), binding to conformational epitopes on polymerised actin. They were later detected among patients with malignancy, infectious and rheumatic disorders as well as heroin abuse. ASMA, in addition to hepatic tissue also react with intestinal, endothelial, and other cell types. SMA autoantibodies present in non-AIH diseases recognise structures of the cytoskeleton such as actin, troponin, vimentin and tropomyosin. It was, however later discovered that the ASMA were specific towards actin filaments. SMA with actin specificity (anti-actin antibodies, AAA) are usually present in AIH, while those directed against other cytoplasmic constituents are more common in viral infections such as measles, mumps and hepatitis (Toh *et al*, 1979; Liebovitch *et al*, 1995). AAA are specific for AIH-1 and are closely associated with HLA phenotypes B8 and DR3, which are associated with poor prognosis in AIH.

2.6.4 LKM autoantibodies

Autoantibodies against liver kidney microsomes, LKM, were originally detected by a typical immunofluorescence staining on liver and kidney slides (Wies 2006). LKM subtypes have been classified according to their different specificities. LKM-1 is the characteristic serological marker for AIH-2 and tests for this autoantibody are routinely carried out. Its major target antigen is cytochrome p4502D6 (CYP2D6), a 50 kDa enzyme responsible for the metabolism of many drugs and environmental chemicals. LKM-2 and LKM-3 show a similar staining pattern to LKM-1 in IF, but are of less diagnostic value for AIH-2. LKM-2 antibodies, which are directed against another p450 cytochrome isoform, cytochrome p4502C9, have only been found in some cases of drug induced hepatitis caused by tienilic acid. Since this drug has not been used for 20 years, LKM-2 autoantibodies are mainly of historical interest.

However, LKM-2 autoantibodies have been the subject of research since they target the enzyme which is presumed to catalyse the metabolic oxidation of the disease-inducing drug (Zachou *et al* 2004). It was suggested that transformation of the drug to a reactive metabolite allows its close interaction with the cytochrome molecule, creating new epitopes composed of enzyme and drug. These so called neoepitopes may be immunogenic and thus could trigger the autoimmune response. LKM-3 autoantibodies can be found in 5-10% of AIH-2 patients, alone or in combination with LKM-1. These autoantibodies are typically present in patients with chronic hepatitis D (about 13%). LKM-3 autoantibodies target family 1 of UDP glucoronosyl-transferases (UGT1), which are involved in drug metabolism. In IIF tests LKM-3 autoantibodies show additional fluorescence in pancreas, adrenal gland, thyroid and stomach sections. LKM1 autoantibodies could be found in 11% of patients with HCV infection (Dalekos *et al* 2002; Vergani *et al* 2004b).

Table 3: The International Autoimmune Hepatitis Group Diagnostic Criteria

Requisites	Definite	Probable
No genetic liver disease	Normal alpha-1AT Normal serum ceruloplasmin, Fe, and ferritin	Partial alpha-1AT def. Non-specific serum Cu, ceruloplasmin, Fe, and/or ferritin
No active viral infection	No markers of current infection with Hepatitis A, B, C	No markers of current infection with Hepatitis A, B, C
No toxic or alcohol injury	Daily alcohol<25g/d and no recent use of hepatotoxic drugs.	Daily alcohol<50g/d and no recent use of hepatotoxic drugs.
Laboratory features	Predominant aminotransferase abnormality. Globulin, gamma-globulin or IgG level ≥1.5x normal.	Predominant aminotransferase abnormality. Hypergammaglobulinaemia of any degree.
Autoantibodies	ANA, ASMA, or anti-LKM1≥1:80 in adults and 1:20 in children; No AMA	ANA, ASMA, or anti-LKM1>/=1:40 in adults or other Autoantibodies(including pANCA, AAA, SLA/LP, LC1, ASGPR
Histologic findings	Interface hepatitis No biliary lesion, granulomas, or prominent changes suggestive of another disease	Interface hepatitis No biliary lesion, granulomas, or prominent changes suggestive of another disease

AT = antitrypsin.
X=times

2.6.5 SLA/LP autoantibodies

These are directed against several liver antigens including cytokeratins 8 and 18 (Wachter *et al.* 1990) and subunits of glutathione S-transferases (Wesierska-Gadek *et al.* 1998). They may be the only circulating antibodies in some patients with AIH, and their detection may help in diagnosis of patients classified as having cryptogenic chronic hepatitis.

SLA/LP autoantibodies have been shown to be an outstanding marker for AIH as these are the only autoantibodies which are 100% specific. Originally described independently in the late 1980s by different groups, the autoantibodies reacting with soluble liver antigen (anti-SLA) and the autoantibodies against liver pancreas antigen (anti-LP) were thought to be different. In the year 2000 evidence was provided that anti-SLA and anti-LP are in fact the same autoantibodies (Wies *et al.* 2000). Because of this they were named SLA/LP. Until the year 2000, when the sequence of the target antigen was identified by molecular cloning, the target antigen was not available for use in a standardised, universally available diagnostic test. Testing with IIF is impossible on common substrates.

Screening of cDNA expression libraries identified the SLA/LP target antigen, a previously unknown amino acid sequence which was revealed to be a UGA suppressor tRNA-associated protein of unknown function (Weis *et al* 2000). During translation this special tRNA codes for the insertion of selenocysteine into the growing polypeptide chain if a UGA codon is present.

Despite the fact that the protein's sequence was identified in the year 2000, its physiological function is still unclear as is its role in the pathogenesis of AIH. When the identified target antigen was produced by recombinant means it was found to be

highly specific for both SLA and LP autoantibodies, demonstrating that both recognise the same antigen and are therefore identical. The availability of cloned SLA/LP antigen now allowed the development of a reliable standardized ELISA test system. SLA/LP has been found neither in AIH-2 nor in other autoimmune liver diseases (primary biliary cirrhosis, primary sclerosing cholangitis), chronic viral hepatitis, alcoholic liver disease and non-hepatic autoimmune diseases when standardised ELISAs using reference autoantibody or the recombinant antigen are used (Weis *et al* 2000). It is thus a highly specific marker for AIH-1 with a sensitivity of about 30% if all AIH patients are included.

Testing for SLA/LP is extremely important in those patients who are seronegative for other autoantibodies, as it may help to identify additional patients with AIH who were thought to be suffering from chronic hepatitis and/or cirrhosis of unknown origin.

2.6.6 *Anti-neutrophil cytoplasmic antibodies (ANCA).*

These are a group of autoantibodies which recognize neutrophil proteins. They are of two types, the cytoplasmic or cANCA (cytoplasmic pattern on immunofluorescence) directed against serine protease 3 and perinuclear or pANCA (perinuclear pattern on immunofluorescence) directed against myeloperoxidase (MPO).

An atypical form of p-ANCA may be found in autoimmune hepatitis (AIH), also referred to as x-ANCA, may be directed against a number of antigens including lactoferrin, cathepsin G, and bactericidal/permeability-increasing protein (Semrad *et al* 1998).

2.6.7 *Liver Cytosol-1 autoantibodies (Anti-LC-1)*

LC-1 autoantibodies are directed against formiminotransferase cyclodeaminase (FCTD), a liver specific enzyme whose role in AIH-1 pathogenesis is however still unknown (Rigopoulou et al 2007). With IIF, the LC-1 pattern is usually masked by the concurrent presence of LKM. LC-1 autoantibodies on their own indicate AIH-2.

2.6.8 Atypical pANCA

Atypical anti-neutrophilic cytoplasmic autoantibodies are named because of their perinuclear staining of neutrophils, but they do not detect the classical pANCA antigens. They are presumed to bind to nuclear membrane components. Atypical pANCA can be found in 50-96% of AIH-1 patients but not in AIH-2 patients (Wies 2006). They can also be found in patients with primary sclerosing cholangitis, ulcerative colitis and Crohn's disease. Due to the lack of specificity, detection may only be useful for ANA/SMA/LKM negative patients when attempting to diagnose AIH-1 (Savige *et al* 1994).

2.6.9 *Anti-Asialoglycoprotein receptor (ASGPR)*

These are autoantibodies against asialoglycoprotein receptor (ASGPR), is a liver-specific glycoprotein. They are found in AIH, PBC, chronic viral hepatitis B and C as well as in alcoholic liver disease. They are not specific for AIH. Nevertheless, about 88% of AIH patients are anti-ASGPR positive (Strassburg and Manns 2002). It is believed that anti-ASGPR represents a general marker of liver disease and may be diagnostically helpful if other autoantibodies are not detected, yet AIH is suspected (Wies 2006).

2.6.10 Hepatocyte membrane antigen (HMA)

Autoantibodies to hepatocyte membrane have been demonstrated in the sera of patients with AIH and are divided into two (Wies 2006). These autoantibodies are yet to be well characterized in terms of significance in AIH. A study however suggested that anti-HMA was tightly associated with the degree of hepatocyte inflammation and that the measurement of anti-HMA may have some advantage in clinical evaluation of some of non-B, non-C hepatitis patients (Sasaki *et al* 2001).

2.7 *Other Autoimmune Disorders Among Nigerians.*

A number of autoimmune disorders have been reported at various times among Nigerians in literature. The disorders include, rheumatoid arthritis (Adelowo et al 1998; Adelowo et al, 2010), systemic lupus erythematosus (Adelowo et al, 2009), myasthenia gravis (Ayanru 1978; Ojini et al 2004) , autoimmune haemolytic anaemia (Salawu and Durosinmi, 2002), diabetes (Akinsola and Salimonu. 1985; Akinlade *et al* 2001), Guillain-Barre syndrome (Sunmonu *et al.* 2008), and autoimmune thyroiditis (Oli et al 1981; Cardoso et al 1995; Ogbera et al 2007). Multiple autoimmune disorder was reported in a particular case (Talabi *et al.* 2003). One case report of autoimmune liver disease was reported (Otegbayo *et al*, 2010).

In view of the enormous burden of liver diseases in Nigeria (Bojuwoye, 1997), and the need for literature on autoimmune liver diseases and response of autoimmune liver diseases to steroids and other novel immunosuppressive therapies, it is auspicious to embark on this study so that our patients with autoimmune liver disease will receive prompt and effective therapy.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study design and study population.

The study was a prospective, case controlled study. Samples were collected between January 2004 and December 2005. Test subjects were recruited from patients attending the Medical Outpatients' Clinic and those on admission in the Liver Unit on the Medical Wards at the University College Hospital (UCH), Ibadan, Nigeria, under the management of a consultant Physician/Gastroenterologist. They consisted of volunteer patients diagnosed with acute or chronic liver diseases such as viral hepatitis, liver cirrhosis and primary liver cell carcinoma, who fulfilled the inclusion and exclusion criteria for the study.

Diagnosis of liver diseases was made by relevant clinical features and laboratory tests such as liver function tests, prothrombin time/international normalized ratio (INR), liver biopsy and liver ultrasonography. Liver function tests consisted of serum bilirubin, alanine transaminase, aspartate transaminase, gamma glutamyl transpeptidase, alkaline phosphatase, total protein and albumin.

A questionnaire (Appendix I) was used to collect the clinical and laboratory data.

Control subjects were apparently healthy individuals who also fulfilled the inclusion and exclusion criteria for the study. They were all physically examined and not found to have any clinical features of acute or chronic disease. Specifically, hepatomegaly, splenomegaly, ascites, jaundice and peripheral stigmata of chronic liver disease were sought for and excluded. Biochemical features of liver were excluded by determination of serum alanine and aspartate transaminases. Most of the control subjects were recruited from consenting relations of patients, administrative staff of

the hospital, doctors, nurses, medical students and unremunerated or non-commercial blood donors.

Inclusion criteria for cases

1. Adults aged 18 years and above.
2. Volunteer patients with established acute or chronic liver diseases eg viral hepatitis, liver cirrhosis and hepatoma.
3. Biochemical evidence of liver disease (raised ALT, AST and bilirubin).
4. Absence of hepatic encephalopathy.

Inclusion criteria for controls

1. Adults aged 18 years and above.
2. Apparently healthy individuals.
3. Ability to give consent.
4. No known clinical or biochemical features of liver disease either in the past or presently.

Exclusion criteria for cases

1. Unwillingness to participate.
2. Pregnancy.
3. Inability to give consent.

Exclusion criteria for controls

1. Unwillingness to participate.

2. Pregnancy.

3. Inability to give consent.

4. Known liver disease.

A questionnaire was administered on each patient to collect biodata, alcohol and drug history, as well as history of hepatitis B immunization, tribe, smoking, educational level, past medical history of jaundice and family history of liver disease.

Ten milliliters of venous blood was collected at least one hour after the last meal to avoid lipaemia, from each subject from the antecubital vein into a plain specimen tube by approved venipuncture procedures. Samples were allowed to clot at room temperature within 15-20 minutes to avoid haemolysis and centrifuged at 1000rpm. Sera were separated after clot retraction, into 5 aliquots in 2ml plain eppendorf tubes, stored immediately at $-80^{\circ}C$ till analysed. The specimens were analysed at the Institute of Immunology, Laboratoire de Sante, Luxembourg Each of the 5 aliquots was used for different analytes. Liver function tests (LFT), prothrombin time, alphafetoprotein and abdominal ultrasonography were carried out at the University College Hospital, Ibadan, Nigeria.

Ethical consideration

Ethical approval was sought and obtained from the UCH/UI Institutional Review Board (IRB) (Appendix II).

3.2 Biochemical Analysis

Liver function tests, which comprised Total bilirubin, aspartate transaminase (AST), Alanine transaminase (ALT), gamma glutamyl transpeptidase (γ-GT), alkaline phosphatase (ALP), albumin, globulin and total protein were done with automated

Saturno 150 machine (colorimetric method). Prothrombin time/international normalized ratio (INR) as well as alphafetoprotein were also analysed.

3.3 Detection of autoimmune markers

Autoimmune markers

Anti-nuclear antibodies (ANA), anti-mitochondrial antibodies (AMA), anti-Liver–kidney microsome type-1 antibodies (anti-LKM-1), anti-soluble liver antigen (Anti-SLA), and perinuclear antineutrophil cytoplasmic antibodies (pANCA) were analysed using the Enzyme-linked Immunosorbent Assay (ELISA) method according to manufacturer's specification (AESKU DIAGNOSTICS. GmBH, Germany).

Principle of the test

After incubation of diluted sera (1:101) in microplates coated with specific antigen, patient's antibodies, if present in the specimen, bind to the antigen. Unbound fraction is washed off. Incubated anti-human immunoglobulins conjugated to Horseradish peroxidase (conjugate) reacts with the antigen-antibody complex of the samples in the microplates. Unbound conjugate is washed off. Addition of TMB-substrate generates an enzymatic colorimetric (blue) reaction, which is stopped by diluted acid (colour changes to yellow).

The rate of colour formation from the chromogen is a function of the amount of conjugate bound to the antigen-antibody complex and this is proportional to the initial concentration of the respective antibody in the patient's sample.

Materials: Shown in appendix III

Methods:

For each of the autoimmune antibody analysed, a plate scheme was prepared with wells allocated for negative control, positive control, cut-off control, calibrators in varying dilutions, for test and control samples. Sera for analyses and kits equilibrated to room temperature before the procedure was carried out. Sera were diluted to 1:101 using the sample buffer provided (Tris NaCl, Tween, Na azide <0.1% and thimerosal 0.01%).

100ul of diluted serum was pipetted into designated microwells while 100ul of calibrators or cut-off control and positive and negative controls into the designated wells. The plate was incubated at room temperature (20°-26°C) for 30 minutes. This was washed 3 times with washing buffer. 100ul of conjugate was added to each well and incubated again at room temperature (20°-26°C) for 15 minutes.

A second run of washing with washing buffer was done 3 times, after which 100ul of TMB substrate was added into each well and incubated at room temperature (20°-26°C) in the dark for 15 minutes. At the end of the third incubation period, 100ul of stop solution (1M HCl) was added to stop the reaction. This was incubated for 5 minutes, after which the plate was agitated for 5 seconds. The absorbance was read with a microplate reader (Appendix IV) at 450 nanometer within 30 minutes.

3.4 Determination of serological viral markers.

Serum levels of IgG anti-HCV, HBsAg, HBeAg, Anti-HBe and Total and IgM anti-HBc were determined using ELISA technique (ABBOT Murex Diagnostics, Germany) according to the manufacturer's protocol.

Hepatitis B surface antigen determination

Principle of the procedure:

Immobilised antibody (anti-HBs) solid phase specific for HBsAg in a 96 polystyrene microtitre plate wells is used. The sample to be analysed for HBsAg is added to the well and the antigen captured to form an antibody-antigen complex. Unbound molecules are removed by washing. An HBs-antibody labelled with the enzyme Horseradish peroxidase is added to form an antibody-antigen-antibody/enzyme conjugate complex, followed by a wash step.

A substrate solution was added to produce a colour change which is proportional to the amount of bound enzyme. Thus samples which do not contain HBsAg will not form a complex and therefore no colour reaction will take place. Wells that contain samples that do contain HBsAg will show a colour change corresponding to the number of individual complexes formed. A spectrophotometer measures the colour produced to give a numerical reading.

Materials: Shown in appendix IV

Methods:

A plate scheme was prepared to designate wells for negative and positive controls as well as for test and control sera. 25ul of sample diluent was added to each well, after which 75ul of serum (sample) was added to designated wells. Similarly, 75ul of negative control reagent was added to wells A1 and 1B, while the positive control reagent was added to well 1C. The plate was covered and incubated for 60 minutes at 37°C. After incubation, 50ul of conjugate was added into each well. This was agitated

gently, by tapping the sides of the plate for 10 seconds and incubated again at 37°C for 30 minutes. The wells were washed 5 times with wash solution at the end of which the plate was inverted on an absorbent paper to ensure dryness of the wells. At the end of the washing, 100ul of substrate solution was added to each well and incubated for another 30 minutes at 37°C.

After the third incubation period, 50ul of stop solution (1M H_2SO_4) was added to inactivate the reaction, and the absorbance/optical density (OD) was read at 450nm wavelength in a spectrophotometer (SpectraMax plus, Appendix III) within 15 minutes, using 650nm as reference wavelength. The result was interpreted by calculating the cut-off value thus:

Cut-off value = 0.05 + mean of Negative control replicates.

Interpretation of result: Samples with absorbance equal to or greater than the cut-off value were considered reactive (positive), while those less than the cut-off value were considered non-reactive (negative).

3.5 *Determination of Hepatitis B e Antigen*

Principle of the test:

To detect HBeAg, test and the control sera are incubated with a second monoclonal antibody conjugated to horseradish peroxidase in microwells coated with monoclonal antibody to HBeAg. HBeAg, if present, simultaneously binds to both antibody on the solid phase and the conjugate, creating an antibody-antigen-antibody 'sandwich'. After washing to remove unbound conjugate and excess sample, a solution containing TMB and hydrogen peroxide is added to the wells. Wells with bound conjugate

develop a purple colour which is converted to an orange when the reaction is terminated with H_2SO_4. Colour intensity is directly related to HBeAg concentration.

Materials: Shown in appendix V

Methods:

Same as for HBsAg determination except for the difference in the conjugate and neutralizing antigen use.

Cut-off values were determined according to reagent manufacturer's specification and positive and negative samples were noted.

Determination of Antibody to Hepatitis e Antigen

Principle of the test:

To detect anti-HBeAg, test and the control sera are incubated with a second monoclonal antibody conjugated to horseradish peroxidase in microwells coated with monoclonal antigen to react with HBeAg. Anti-HBe, if present, simultaneously binds to both antigen on the solid phase and the conjugate, creating an antibody-antigen complex. After washing to remove unbound conjugate and excess sample, a solution containing TMB and hydrogen peroxide is added to the wells. Wells with bound conjugate develop a purple colour which is converted to an orange when the reaction is terminated with H_2SO_4. Colour intensity is directly related to anti-HBeAg concentration.

Materials Shown in appendix VI

Methods:

Same as for HBsAg determination except for the difference in the conjugate and neutralizing antigen use.

Cut-off values were determined according to reagent manufacturer's specification and positive and negative samples were noted.

3.6 *Total Antibody to Hepatitis B core Antigen determination:*

Principle of the test:

Microwells coated with recombinant HBc antigen (rHBcAg) and samples are incubated, and any anti-HBc present in the sample binds to the rHBcAg. Excess antibody is removed by washing. Conjugate (monoclonal anti-HBc conjugated to Horseradish peroxidase) is added to the wells, followed by a second incubation, during which the conjugate binds to any rHBcAg on the well surface which has not been blocked by human anti-HBc in the test sample. Washing removes unbound conjugate, and a solution containing TMB and hydrogen peroxide is added. Wells without anti-HBc and bind conjugate, with development of a blue/green colour which is converted to orange when enzyme reaction is stopped with H_2SO_4. The intensity of colour is measured spectrophotometrically.

Materials: Shown in appendix VII

Methods:

A plate scheme was prepared and wells were designated for negative and positive controls as well as for test and control sera. Sample and test kit were brought to room temperature.

50 ul of sample diluent was added to each well, this was followed by addition of 50ul of test and control sera into designated wells, using separate tips. The plate was then covered with a lid and then incubated for 30 minute at 37 0C. At the end the incubation period the plate was washed manually five times with the wash solution provided in the kit. After washing, 50ul of conjugate was added to each microwell, covered with a lid and then incubated the second time for 30 minutes at 37oC. A second run of washing was done 5 times after the incubation period and 100ul of substrate solution was added to each well. The plate was then incubated the third time at 37 0C for 30 minutes. The reaction was stopped by addition of 50ul of 1M H2SO4. The OD was then read in a spectrophotometer within 15 minutes at a wavelength of 450nm, using 690nm as reference wavelength.

Cut-off values were determined according to reagent manufacturer's specification and positive and negative samples were noted.

3.7 Immunoglobulin G Antibody to Hepatitis C Virus determination:

Principle of the test.

The assay procedure is a three-stage test carried out in a microwell coated with a combination of recombinant hepatitis C virus antigen (c22-3, c200, and NS5).

Incubation of sera in the microwell yields antigen-antibody complexes if antibody reactive to any of the 3 antigens is present in the specimen. O'phenylenediamine (OPD) is used for colour generation, with sulphuric acid used as a stop agent. The intensity of colour is dependent on the amount of bound conjugate, and therefore is a function of the concentration of anti-HCV present in the specimen. The colour intensity is measured with a microwell reader.

50 ul of sample diluent was added to each well, this was followed by addition of 50ul of test and control sera into designated wells, using separate tips. The plate was then covered with a lid and then incubated for 30 minute at 37 0C. At the end the incubation period the plate was washed manually five times with the wash solution provided in the kit. After washing, 50ul of conjugate was added to each microwell, covered with a lid and then incubated the second time for 30 minutes at 37oC. A second run of washing was done 5 times after the incubation period and 100ul of substrate solution was added to each well. The plate was then incubated the third time at 37 0C for 30 minutes. The reaction was stopped by addition of 50ul of 1M H2SO4. The OD was then read in a spectrophotometer within 15 minutes at a wavelength of 450nm, using 690nm as reference wavelength.

Cut-off values were determined according to reagent manufacturer's specif cation and positive and negative samples were noted.

3.7 Immunoglobulin G Antibody to Hepatitis C Virus determination:

Principle of the test.

The assay procedure is a three-stage test carried out in a microwell coated with a combination of recombinant hepatitis C virus antigen (c22-3, c200, and NS5).

Incubation of sera in the microwell yields antigenantibody complexes if antibody reactive to any of the 3 antigens is present in the specimen. O'phenylenediamine (OPD) is used for colour generation, with sulphuric acid used as a stop agent. The intensity of colour is dependent on the amount of bound conjugate, and therefore is a function of the concentration of anti-HCV present in the specimen. The colour intensity is measured with a microwell reader.

Materials: Shown in appendix VIII

Methods:

A plate scheme was prepared. All reagents were equilibrated to room temperature (20-26 °C) 30 minutes before the procedure. 200ul of sample diluent was added to each well, after which 20ul of samples and controls were added into appropriate wells (1 reagent blank well was excluded). The plate was incubated at 37°C for 30 minutes, followed by manual washing of the wells 5 times with the wash buffer. After the washing, 200ul of conjugate was added to each well, and the plate was incubated for 30 minutes at 37 °C. At the end of the incubation period, the wells were washed 5 times with the wash buffer. 200ul of substrate solution was subsequently added to each well and incubated at room temperature in the dark for 30 minutes (Appendix IV). To stop the reaction, 50ul of 4N H_2SO_4 was added to each well after which the optical density (OD) was read on a spectrophotometer at 490nm wavelength within an hour of addition of the stop solution.

Cut-off values were determined according to reagent manufacturer's specification and positive and negative samples were noted.

3.8 *Determination of molecular markers of Hepatitis B virus:*

HBV DNA extraction.

Principle of the procedure

Cells are lysed during a short incubation with proteinase K in the presence of chaotropic salt (guanidine HCl). The incubation process inactivates all nucleases and

enhances the binding of DNA onto a glass surface. Subsequent serial washings remove contaminants.

Materials. Shown in appendix IV

Methods.

Sera from liver disease patients and controls were thawed and equilibrated to room temperature (20–26°C). The heating block was heated to a temperature of 56°C.

20 µl of QIAGEN Protease was pipetted into 1.5 ml microcentrifuge tubes (23 tubes at a time). 200 µl serum sample was added to each microcentrifuge tube, followed by addition of 200 µl Buffer AVL. The mixtures were mixed thoroughly by pulse-vortexing for 15s and then incubated at 56°C on the heating block for 10 minutes. 200ul of 100% ethanol was added to each sample, mixed again by pulse-vortexing for 15 seconds and briefly centrifuged.

The mixture was pipetted into the QIAamp spin column (in a 2ml collection tube), and spun at 8000 rpm for 1 minute. QIAamp spin column was placed in another 2ml collection tube and the filtrate was discarded. 500ul of AW1 buffer was added to the spin column and again centrifuged at 8000 rpm for 1 minute. Spin column was placed in 2 ml collection tube and the filtrate was discarded. 500ul of buffer AW2 was added and spun at 14,000 rpm for 3 minutes. The QIAamp Spin Column was placed in a clean 1.5ml microcentrifuge tube and the filtrate discarded. This was followed by addition of 200 µl Buffer AE and subsequent incubation at room temperature for 1 min. The tubes were centrifuged at 8000 rpm. The filtrate, which is the DNA extract was frozen at –25°C till amplified by polymerase chain reaction.

3.9 *Polymerase Chain Reaction for S-gene of HBV:*

Principle of the test

Heating up the DNA template to 94°C, leads to denaturation of the double strands into single strands. Cooled to about 54°C causes annealing. Heating up again to 72°C leads to extension by using polymerase enzyme. The cycle of denaturation, primer annealing and primer extension is repeated over and over again. During repeated rounds of these reactions, the number of newly synthesized DNA strands increases exponentially.

Materials: Shown in appendix X

Methods:

Premix preparation: 23ul of premix consisting of 1-8 above was carried out in the premix room inside a laminar flow hood according to calculated concentration and volume in the protocol (Appendix XI) used at the Laboratoire de Sante, Institute of Immunology, Luxembourg. A plate scheme for 24 samples and, positive and negative controls was prepared. 23ul of the premix was pipetted into each of the 26 designated microwells, followed by addition of the positive and negative controls as well as sera into the microwells in the PCR plate and covered with cap strips. The mixture was vortexed and centrifuged briefly to remove droplets on the tubes

5ul of HBV-DNA template was added into each well in the PCR plate and vortexed, followed by a brief centrifuging. The PCR plate was placed in the PCR machine (Opticon 2^{R} – Appendix IX) and allowed to run according to configured protocol (Appendix XI), which involved the use of a thermal cycler for the process of

denaturation, annealing and extension for the first PCR run of 40 cycles. To further amplify the product, a second PCR run was carried out with the first PCR run product as template. The protocol/conditions of the second PCR run were partially varied (Appendix XI) from the first run in that the concentration of the magnesium chloride ($MgCl_2$) was increased and the reverse primer and template were altered.

3.10 *Molecular markers of Hepatitis C Virus:*

HCV RNA extraction.

Principle of the test: Essentially as for HBV DNA.

Materials: Shown in appendix XI

Methods:

Purification of Viral RNA (Spin Protocol)

140 µl of sera from cases and controls and buffer AVE were equilibrated to room temperature (15–25°C) for eluting. Carrier RNA reconstituted in Buffer AVE to Buffer AVL. 560 µl of prepared Buffer AVL containing the carrier RNA was added into a 1.5 ml microcentrifuge tube. 140 µl serum was added to the Buffer AVL–carrier RNA in the microcentrifuge tube. This was mixed by pulse-vortexing for 15s to yield a homogeneous solution.

The mixture was incubated at room temperature for 10 minutes, to ensure complete viral particle lysis. Briefly centrifugation was done to remove drops from the inside of the lid. 560 µl of ethanol (100%) was added and mixed by pulse-vortexing for 15s, followed by brief centrifugation to remove drops from inside the lid. 630 µl of the

solution from above was applied to the QIAamp Mini column in a 2 ml collection tube.

The cap was closed and tube centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini column was placed into a clean 2 ml collection tube. The tube containing the filtrate was discarded and process repeated until all of the lysate had been loaded onto the spin column.

The QIAamp Mini column was opened and 500 µl of Buffer AW1 was added and centrifuged at 6000 x g (8000 rpm) for 1 min and placed in a clean 2 ml collection tube. The filtrate was then discarded.

The QIAamp Mini column was opened again and 500 µl of Buffer AW2 was added before being centrifuged at 20,000 x g; (14,000 rpm) for 3 min. The QIAamp Mini column was again placed in a clean 1.5 ml microcentrifuge tube. Filtrate was discarded. 60 µl of Buffer AVE was added and allowed to equilibrate at room temperature. This was incubated for 1 min before being centrifuged at 6000 x g (8000 rpm) for 1 min. The RNA eluate was stored at -20^{0}C till PCR analysis.

3.11 *HBV DNA electrophoresis:*

Principle of the test

Electrophoresis is a technique used to separate and sometimes purify macromolecules, especially proteins and nucleic acids that differ in size, charge or conformation. When charged molecules are placed in an electric field, they migrate toward either the positive or negative pole according to their charge. In contrast to proteins, which can have either a net positive or net negative charge, nucleic acids have a consistent negative charge imparted by their phosphate backbone, and migrate toward the anode.

Materials: Shown in appendix XII

Methods:

Agarose Gel preparation:

2g of agar was weighed into conical flask and 100mls Tris-Acetate-EDTA (TAE) buffer was added. The flask was shaken gently to mix and placed in a microwave oven till boiled and turned into a bubble-free colourless gel. This was allowed to cool slightly at room temperature. 2mls of ethidium bromide was added and shaken gently. The mixture was poured into the gel casting chamber (Appendix V) composed of UV-transparent plastic and contains a sample comb (electrophoretic plate) while avoiding bubble formation. Appropriate combs were placed in the gel chamber to make wells and the gel allowed to set. The 2 combs were removed after setting to reveal the wells.

Agarose Gel Electrophoresis:

2ul of loading dye was added into each well in the PCR plate (Appendix VI) after which 5ul of PCR product was added. The mixture was centrifuged for about 10 seconds to remove droplets on the wall of the wells in the PCR plate. The gel plate was set in the gel-running chamber containing TAE buffer solution. 7ul of the mixture in the PCR plate was pipetted into wells in the gel. 1Kb DNA ladder was added into each well. The negative and positive electrodes were connected and the current run for 30 minutes at a voltage of 80mAmp. The gel was removed from plate and placed in the UV camera chamber where photographs of the gel was taken and stored. Appendix VII Wells that were positive on the photographs (Appendix VIII) were noted and selected for HBV-DNA quantification (Viral load) and sequencing. HBV-

DNA viral load was quantified using a computer software on the Opticon^R DNA amplification machine (Appendix IX).

3.12 *HBV-DNA sequencing and genotype determination.*

Samples that were positive on agarose gel electrophoresis (Appendix X) were purified and further subjected to PCR amplification. The PCR products were purified by using a JetQuick Purification Spin kit (Genomed GmbH). The purified DNA was quantified with Picogreen (Invitrogen) by using a Genios Plus fluorescence reader (Tecan). Purified DNA (50 ng) was sequenced in both directions on an An ABI Prism 3010 capillary sequencer (Applied Biosystems). Briefly, 5 ml DNA was amplified in a 10 ml reaction volume containing 4 ml premix (BigDye Terminator Cycle Sequencing Ready Reaction kit; Applied Biosystems) and 1 ml of each sequencing primer was added. One hundred and eighty sequences were obtained either with primers C1/C2 or C1/preA or C1/preS and included the entire preC/C gene with a total length of 517–584 bp, depending on the genotype (genome positions 1814–2331; numbering according to GenBank accession no. X75657). In addition, three complete genomes and four preS fragments (positions 2455–159) were sequenced. Nucleotide sequences were analysed by using ABI Sequencing Analysis (version 3.4.1) and Sequence Navigator (version 1.0.1), aligned with CLUSTAL W software and checked by visual inspection. Phylogenetic trees were constructed with the MEGA 3.1 software, using the neighbour-joining and Kimura 2 parameter method and including reference strains of genotypes A–G and all known A and D subtypes. Sequences were submitted to GenBank/ EMBL/DDBJ under accession numbers AM11079.1–

AM110915 for the preC/C gene and AM180623-AM180628 for the complete genome and preS segment sequences.

3.13 *HCV RNA amplification*

For amplification of the core/E1 region of HCV, a semi-nested PCR was performed in a 25-IL reaction containing 0.5 IL cDNA, 2.5 mM MgCl2, 200 nM dNTPs, 50 nM each primer (fw290utr(+), 5¢-TGCCTGATAGGGTGCTTGCGAG, pos. 290–311; 1321e1, 5¢-ACCAGTTCATCATCATATCCCATG CCAT, pos. 1293–1320), and 1 U Platinum Taq DNA polymerase with 1 · PCR buffer. PCRs comprised 95°C for 5 mins, followed by 40 cycles of 95°C for 1 min, 63°C for 1 min and 72°C for 1 min. Nested PCRs were performed using the same conditions with 5 IL of the first-round product diluted 1:100, but with a different forward primer (fw480c(+), 5¢ CGCGCGACTAGGAAGACTTC, pos. 480-499; rv1321c1), 0.10 IM each primer, 2 IM MgCl2 and an annealing temperature of 62°C. The product of the first round S fragment PCR was cloned using the pCR4-TOPO kit (Invitrogen, Hilden, Germany) according to the manufacturer's protocol.

3.14 Statistical analyses

Statistical analysis was carried out on generated data using SPSS statistical software version 11.0 for windows. Prevalence rates of autoimmune and specific viral markers were calculated to reflect the relative frequency of each disease. Odds ratio (OR) and ninety five percent confidence interval (95% CI) was calculated using the Fischer's Exact Test to estimate the strength of the association between each marker and possible risk factor. Pearson Chi square was used to compare proportions while

students' t-test was used to compare means. Where numerical values were low, median was used. Significant statistical difference was specified at $p < 0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 Age and sex distribution of cases and control subjects

A total number of 145 adult patients were recruited, but 126 samples from 91 (72.2%) males and 35 (27.8%) females were considered for analyses. Samples from nineteen patients were excluded because of spillage and insufficient volumes. The patients consisted of HCC 77 (61.1%), liver cirrhosis 32 (25.4%), chronic hepatitis 10 (7.9%), acute viral hepatitis 4 (3.2%), alcoholic cirrhosis 1 (0.8%) and primary biliary cirrhosis 2 (1.6%).

Eighty two (82) apparently normal individuals consisting of 59 (72%) males and 23 (28%) females were recruited over the study period to serve as controls. The mean ages of the cases and the controls were 47.5±14.4 vs 39.6±16.5 respectively. There was no statistically significant difference in the sex distribution between the cases and control subjects ($p>0.05$). Not surprisingly, majority of the patients were Yoruba constituting 103 (81.7%), while Hausa, Ibo and other tribes constituted 3 (2.4%), 4 (3.2%) and 16 (12.7%) respectively.

4.2 Biochemical parameters and clinical presentation among subjects with Liver Disease.

As shown in Table 4, the mean levels of bilirubin, gamma-glutamyl transferase, alkaline phosphatase, globulin, albumin, PTR and alpha-fetoprotein were high among some of the liver cases. Higher ALT 123 iu/l vs 12.2 and AST 196.6 iu/l vs 24.3 were recorded among cases compared with controls ($p<0.01$).

Among the test subjects, hepatomegaly occurred in 99 (78.6%), ascites in 72(57.1%),

jaundice in 62 (49.2%) among others. See Table 4. Fifty two (41.3%) subjects with liver disease consumed significant alcohol. Significant alcohol consumption was 50g of alcohol per day for five years in men and 40g in women. In order to standardize and align with SI unit, many authorities have recommended conversion to grammes of alcohol consumed. To convert concentrations of alcohol, usually listed in volume percent (equivalent to the volume of solute/volume (%v/v) is multiplied by the specific gravity of alcohol, 0.79g/ml (Turner 1990, Hrick, 2006, & O'Shea *et al;* 2010).

4.3. Prevalence of serologic autoimmune markers among cases and controls

One hundred and twenty six cases and 82 controls were analysed for autoantibodies (see Table 5), except for anti-nuclear antibodies (ANA) for which only 107 cases and 67 controls were analysed due to insufficient volume of some samples.

Of the 5 autoimmune serologic markers tested, only antimitochondrial antibodies (AMA) was found to be significantly higher among cases compared with controls (See Table 5). Antimitochondrial antibodies were present in 76 (60.3%) of the cases compared with 36 (43.9%) controls ($p<0.05$), while antinuclear antibodies (ANA) were present in 42 (39.3%) of cases compared with 27 (39.7%) controls ($p=0.68$). Anti-soluble liver antigen (anti-SLA/LP) and perinculrophil cytoplasmic antibodies (pANCA) were completely absent among cases and controls (See Table 5).

Table 4: Biochemical and Clinical parameters among subjects with liver disease.

Biochemical parameter	n	Range	Mean (SD)	Median
Total Bilirubin (mg/dl)	126	0.40	9.5 ± 10.2	5.3
ALT(iu/l)	126	0.6-963	123.4±154	87.5
AST(iu/l)	126	2.5-882	196.6±176.1	139.0
γ-GT (iu/l)	126	27-1009	341.9±300.9	236
Alk Phosphatase (iu/l)	126	26-1226	338.9±267.8	249
Total Protein (g/dl)	126	4.4-10.7	7.9±1.3	-
Albumin (g/dl)	126	1.3-4.6	2.8±0.4	-
Globulin (g/dl)	126	2.1-7.2	4.9±0.96	4.9
Prothrombin time ratio	126	0.65-3.26	1.51±	1.26
Alphafetoprotein (uk/L)	38	4.5-711.5	135.2±249.2	11.7

Clinical Parameters		
Clinical sign	Number	Percentage
Hepatomegaly	99	78.6
Ascites	72	57.1
Jaundice	62	49.2

NB: Among controls ALT was 0-32±3.6; AST was 0-37±2.2

Table 5: Prevalence of viral markers and autoantibodies among cases and controls

Markers	Cases N= 126	Controls N= 82	X^2	p-value
ANA	42(39.3)	27(39.7)	0.75	0.68
AMA	76(60.3)	36(43.9)	5.37	0.02
LKM-1	1(0.8)	1(1.2)	0.09	Fischer's exact 1.0
pANCA	0	0	-	-
Anti-SLA/LP	0	0	-	-
HBsAg	103(81.7)	49(59.8)	12.21	0.000
HBcAg	27(21.4)	1(1.2)	17.41	0.000
Anti-HBc	60(47.6)	20(24.4)	11.32	0.001
Anti-HBc	118(93.7)	60(73.2)	16.88	0.000
Anti-HCV	45(35.7)	8(9.76)	17.63	0.000
DNA	58(46%)	1 (1.2%)	19.58	0.000
PreS PCR	53 (42.1%)	1 (1.2%)	16.24	0.000

NB. Percentage in parenthesis, ANA analysed: cases =107, controls=67

*P values less than 0.05 ($p<0.05$) in front of data show that there are significant differences between cases and controls

4.4. Prevalence of serologic viral markers among cases and controls.

All the tested serologic viral markers were significantly different between the cases and control subjects. Hepatitis B surface antigen was positive in 103 (81.7%) cases compared with 49 (59.8%) controls ($p<0.05$). Similarly, HBcAg, Anti-HBc, anti-HBc (total) and anti-HCV were significantly higher among cases compared with controls, suggesting a strong association of the viral agents with liver disease in this environment (Table 5). Among the cases, HBsAg and anti-HBc had the highest frequencies, being present in 81.7% and 93.7%, respectively. These parameters were also noted to be high among control subjects, though at a relatively lower rates. Table 5.

4.5 Sex distribution of autoimmune and viral markers among cases and controls

Anti-mitochondrial antibody was more prevalent in males (68.4%) compared with females (31.6%) among the cases. Similarly, among the controls, AMA was more prevalent (83.3%) in males compared with females (16.7%). However, among cases, the proportion of females positive for AMA was higher when compared with the control group. Fifty two (57.1%) of the 91 males and 24 (68.5%) of the 35 females among cases were AMA positive but they constitute 68.4% and 31.6% of the 76 positive for AMA (Table 6). Among the controls, 30 (50.8%) of the 59 males and 6 (26.0%) of the 26 females were AMA positive. One positive anti-LKM-1 each occurred among the cases and the controls, and both were males (Table 6).

All the markers of HBV (HBsAg, anti-HBc, HBcAg, Anti-HBc) and HCV (anti-HCV) were also higher in the male gender, but did not reach statistical significance as

depicted in Table 6. For HBsAg, 72.8% of those positive among cases were males compared with 27.2% in females (Table 6).

4.6. Age distribution of subjects positive for autoimmune and viral markers

The highest positivity for AMA among cases was recorded in the age group 30-39 years in contrast to the controls which was in age group less than 30 years. The result presented in Table 7 also showed that the least occurrence of AMA was found in the age group 70 years or more, in both cases 5(6.6%) and controls 2(5.6%).

Anti-LKM was recorded positive in one sample each among cases and controls, and both were below 30 years of age.

The higher prevalences of HBsAg was in the age ranges 30-39, 40-49 and 50-59 years among the cases, while the highest prevalence among the control group was in the age group below 30 years (Table 8). The lower prevalences of HBsAg was found in the ages below 30 years and above 70 years among cases, while the lowest prevalence among the controls was in the age 60 years and above. The HBcAg was most prevalent in the age 30-39 years among the cases. Anti-HBc was more prevalent in the age range 30-39 years among cases, similar to the pattern of HBsAg, but more controls were positive for anti-HBc in the age group less than 30 years (Table 8). The prevalence of anti-HCV was highest among the cases in the age ranges 40 to 49 years. The prevalences of other serological viral markers are shown in Table 8.

Table 6: Sex distribution of subjects with positive autoantibodies and viral markers.

Parameter	Total positive		Total positive (Cases)		Total positive (Controls)			
	Cases	Control	Male	Female	Male	Female	X^2	P-value
ANA*	42 (39.3)	27(39.7)	37(88.1)	5(11.9)	21(77.8)	6(22.2)	1.31	0.253
AMA	76(60.3)	36(43.9)	52(68.4)	24(31.6)	30(83.3)	6(16.7)	2.77	0.096
LKM-1	1(0.8)	1(1.2)	1(100)	0	1(100)	0	-	-
HBsAg	103(81.7)	49(51.8)	75(72.8)	28(27.2)	34(69.4)	15(30.6)	0.19	0.661
HBcAg	27(21.4)	1(1.2)	20(74.1)	7(25.9)	1(100)	0	0.35	0.556
Anti-HBe	60(47.6)	20(24.4)	44(73.3)	16(26.7)	11(55)	9(45)	0.35	0.128
Anti-HBc	118(93.6)	60(73.1)	88(74.6)	20(25.4)	43(71.7)	17(28.3)	2.16	0.141
AntiHCV	45(35.7)	8(9.7)	37(82.2)	8(17.8)	6(75.0)	2(25.0)	0.23	0630

Cases =126; Controls = 82

NB. Percentage in parentheses. *ANA=107 cases; 67 controls.

• P values less than 0.05 ($p<0.05$) in front of data show that there are significant differences between cases and controls.

Table 7: Age group distribution of subjects positive for autoimmune markers.

Age grp (yrs)	AMA		Anti-LKM-1		ANA	
	Cases	Control	Cases	Control	Cases	Control
<30	10 (13.2)	15 (41.7)	1 (100)	1 (100)	6(14.3)	8(29.6)
30-39	19 (25)	7 (19.4)	0	0	8(19.0)	5(18.5)
40-49	14 (18.4)	3 (8.3)	0	0	12(28.3)	2(7.4)
50-59	15 (19.7)	6 (16.7)	0	0	9(21.4)	4(14.8)
60-69	13 (7.1)	3 (8.3)	0	0	4(9.5)	2(7.4)
≥70	5 (6.6)	2 (5.6)	0	0	3(7.1)	6(22.2)

Nb: Number with percentages in parenthesis.

Table 8: Age group distribution of subjects positive for viral markers.

Age GP (Yrs)	HBsAg		HBeAg		Anti-HBc		Anti-HDc		Anti-HCV	
	Cases	Control	Cases	Control	Cases	Control	Cases	Control	Cases	Control
<30	10	18	3	0	8	3	12	19	4	2
	(9.7)	(36.7)	(11.1)		(13.3)	(15.0)	(10.2)	(31.7)	(30.8)	(6.5)
30-39	23	7	9	1	14	4	26	10	10	0
	(22.3)	(14.3)	(33.3)	(100)	(63.3)	(20.0)	(21.0)	(16.7)	(33.3)	
40-49	23	8	3	0	14	3	26	11	12	2
	(22.3)	(16.3)	(11.1)		(23.3)	(15)	(22)	(18.3)	(46.2)	(16.7)
50-59	23	6	6	0	9	5	26	8	10	0
	(22.3)	(12.2)	(22.2)		(15)	(25)	(22)	(13.3)	(37)	
60-69	16	5	4	0	10	4	20	6	5	3
	(15.5)	(10.2)	(14.8)		(16.7)	(20)	(16.9)	(10)	(22.7)	(37.5)
≥70	8	5	2	0	5	1	8	6	4	1
	(7.8)	(10.2)	(7.4)		(8.3)	(5)	(6.8)	(10)	(30)	(16.7)

NB: Number with percentages in parenthesis.

4.7. Frequency of viral and autoimmune markers among liver cases.

Among the autoimmune markers tested, only AMA was significantly higher in cases compared with controls, $p<0.05$ (Figure 12). Chronic hepatitis had the highest frequency of AMA, being positive in 9 (90%) of the 10 cases, this was followed by HCC, 48(62.3%) of the 77 cases tested were positive for AMA. The only patient with alcoholic cirrhosis was also positive for AMA.

All the viral markers were significantly higher among liver disease cases compared with controls (Tables 5, & Figure 12). Hepatitis B surface antigen was positive in 103 (81.7%) vs. 49 (59.8%), HBeAg was positive in 27 (21.4%) vs. 1 (1.2%), anti-HBe in 60 (47.6%) vs. 20 (24.4%), anti-HBc in 118 (93.7%) vs. 60 (73.2%), anti-HCV in 45 (35.7%) vs. 8 (9.8%), preS PCR in 53 (42.1%) vs. 1 (1.2%) and HBV-DNA in 58 (46%) vs. 1 (1.2%) among test and control subjects respectively (Table 5).

Total anti-HBc had the highest prevalence among the different types of liver diseases, except for PBC in which it was negative. Total anti-HBc was positive in all cases with acute viral hepatitis and alcoholic liver disease. It was also present in 31(96.9%) of liver cirrhosis, 73(94.8%) of HCC, and 9(90%) of chronic hepatitis (Table 9). Prevalence of Hepatitis B surface antigen was highest among cases with HCC, 70 (90.9%) of the 77 cases. This was followed by chronic hepatitis 8 (80%), acute viral hepatitis 3 (75%) and liver cirrhosis 21(65%). Prevalence of HBeAg, anti-HBe and anti-HCV among cases are shown in Figure 12. The percentage frequencies of viral markers and autoantibodies are shown in Figure 13.

4.8. Prevalence of viral markers in samples positive for autoantibodies.

Among cases, anti-HBc had the highest frequency 70 (92.1%) followed by HBsAg 62 (81.6%) among the 76 cases who were positive for AMA. Anti-HCV was positive in

60% while Anti-HBc and HBeAg were positive in 53.9% and 21.1% respectively (Table 9). Prevalence among the controls who were AMA positive was similar to findings among cases, anti-HBc had the highest frequency of 25 (69.4%), but unlike among cases, anti-HCV ranked second with HBsAg having the third highest frequency (Table 10). HBeAg was completely negative among controls who were AMA positive.

The positive ANA, AMA and all the serological HBV markers were significantly higher than the negative counterparts when compared (Table 11). On the other hand, when the positive ANA and AMA among cases were compared with the positive controls, there was no significant difference observed (Table 12). However, comparison of negative pANCA, anti-LKM-1 and anti-SLA/LP among cases and controls showed significant difference (Table 12), with higher values among cases for anti-LKM-1 and anti-SLA/LP, and higher values among controls for AMA and pANCA (Table 12).

On comparative analysis of the numerical values of positivity and negativity of the autoantibodies and viral markers, positivity of HBsAg and anti-HBc were significantly higher among cases compared with controls, p 0.00 (Table 13).

This would suggest that HBsAg and anti-HBc among cases were in greater concentrations in positive samples among cases compared with positive samples among the control group.

Similarly, HBsAg and anti-HCV negativity was significantly different among cases compared with controls, p 0.02 (Table 13). However, no significant difference was found for negative anti-HBc, positive anti-HBc and positive anti-HCV when compared, p>0.05 (Table 13).

Table 9: Frequency of viral and autoimmune markers among liver cases.

Type of liver disease	No tested	HBsAg	HBeAg	Anti-HBc	Anti-HBe	Anti-HCV	ANA	AMA	Anti-LKM-1
Acute hepatitis	4(3.2)	3(75)	1(25)	2(50)	4(100)	1(25)	1(25)	2(50)	0
Chronic hepatitis	10(7.9)	8(80)	4(40)	6(60)	9(90)	6(60)	4(40)	9(90)	0
Liver cirrhosis	32(25.4)	21(65.6)	5(15.6)	14(43.8)	31(96.9)	11(34.4)	11(34.4)	15(46.9)	0
Alcoholic cirrhosis	1(0.8)	0	0	1(100)	1(100)	0	0	1(100)	0
PBC	2(1.6)	1(50)	1(50)	1(50)	0	1(50)	0	1(50)	0
HCC	77(61.1)	70(90.9)	16(20.8)	36(46.8)	73(94.8)	26(33.8)	26(61.9)	48(62.3)	1(1.3)
Total Cases	126	103	27	60	118	45	42	76	1
p-value		0.07	0.55	0.86	.00	060	0.65	0.210	0.99

Percentage in parenthesis.

Anti-SLA and pANCA were negative in all.

Figure 1b:

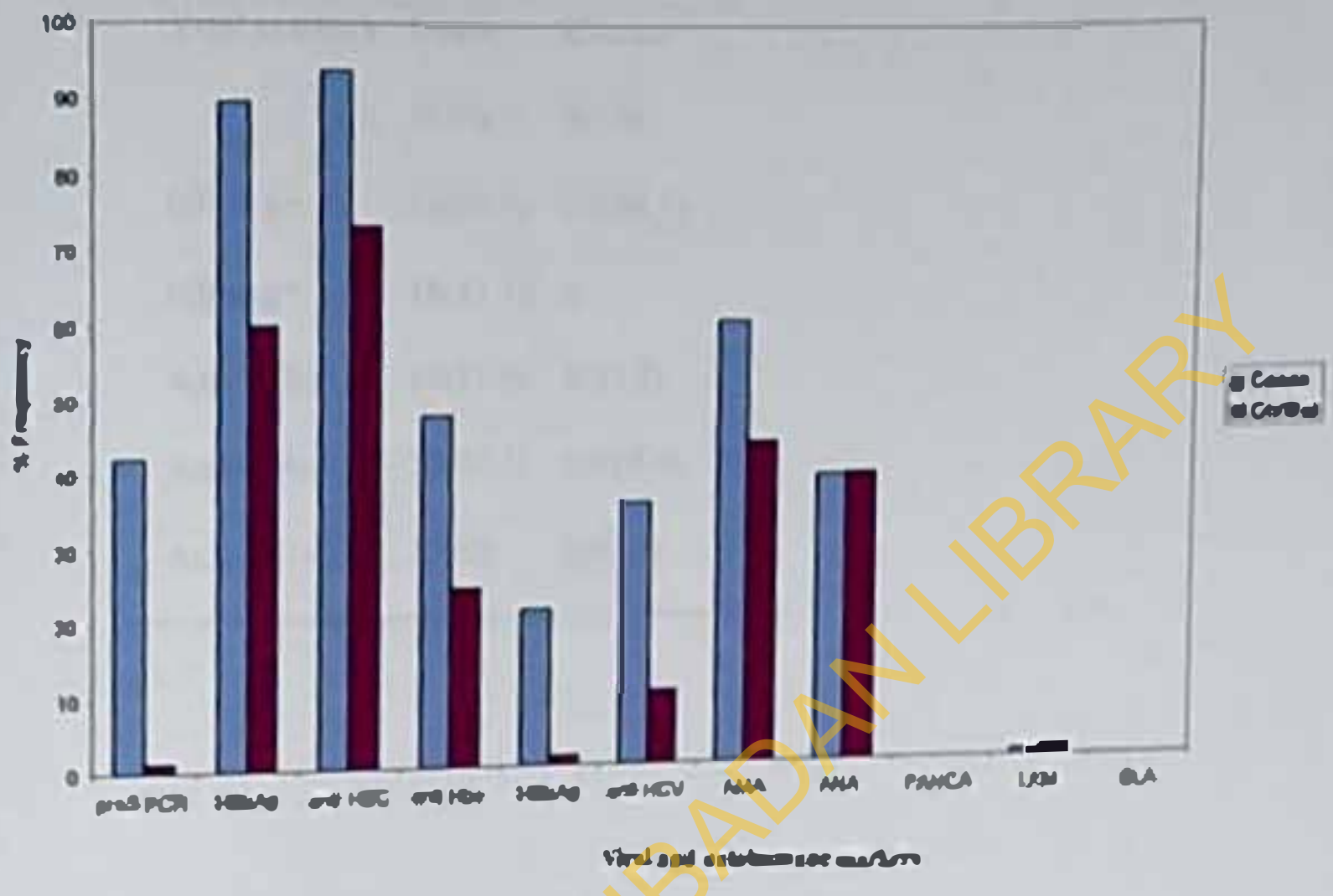

Fig. 13: Percentage frequency of viral and autoimmune markers

Table 10: Prevalence of viral markers in samples significantly positive for AMA autoantibodies

	AMA+	
Viral markers	Cases	Control
	N=76	N=36
HBsAg+	62(81.6)	21(58.3)
HBeAg+	16(21.1)	0
Anti-HBe+	41(53.9)	8(22.2)
Anti-HBc+	70(92.1)	25(69.4)
Anti-HCV+	27(60)	5(62.5)

Table 11 Relative strengths of positive and negative autoimmune and viral markers

Cases					Controls			
Status	n	Mean±SD	t	p	n	Mean±SD	t	p
ANA								
Pos	43	[illegible]	[illegible]	[illegible]	28	[illegible]	9.5	0.00
Neg	42	[illegible]			50	[illegible]		
ANA								
Pos	76	[illegible]	15.9	0.00	36	[illegible]	14	0.00
Neg	30	[illegible]			42	[illegible]		
HBsAg								
Pos	103	[illegible]	8.3	0.00	19	[illegible]	3.0	0.00
Neg	23	[illegible]			59	[illegible]		
Anti-HBc								
Pos	118	[illegible]	20.4	0.00	60	[illegible]	16.5	0.00
Neg	8	[illegible]			22	[illegible]		
Anti-HBe								
Pos	80	[illegible]	11.8	0.00	20	[illegible]	6.167	0.00
Neg	66	[illegible]			62	[illegible]		
HBeAg								
Pos	27	[illegible]	12.8	0.00	1	[illegible]	3.0	0.00
Neg	99	[illegible]			81	[illegible]		
Anti-HCV								
Pos	45	0.9±0.7	10.1	0.00	8	[illegible]	12.3	0.00
Neg	81	[illegible]			74	[illegible]		

*P values less than 0.05 ($p<0.05$) in front of data show that there are significant differences between the positive and the negative markers.

Table 12: Relative strength of positive and negative autoimmune markers among liver cases compared with controls

	n	Mean±SD	T	P
ANA Positive				
Cases	33	88[illegible]	-1.6	0.11
Controls	28	1021.7±435.7		
ANA		Negative		
Cases	42	305.4±95.8	2.18	0.16
Controls	36	250.3±89.1		
ANA Positive				
Cases	76	1431.7±358.0	0.9	0.35
Controls	30	1369.4±307.9		
ANA Negative				
Cases	50	353.9±220.7	-2.61	0.01
Controls	46	637.3±158.4		
p-ANCA Negative				
Cases	126	191.60±77.17	-3.10	0.00
Controls	82	226.93±88.19		
c-ANCA Negative				
Cases	123	212.12±88.56	2.65	0.01
Controls	81	176.64±88.23		
SCA Negative				
Cases	126	235.81±93.31	2.16	0.03
Controls	82	170.74±65.[illegible]		

* P values less than 0.05 (p<0.05) in front of data show that there are significant differences between cases and controls

Table 13: Relative strength of positive and negative viral markers among liver cases compared with controls

HBsAg Positive	n	mean±SD	t	p
Cases	163	2.04±1.6	9.8	0.00
Controls	19	0.53±0.3		
HBsAg Negative				
Cases	23	0.13±0.02	-2.37	0.02
Controls	33	0.14±0.02		
Anti-HBsAg Positive				
Cases	118	0.71±0.07	-3.7	0.00
Controls	60	0.2±0.1		
Anti-HBc Negative				
Cases	8	0.47±0.35	-1.82	0.08
Controls	22	1.35±0.32		
Anti-HBe Positive				
Cases	60	0.3±0.2	-1.5	0.14
Controls	20	0.3±0.2		
Anti-HBe Negative				
Cases	66	1.69±0.91	3.34	0.01
Controls	62	1.29±0.22		
Anti-HCV Positive				
Cases	13	0.94±0.60	1.45	0.15
Controls	8	0.58±0.38		
Anti-HCV Negative				
Cases	81	0.13±0.10	1.37	0.00
Controls	74	0.06±0.01		

* P values less than 0.05 (p<0.05) in front of data show that there are significant differences between the positive and the negative markers.

The only LKM-1 positive case was also positive for all the tested viral markers except anti-HCV, while the only LKM-1 positive control was negative for all viral markers except anti-HBc (Table 10).

4.9. Hepatitis B virus DNA among cases and controls.

Overall, HBV-DNA was detected in the sera of 58 (46.0%) of 126 patients with liver diseases compared with only one among the 82 (1.2 %) controls (X^2=51.53, p=0.000). Thirty-five (60.3%) of the 58 cases that were HBV-DNA positive were concomitantly AMA positive. The only LKM-1 positive case was also HBV-DNA positive in contrast to the HBV-DNA negative LKM-1 positive found in the control group (Table 10). Among the liver cases, HBV-DNA was found to be positive in all the anti-SLA and pANCA negatives, similarly, among the control group the only HBV-DNA positive was found among the pANCA and anti-SLA negative.

4.10. Frequency of HBV DNA positivity compared with serological viral markers

Among the cases, HBsAg positive samples had the highest level of HBV-DNA positivity, with 57(98.3%) of the 58 DNA positives occurring in HBsAg positives, compared with 1 (1.7%) among HBsAg negatives. This showed that, of the 103 HBsAg positives 57 (55.3%) had HBV-DNA, while of the 23 HBsAg negatives, 1 was HBV-DNA positive, suggesting the incidence of occult HBV infection of 4.3%. The only HBV-DNA positive sample among the controls was also HBsAg positive. Twenty-three (85.2%) of the HBeAg positive cases however were HBV-DNA positive with a lower HBV-DNA positivity (35.4%) being recorded among cases who

are negative for HBcAg (Table 15). The DNA positivity was however too low to be compared statistically. It is obvious that there is a stronger association of HBcAg with HBV-DNA.

High frequency of HBV-DNA positives were also observed among anti-HBe (53.3%), anti-HBc (47.5%), and anti-HCV (50%) positives but lower than that that in HBsAg positives. The corresponding frequencies of HBV-DNA in anti-HBe (39.4%), anti-HBc (25%) and anti-HCV (45.9% negatives were relatively lower with significant statistical difference attained only for anti-HCV (Table 14). There was also no statistically significant difference in the HBV-DNA positivity among cases positive for Anti-HBe and anti-HBc ($p=0.12$ and 0.22 respectively), (Table 15).

4.11 Hepatitis B viral load among liver cases

The mean viral load using the s-plasmid for the surface antigen was highest among the cases with acute viral hepatitis, with a mean greater than 3.5 million (Geometric mean of 751.86) and maximum of [illegible] million copies/uL. This was followed by patients with liver cirrhosis with a mean viral load over 1.3 million (Geometric mean of 63.6) but a maximum viral load greater than that of patients with acute viral hepatitis (22.2 million copies/ul). Hepatocellular carcinoma had a mean of 214, 978, (Geometric mean 43.15), while CH had 15, 119 (Geometric mean 7.17), (Table 16). The levels were undetectable in PBC and alcoholic cirrhosis. Table 17 shows the number of cases and controls that have detectable viral DNA on polymerase chain reaction.

4.12: Frequency of HBV-DNA in clinical diagnosis groups

Hepatitis B virus DNA detection varied in the various classes of liver disease. HBV-DNA detection was highest among cases with acute viral hepatitis (75%) with none found among cases with alcoholic liver disease and primary biliary cirrhosis. Hepatocellular carcinoma and liver cirrhosis showed a serum HBV-DNA prevalence of 50.6% and 40.6% respectively (See Table 17). Only 3 of the 10 patients with chronic hepatitis had detectable HBV-DNA.

4.13. Phylogenetic analyses of HBV

Out of the 56 samples sequenced (55 cases, 1 control there were 53 (94.6%) genotype E and 2 (3.6%) genotype A. The only control was genotype E.

4.14. HCV-RNA

HCV-RNA was negative in all subjects who were positive for anti-HCV and in those who were negative for anti-HCV.

Table 14: Prevalence of HBV-DNA positivity compared with autoimmune markers among subjects

	Cases			Controls		
Autoantibodies	DNA+	DNA-	Total	DNA+	DNA-	Total
AMA+	35	41	76	1	35	36
AMA-	23	27	50	0	46	46
Total	58	68	126	1	81	82
P	0.00			1.294		
LKM+	1		1	0	1	1
LKM-	57		125	1	80	81
Total	58		126	1	81	82
Fischer's exact test	0.460			1.000		
pANCA+	0	0	0	0	0	0
pANCA-	58	68	126	1	81	82
Total	58	68	126	1	81	82
Anti-SLA+	0	0	0	0	0	0
Anti-SLA-	58	68	126	1	81	82
Total	58	68	126	1	81	82

*P<0.05 is considered significant.

Table15: Frequency of HBV-DNA positivity compared with viral markers

	Cases			Controls		
	DNA+	DNA-	Total	DNA+	DNA-	Total
HBsAg+	57(98.3)	46(67.6)	103	1(100)	48(59.3)	49
HBsAg-	1(1.7)	22(32.4)	23	0	33(40.7)	33
Odds ratio	27.26	-	-	-	-	-
Total	58	68	126	1	81	82
P	0.00			1.00		
HBeAg+	23(39.7)	4(5.9)	27	0	48(59.3)	49
HBeAg-	35(60.3)	64(94.1)	99	1(100)	33(40.7)	33
Odds ratio	10.51					
Total	58	68	126	1	81	82
P	0.00			1.00		
Anti-HBc+	32(55.2)	28(41.2)	60	1(100)	19(23.5)	20
Anti-HBc-	26(44.8)	40(58.8)	66	0	62(76.5)	62
Odds ratio	1.76					
Total	58	68	126	1	81	82
P	0.12			1.0		
Anti-HBc+	56(98.2)	62(91.2)	118	1(100)	59(72.8)	60
Anti-HBc-	2(1.8)	6(8.8)	8	0	22(27.2)	22
Odds ratio	2.71					
Total	58	68	126	1	81	82
P	0.22 Fisher's			1.00		
Anti-HCV+	2(1.8)	2(2.9)	4	-	-	-
Anti-HCV-	56(98.2)	66(97.1)	122	1(100)	81(100)	82
Odds ratio	1.20					
Total	58	68	126	1	81	82
P	0.03			1.00		

*P<0.05 is considered statistically significant.

Table 16: Pre-S-plasmid viral load in diagnosis groupings (copies/uL).

Diagnosis	No.	Mean (Geometric)	Min	Max
Alcoholic cirrhosis	1	1.00	0.00	0.00
HCC	74	43.15	0.00	6,170,000.00
Cirrhosis	30	63.60	0.00	22,200,000.00
PBC	2	1.00	0.00	0.00
CH	10	7.17	0.00	151,097.30
AH	4	751.86	0.00	14,000,000.00
Total	121			

Only 1 control was positive for s-plasmid viral load (0.025copies/uL).

Key: HCC= Hepatocellular carcinoma; PBC= Primary biliary cirrhosis; CH= Chronic hepatitis; AH= Acute hepatitis.

Table 17: Frequency of HBV-DNA detection in clinical diagnosis group

Clinical diagnosis	HBV DNA+ (%)	HBV DNA- (%)	Total
Alcoholic cirrhosis	0	1(100)	1
HCC	39 (50.6)	38(49.4)	77
Liver cirrhosis	13(40.6)	19(59.4)	32
PBC	0	2(100)	2
Chronic hepatitis	3(30)	7(70)	10
Acute hepatitis	3(75)	1(25)	4
Total	58	68	126

CHAPTER 5

5.0. DISCUSSION

The scourge of liver diseases have contributed immensely to morbidity and mortality in clinical medicine. Autoimmune and viral-related liver diseases have been well known to be major drain on the health budgets worldwide especially in developed countries where vital health statistics are available. In Africa and indeed in Nigeria, despite the evidences for the presence of clinically appreciable autoimmune disorders, there has been no remarkable effort directed at unraveling the presence and magnitude of autoimmune liver diseases.

This study focused on autoantibodies associated with autoimmune liver diseases among Nigerian patients with liver diseases, examined the prevalence of autoimmune antibodies related to liver diseases, and then related these with hepatitis B and C viral markers, which are well established causes of liver diseases globally and in Nigeria.

The spectrum of liver diseases found during the study period suggested that, in the hospital setting most of the liver diseases seen were HCC (61.1%) and liver cirrhosis (25.4%), with autoimmune liver diseases being uncommon constituting only about 1.6%. This confirms the age long suspicion that autoimmune liver diseases are rare among Nigerians, compared with Caucasian populations (Greenwood 1968). There were no readily available data among other African countries to compare our findings with because there are no published data on autoimmune liver diseases. This study which was carried out over a twenty-four month period showed that males were more afflicted significantly with liver diseases than females in keeping with findings in previous studies (Armstrong et al. 2000; Bell *et al.* 2008, Clark *et al.* 2003; Fischer *et al.* 2009). This gender difference in prevalence of liver diseases is thought to be

multifactorial as the male gender is more affected with hepatitis viruses and are more likely to indulge in alcohol consumption, even though. women die more of alcohol-related liver disease (Ashley *et al.* 1977; Becker *et al* .1996). This is because women are less resistant to the damaging effect of alcohol on the liver (Baraona *et al.* 2001).

The major clinical examination findings were those well known to be associated with chronic liver disease, that is, liver disease that has lasted for more than six months, and they include hepatomegaly, ascites and jaundice in order of frequency (Table 4). Alcohol did not play a major role in the causation of liver disease among our cohort of patients, in spite of over half of the patients having imbibed significant alcohol. According to the guidelines on alcoholic liver diseases by the American College of Gastroenterology (ACG) and other studies, alcohol consumption is significant if amounts consumed is up to 80g of alcohol per day for ten years in men (Lindros, 1995), and 60g or more per day for women (Lelbach *et al.* 1975; McCullough & O'Connor, 1998). Significant alcohol consumption means the amount of alcohol consumed per day that would definitely lead to liver damage over a specified period of ten years, which might be alcoholic steatosis or steatohepatitis, alcoholic hepatitis and alcoholic cirrhosis. The amount is lower in women because of the lower levels of gastric epithelial alcohol dehydrogenase among women, which makes them develop liver disease at a lower rate of alcohol consumption (Baraona *et al.* 2001). Alcohol dehydrogenase is a group of enzymes found in the gastric epithelium and involved in the breakdown of alcohol before its absorption. In line with common knowledge, more men consumed significant alcohol compared to women.

The use of ELISA technique to measure the liver-related autoantibodies is unique and novel in that hitherto, the cumbersome indirect immunofluorescence technique with

use of rat kidneys was employed in most studies (Kerkar *et al*; 2002; Vergani & Micli-Vergani, 2004). The novel use of ELISA, which has been found to be sensitive, specific, objective and rapid would facilitate standardized approach to measurement of autoantibodies and afford comparability of studies globally.

In this study, antimitochondrial antibodies (AMA) were the only autoantibodies found to be statistically significantly higher among the patients with liver disease (60.3%) compared with the apparently normal control group (43.9%). The enigma of this finding is in the fact that even those who were apparently healthy also had a relatively high percentage of ANA though its presence is supposed to be diagnostic of primary biliary cirrhosis (Hu *et al* 2010). Similarly, the lack of significant difference in the levels of ANA and LKM-1 in liver cases compared to controls would suggest that there is a mechanism responsible for erratic production of liver-related autoantibodies in the Nigerian population, regardless of health status of the liver. This is contrary to what obtains among the Caucasians in whom the presence of ANA correlates well with systemic or organ specific autoimmune disease, being the most common autoantibodies in autoimmune hepatitis (Hahn, 1998). In the same population, ANA has been found to be predictive of autoimmune diseases (Tan. *et al* 1988, Tan. 1989), while LKM-1 is a useful laboratory tool in the diagnosis of type-2 autoimmune hepatitis, which is commoner among children and young adults (Mieli-Vergani and Vergani. 2009).

In the sixties, Greenwood postulated that the rarity of autoimmune disorders among Nigerians may be due to the presence of several environmental parasitic antigens stimulating the immune system. His study at the UCH, Ibadan showed that diseases in which autoimmune processes were thought to be involved are uncommon in

Western Nigeria (Greenwood, 1968), and suggested that the infrequent occurrence of autoimmune disease in parts of tropical Africa is related to the immunological disturbance produced by multiple parasitic infections. He also described a low incidence of autoantibodies in rheumatoid arthritis and high incidence of rheumatoid factor among apparently healthy Nigerians (Greenwood et al 1970). Evidence abound that some of the immunological changes noted in apparently healthy Africans are related to infection with malaria (McGregor et al, 1956, Greenwood *et al*; 1970). Similar evidence of malaria infection affecting immunological response has also been documented in mice infected with malaria (Greenwood and Voller; 1970). In 1995, Skalsky et al, in a study of chronic liver diseases in rural south-west Cameroun found that serum autoantibodies were frequently found and were not correlated with the presence of autoimmune liver disease. The complete absence of anti-SLA/LP in both the test and the control subjects further validates the rarity of type-1 autoimmune hepatitis among our patients with liver diseases, as these autoantibodies had been found to be 100% specific for AIH (Bakker-Jonges *et al*. 2006). Similarly, pANCA were also completely negative in both cases and controls in this study, further substantiating the rarity of autoimmune liver diseases in our cohort of patients with liver diseases. These findings are in contradiction to studies in Caucasian populations (Zauli *et al*. 1997; Bogdanos et al 2009; Washington 2007, Bakkerjonges *et al*. 2006) and in India (Choudhuri *et al*. 2005), a developing country, where they have been found to be useful in the diagnosis of autoimmune liver disease, and sometimes used for prognostication (Pokorny *et al*. 1991). There was no published study on autoimmune liver disease to compare our study with in Nigeria, making this study a pioneering effort in Nigeria and in most countries of Africa. If the postulation of

Greenwood about four decades ago, that malaria was responsible for the erratic production of autoantibodies among our population, it would suggest that the battle against malaria is far from over in spite of the huge investment being made into research and pharmacotherapeutics. It is however tempting to postulate that autoimmune diseases will emerge in our population, if and when the scourge of malaria is stemmed.

Unlike the serological autoantibodies, serological and molecular viral markers were by far commoner and significantly higher among patients with liver diseases compared to controls. The finding of HBsAg in 81% of the liver cases compares favourably with previous studies in Nigeria and the rest of sub-sahara Africa. Ndububa *et al.* 2005 at Ile-Ife (Nigeria) found HBsAg positivity in 77.4% of symptomatic patients with liver disease; same study found 100% HBsAg in asymptomatic patients with liver disease. Similarly, Ojo *et al.* (1995) in a study at Ile-Ife, found HBsAg to be present in 62% of their patients with chronic liver disease. In Lagos Nigeria, Lesi et al (2004), found 58% of patients with chronic liver disease to be HBsAg positive, while in Ibadan Nigeria, Olubuyide *et al.* (1997) found it in 59% of patients with hepatocellular carcinoma, the commonest chronic liver disease in Nigeria. Similarly, Ola *et al.* (2002) found the prevalence of HBsAg to be 84.4% among patients with acute viral hepatitis. The prevalence of HBsAg among the control group is rather high, this is likely due to the fact that most of the control group were recruited from hospital staff like ward maids, nurses, doctors and relations of patients on admission on the wards or clinics of the hospital. Olubuyide *et al.* (1995), had previously documented a high risk of HBsAg among doctors and dentists in the hospital, while Otegbayo *et al.* (2002) found a high risk of exposure to hepatitis B

among doctors. However, similarly high HBsAg carriage among normal population in both rural and urban centers have been documented in 1993 by Olubuyide *et al.* A prevalence rate of 47% - 49% was documented among apparently normal individuals in this study. All these prevalences are much higher than in low and intermediate endemic continents of the world like the USA and the Mediterranean respectively. The prevalence of HBsAg in the USA is about 0.5% of the normal population. (McQuillan *et al.* 1999), while the prevalence is 4-8% in the Mediterranean (Nothdurft *et al.* 2007).

Hepatitis B core antibody was the most prevalent HBV marker among liver cases and controls. This is due to the high rate of exposure of individuals to the virus in an endemic area like Nigeria. Similar results were found in other hyperendemic areas of the world like South Africa (Kew, 1996), Gambia (Edmunds *et al* 1996), the rest of sub-saharan Africa, South-East Asia and South America. The higher prevalence of other HBV markers such as HBeAg and anti-HBc, in addition to anti-HCV among liver cases compared to controls shows their strong association with liver disease and are therefore likely pathogenic. In particular HBeAg, a marker of viral replication, has been shown to be a surrogate marker for Hepatitis B x antigen (HBxAg), a transactivating protein implicated in the pathogenesis of liver cancer, HBeAg being also identified as a risk factor for the development of hepatocellular carcinoma (Yang *et al* 2002).

Although, in this study, gender distribution of AMA showed that more males were positive compared to females among cases and the control group, the proportion of females who were positive for AMA among cases was higher than that of the

controls. This would suggest that more females with liver disease are likely to be positive for AMA compared to apparently normal individuals.

Given the finding that autoimmune liver disease was uncommon in this study, it is not surprising that anti-LKM-1 was detected in only one case and one control in our patients, since these autoantibodies are usually associated with type II autoimmune hepatitis, which is mainly a disease of children in the paediatric age group.

In contrast to non-pathologic autoantibodies, which are usually found in the older age groups, AMA in our study was found more in the younger age groups. The significance of this is not yet clear. However, anti-nuclear antibodies on the other hand did not demonstrate any particular pattern of age difference.

The higher prevalence of HBsAg in the older age groups of 30-59 years among cases compared to less than 30 years among controls would be in keeping with the established fact that HBV was transmitted horizontally within the family early in life (Szmuness *et al.* 1975; Bernier *et al* 1982) and becoming pathogenic and leading to liver diseases a decade or more later (de Franchise *et al.* 1993; Villeneuve *et al.* 1994). Persons aged above seventy years also had lower prevalence of the virus. This might suggest that the disease states caused by HBV are associated with mortality leading to premature deaths and reducing survival to old age.

Although HBeAg was generally low in both cases and controls, it was highest in the age group below forty years. This might be due to the loss of the antigen in the evolution of the infection. It has been observed that persons of African descent tend to have low HBeAg levels due to the development of hepatitis B pre-core and core mutants, a condition associated with inability to secrete the envelope antigen (Carman *et al.* 1989), and also associated with poor response to interferon therapy.

Cases that were positive for anti-HCV were found to be a decade or two older (<50 years) suggesting a late pathogenetic effect of HCV compared to HBV. This observation has been previously documented by Okuda *et al* (1984) and Shiratori *et al* (1995), who found that patients with HBV-related liver disease were usually about ten years younger than those with HCV-related liver disease. Serum anti-HBc however, appeared not to have any age preponderance. This is not surprising as it was the most prevalent marker of HBV infection in this study, being the immunological fingerprint of previous contact with HBV infection.

The number of patients diagnosed with primary biliary cirrhosis (PBC) [2 (1.6%)] and alcoholic liver disease [1 (0.8%)] were too few to draw any reasonable conclusion from. It could be said that PBC, a form of autoimmune liver disease and alcoholic liver disease are rare in the South-western part of Nigeria, in spite of a relatively high significant alcohol consumption as found in this study (50.3%). Studies in the Middle belt and Northern Nigeria have however shown higher prevalence of alcoholic liver disease (Okeke *et al* 2002). The low incidence of alcoholic liver disease in spite of significant alcohol consumption confirms the assertion that HBV is the main causative agent of liver disease in our environment, with alcohol having an accelerating effect (Ndububa et al 2005). In the same vein, concentrations of HBsAg and anti-HBc positivity were significantly in higher concentrations among cases compared with controls. This, further goes to strengthen the the observation that increase in viral load of HBV is strongly associated with severity of liver disease (Chen *et al*, 2006; Viana et al 2009).

When viral markers were evaluated among cases and controls that were AMA positive, all the HBV markers were equally high in both groups. This would further

strengthen the suspicion that the measured autoantibodies do not really have any pathogenic role, since viral markers have been strongly associated with liver diseases among cases.

Apart from HBsAg, anti-HCV and AMA, another marker of HBV found to be significantly higher among cases compared to controls and strongly associated with liver disease is the HBV-DNA. This molecular marker of HBV is one of the markers of replication of HBV besides HBeAg and has been found to be strongly associated with liver diseases such as acute and chronic hepatitides, liver cirrhosis and hepatocellular carcinoma (Chen *et al*, 2006; Viana et al 2009) and higher HBV-DNA has been associated with more severe liver disease as was found in this study.

The occurrence of HBV-DNA in the serum in one case of HBsAg negative among cases would suggest a low incidence of occult HBV among our patients with liver diseases in this study. Occult HBV, a phenomenon in which HBV-DNA is present in the serum in the absence of serum HBsAg, is a recognized occurrence in the complex biology of HBV, and has been found to be as high as 3.8% to 30% (Chemin et al 2001; Minuk *et al* 2004) or even higher (Pollicino *et al* 2004; Kew *et al* 2008) in some studies both in Nigeria and other parts of the world. The condition has however been associated with liver diseases (Pollicino *et al* 2004). High frequency of HBV-DNA, similar to that found in HBsAg was also found in cases positive for anti-HBc and also in the only one subject among controls that had detectable HBV-DNA. The finding of only one case (4.3%) of detectable HBV-DNA among controls would suggest that HBV-DNA levels were generally low among subjects with HBsAg without liver disease, further strengthening the strong association of HBV-DNA with liver disease. It would also suggest that development of liver diseases in HBV

infection is directly proportional to serum HBV-DNA. Therefore, serial measurement of HBV-DNA in the serum of normal individuals with HBsAg would be a useful tool in monitoring development of liver disease, and thus early treatment. Monitoring of serum HBV-DNA should be the standard practice in the surveillance of subjects with HBsAg in addition to liver ultrasonography and serum alphafetoprotein (Mok *et al* 2005; Ferenci *et al* 2010). A previous study in Ibadan, however showed a prevalence of 7.2% for occult hepatitis B among the patients with viral hepatitis, though the sample size was small relative to that of this study (Ola *et al.* 2009).

Our study showed that subjects with HBV-DNA are about thirty times as likely to develop liver disease (Odds ratio 27.1, Table 11) compared to those without HBV-DNA, while those positive for HBeAg are about ten times as much compared to those who are negative for HBeAg. Such strong association was not however found with anti-HCV and other measured viral markers in this study. Some studies have found odds ratio lower than our finding; Chan *et al* (2009) in Hong-Kong found an odds ratio of 11.1%, while a relative risk of 60.2% was documented for those who were HBeAg positive in addition to HBsAg. In the Gambia, Mendy et al (2010) found a seventeen to thirty nine fold increase in the risk of cirrhosis and hepatocellular carcinoma for patients who were positive for serum HBV DNA. Our findings, therefore, makes it reasonable to check for HBV-DNA, HBsAg and HBeAg among patients with liver disease in our environment.

On quantitative analyses of HBV-DNA, patients with liver cirrhosis, a pre-malignant liver disease, had the highest HBV viral load titre followed by acute viral hepatitis, then hepatocellular carcinoma and chronic hepatitis in that order. This suggests that HBV viral replication tends to be high as in cirrhosis, before the development of

HCC. Therefore, a rising trend in the titres of HBV DNA in patients with CH is a pointer to development of advanced complications of liver disease.

In contrast to the finding of HBV-DNA in a significant number of subjects with HBV infection, all the subjects who were positive for anti-HCV were negative for HCV-RNA on polymerase chain reaction. This could be explained in part by false negativity, recovery from HCV infection or the suppressive effect of HBV on HCV which has been observed in some studies. This may however be a phenomenon peculiar to Nigerian patients with HBV infection or due to the recognized high rate of false positivity to anti-HCV screening in populations with low prevalence of HCV infection, therefore, requiring RIBA and RNA PCR for confirmation (Carson *et al* 1992; Sakugawa *et al.* 1995). Further studies are required in our environment to unravel the phenomenon. It has also, however, been shown that HBV infection has a suppressing effect on HCV replication.

Phylogenetic analysis of HBV in our study showed results in keeping with previous finding by Odeinuyiwa *et al* (2001) who found hundred percent of the twenty patients with HBV to be genotype E, showing the predominance of the E genotype. The genotype E of HBV is known to be endemic in West Africa and has not been found in any other region of the world. The genotype E is known to show resistance to interferon therapy in contrast to other HBV genotypes. The significance of this resistance is that unlike genotypes A, B, C, D, F and G, which are common in the Western World and Asia that readily respond to interferon therapy, other modalities of therapy need to be developed for genotype E. It would therefore be necessary for the pharmaceutical industries to engage in drug development that will be effective in the treatment of HBV genotype E in West Africa.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

Autoimmune liver diseases are uncommon in Ibadan, Nigeria and, the prevalence of autoantibodies to liver antigens is equally high in individuals with or without liver disease. Antimitochondrial antibodies (AMA) were significantly higher among cases with liver disease compared to controls. Antimitochondrial antibodies are the hallmark of primary biliary cirrhosis a condition associated with intense fibrosis of the bile ducts. Since most of the patients in this study had hepatocellular carcinoma and liver cirrhosis which are also associated with fibrosis, there may be a link between serum AMA and fibrosis in the liver.

It could be reasonably concluded that autoantibodies to liver antigens might be unreliable in predicting autoimmune liver disease in the studied population. Antibody to hepatitis B core antigen (Anti-HBc) was the most predominant hepatitis B virus (HBV) marker in the serum of both subjects with liver disease and apparently normal controls occurring in 93.7% and 73.2% respectively. Concentrations of HBsAg and anti-HBc positivity were significantly in higher concentrations among cases compared with controls.

HBV-DNA, HBsAg and anti-HCV were all significantly higher among subjects with liver diseases compared to the control group and therefore strongly associated with liver disease. HCV-RNA was negative in all the subjects that were positive for anti-HCV, revealing a very high rate of false positivity to anti-HCV screening in our population.

Also obvious from this study is that the risk of liver diseases is increased about thirty times fold in subjects with serum HBV-DNA, and its level rises with severity of liver disease.

HBV-DNA prevalence was similar in patients who were negative or positive for AMA, suggesting that AMA might not have any pathogenetic role to play in development of the liver diseases studied. Further studies are required to determine the relevance of AMA among patients with liver diseases other than autoimmune liver diseases.

Occult HBV infection, which is a phenomenon in which HBV-DNA is present in the serum in the absence of HBsAg, was found in a relatively few cases. It may therefore not be a major problem in the pathogenesis of HBV infection in our population.

In spite of the reasonably high prevalence of alcohol consumption among the patients with liver diseases, alcoholic liver disease (ALD) was low (0.8%). This might be due to the high prevalence of HBV infection. In view of the predominance of HBV genotype E in our study, and the attendant known resistance to interferon therapy, alternative effective therapy or therapies need to be developed.

RECOMMENDATIONS

Based on the results from this study, it is recommended that measures to prevent the transmission of HBV infection be entrenched in the population. These preventive measures should combine primary and secondary preventive measures.

Examples of primary and secondary prevention are:

1. Vaccination:
    a. Development of HBV vaccine

b. Hepatitis B Vaccination at birth.

c. Administration of Hepatitis B Immune Globulin (HBIg) to babies whose mothers are positive for HBsAg during antenatal screening.

2. Screening:

a. Screening of blood and blood products before transfusion.

b. Antenatal screening of pregnant women.

c. Pre-school screening for HBsAg

d. Pre-employment screening for HBsAg

e. Pre-marital counseling and screening for HBsAg.

f. Screening of at-risk groups eg prostitutes. Patients with liver disease who are positive to anti-HCV should be screened for HCV-RNA to determine actiology.

g. HBsAg. Anti-HBc and HBcAg should be screened for in all patients with liver disease.

3. Advocacy:

a. Advocacy groups such as non-governmental and community-based organizations (NGO/CBO) with interest in prevention of liver diseases should be encouraged.

4. Health Education:

a. Educating the populace on prevention of viral transmission eg from tattoos, multiple sexual partners, sharing of sharp objects among others.

b. Educating health workers and carriers of HBV infection about HBV-DNA monitoring.

FURTHER STUDIES

1. A larger multicenter community-based study should be carried out in Nigeria to substantiate our findings.
2. The reasons for high prevalence of autoantibodies in the normal population should be further explored.
3. Studies correlating IIF and ELISA-based autoantibody determination should be carried out to compare specificity and sensitivity.
4. Sequencing of HCV isolates for possible vaccine development.
5. Vaccine efficacy in different groups such as HIV, pregnancy, cancer and malaria among others.
6. Phylogenetic analysis of HCV.
7. Quantitation of HBsAg

b. Hepatitis B Vaccination at birth.

c. Administration of Hepatitis B Immune Globulin (HBIg) to babies whose mothers are positive for HBsAg during antenatal screening.

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Appendix I

Evaluation of Autoantibodies in Liver Disease Study Questionnaire

S/N: Hospital No. Age: Yrs. Sex: M/ F

Address: Contact Phone No:

Tribe: Yoruba/Hausa/Others Educational level: None 1°,2°, 3°

Marital Status: Single/Married/Divorced No of Children:

Smoking: Yes/No Alcohol: Yes/No Alc.Qty: g/dl

Alc. Duration: Weight: kg Height: cm BMI:

Family history of liver disease: Yes/No. If yes, relationship:

Presenting Clinical Symptoms:

Clinical Examination Findings:

Clinical diagnosis:

Laboratory Results
LFT: Bil. Total - Conj - Unconj- ALT: IU/L

AST: IU/L Alk phos: IU/L GGT: IU/L

Total Prot. g/dl Alb: g/dl Globulin: g/dl. INR:

Autoantibodies: ANA: AMA: PANCA: Anti-SLA/LP: Anti-LKM-1:
Viral Markers: HBsAg: HBcAg: Anti-HBc:
IgG-HBcAg HBV-DNA:
Anti-HCV: HCV-RNA: HBV genotype

Abd ultrasound report:

Other AID: Vitiligo. RA. SLE. AIHA Others:

Co-morbidities:

Liver biopsy Histology:

APPENDIX II
ETHICAL APPROVAL

INSTITUTE FOR ADVANCED MEDICAL RESEARCH AND TRAINING (IMRAT)
COLLEGE OF MEDICINE, UNIVERSITY OF IBADAN, IBADAN, NIGERIA

UI/UCH INSTITUTIONAL REVIEW COMMITTEE

Principal Investigator: Dr. J. A. Ijaduola

IRC Protocol No:

Protocol Title: NEUROLOGICAL MANIFESTATIONS AND CIRCULATING LEAK IN PATIENTS WITH VIRAL HEPATITIS IN THE AFRICAN REGION OF LIVER DISEASE AND ENCEPHALOPATHY

The UI/UCH Institutional Review Committee has reviewed your protocol titled, "*Neurological Manifestations and Circulating Hepatotoxins and Cytokines in the Pathogenesis of Liver Disease in Nigeria*."

The aim of the study is to investigate the contribution of environmental and established viral causes of liver diseases in Nigerians. Findings from the study will fill the gap in knowledge about the disease in this environment.

[illegible] APPROVED [illegible]

Professor [illegible]

APPENDIX III

Materials for detection of autoimmune markers

ELISA plate, AESKULISA® (AESKU DIAGNOSTICS. GmBH. Germany).

Negative control

Positive control

Cut-off control

Calibrators in varying dilutions

Test and control sera

Tris NaCl. Tween, No azide <0.1% and thimerosal 0.01% (reagent).

Conjugate

Washing buffer

Substrate

Stop solution (1M HCl)

Incubator

Microplate reader

Pipette

Tips

APPENDIX IV

Microplate reader

APPENDIX V

Materials for determination of serological viral markers.

ELISA plate (Murex HBsAg version 3 ABBOT Murex, Germany) was used.

Each well was coated with a mixture of mouse monoclonals specific for different epitopes in the "a" determinant of HBsAg.

Sample diluent (a buffer solution containing detergents and proteins of goat and bovine origin and preservative).

Negative control.

Conjugate, consisting of affinity purified goat antibody to HBsAg conjugated to Horseradish peroxidase.

Wash solution (Glycine/Borate with preservative).

Substrate solution (a mixture of trisodium citrate and hydrogen peroxide as diluent. and 3,3',5,5'-tetramethylbenzidine and preservative as concentrate.

1M H_2SO_4 (Stop solution).

Pipette and Tips, Incubator, Microplate reader.

APPENDIX VI

Materials for determination of *Hepatitis B e Antigen and Antibody to Hepatitis e Antigen*

Same as for HBsAg determination except for the use of:

HBeAg/anti-HBe positive control.

Conjugate (monoclonal antibody to HBeAg conjugated to horseradish peroxidase).

Neutralising antigen (recombinant HBeAg)

APPENDIX VII

Materials for determination of *Antibody to Hepatitis e Antigen*

Same as for HBsAg determination except for the use of:

anti-HBc positive control.

Conjugate (monoclonal antigen for anti-HBeAg conjugated to horseradish peroxidase).

Neutralising antigen (recombinant HBeAg)

APPENDIX VIII

Materials for Total Antibody to Hepatitis B core Antigen determination

-ELISA plate (Murex HBc plate version 3, ABBOT Murex, Germany) was used. The plate consisted of 96 wells, each coated with recombinant hepatitis B core antigen.

-Sample diluent (pH buffered solution)

-Conjugate (monoclonal anti-HBc conjugated to horseradish peroxidase)

-Anti-HBc negative control (a green dye with 0.05% Bronidox® as preservative)

-Anti-HBc positive control (from inactivated human sera)

-Substrate diluent (tri-sodium citrate and hydrogen peroxide)

-Substrate concentrate (3,3',5,5'-tetramethylbenzidine (TMB) and stabilisers)

-Wash fluid

-1M H2SO4 (Stop solution).

-Pipette

-Microplate reader

-Tips

APPENDIX IX

Materials for Immunoglobulin G Antibody to Hepatitis C Virus determination

OrthoR HCV ELISA Kit with SAVe was used (Ortho-Clinical Diagnostics, Inc UK

Wash buffer:

Substrate buffer

OPD tablets (o'phenylenediamine-2HCl)

ELISA plate coated with recombinant HCV antigens c22-3, c200 and NS5.

Incubator

Pipette and Tips

APPENDIX X

Materials for *determination of molecular markers of Hepatitis B virus*

QIAampR DNA Mini Kit (QIAGEN GmbH, Germany).

Heating block

Buffer AVL

Buffer AE

Buffer AW1

Buffer AW2

Protease (QIAGEN GmbH, Germany).

Centrifuge

APPENDIX XI

Materials for *Polymerase Chain Reaction for S-gene of HBV*

1. double distilled water (dd H_2O)
2. x10 TAE Buffer
3. Magnessium Chloride ($MgCl_2$)
4. Desoxyribosenucleotidtriphosphate (dNTPs)
5. Forward primer (Fw primer)
6. Reverse primer (Rv primer)
7. SYBR^R green (S green)
8. Taq polymerase
9. Template Deoxyribonucleic acid (DNA)

APPENDIX XII

Materials for *Molecular markers of Hepatitis C Virus*

As for HBV DNA extraction above, except for the use of carrier RNA and RNAse

APPENDIX XIII

Materials for *HBV DNA electrophoresis*

PCR product
Agar

2% Agarose gel

Loading dye

DNA ladder (1 Kb)

Electrophoresis gel casting chamber.

Electrophoresis gel running chamber.

APPENDIX XIV

Electrophoretic chamber

APPENDIX XV

Gel Camera Chamber

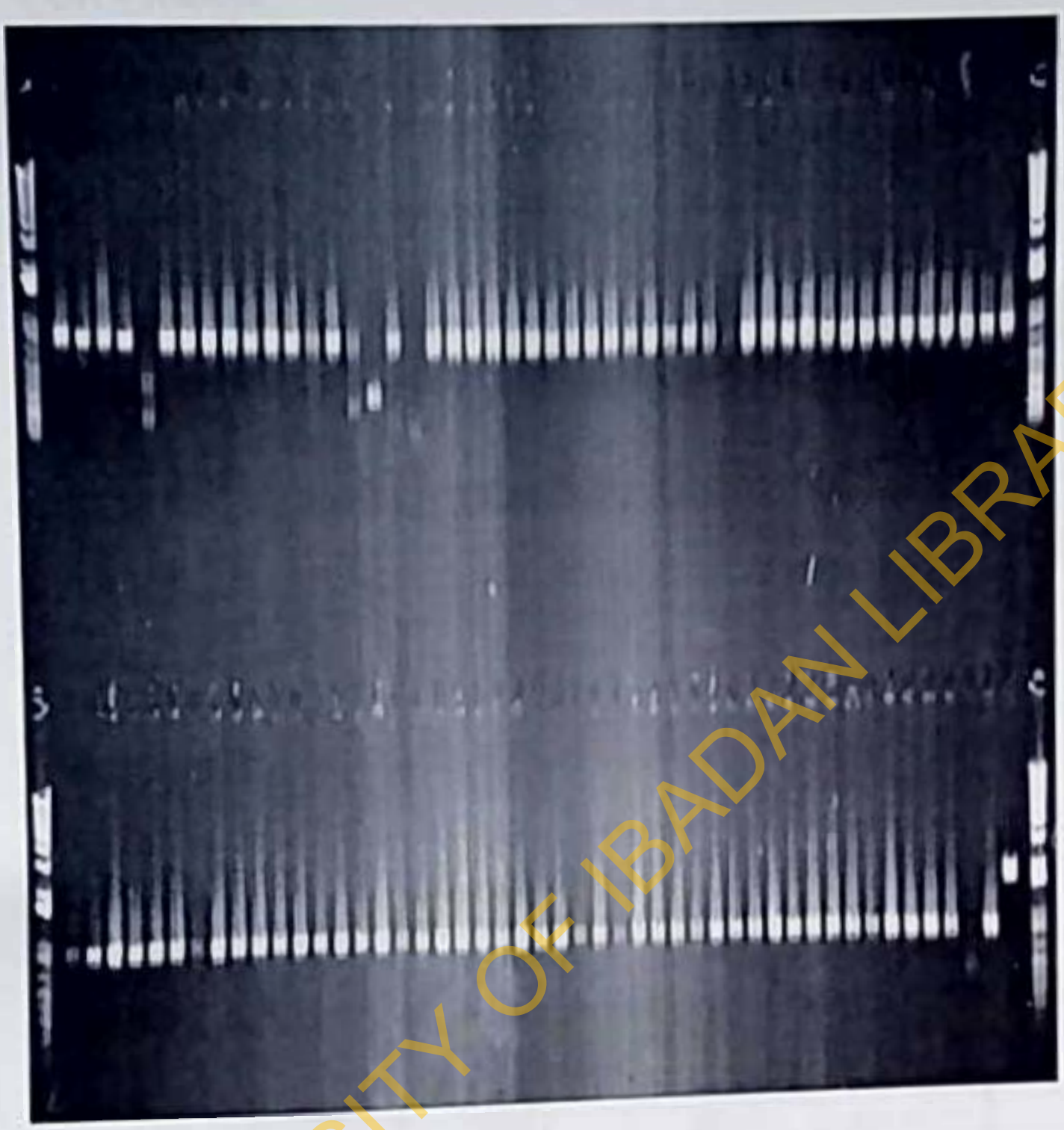
APPENDIX XVI
Positive Wells Photograph

APPENDIX XVII
Opticon[R] DNA amplification machine

Thermal cycler used for further amplification

APPENDIX XVIII

Electrophoretic samples used for sequencing



APPENDIX XIX

PCR Protocol

Exp:	JO3
Date:	20040207

Description: NIE 2005 26xxx Liver patients

Number of Samples: 29

Volume: 25 uL

Cycler: opticon

Conditions:

95	5
95	00 20
60	00 20
72	01 00
72°C	05 00

30

	S
fw primer	P2f
rv primer	Mc2r

Reagent	In PCR	Stocks	ul	Total Mix
ddH20			16.70	642.1
x10 Buffer	1 x	10 x	2.50	72.5
MgCl2	1.5 mM	50 mM	0.75	21.75
dNTPS	200 nM	10000 nM	0.50	14.5
Fw Primer	0.20 uM	50.00 uM	0.10	2.9
Rv Primer	0.20 uM	50.00 uM	0.10	2.9
Sgreen	1 x	100 x	0.25	7.25
Taq	0.02 U/ul	5.00 U/ul	0.10	2.9
Template	2.00 ul		2.00	58

Total			
	w/ template	25.00	725
	w/o template	23.00	667

1:100 dilution of first round

	1	2	3			
A	29021	29033	29041	pos control		
B	29022	29034	29042	neg control		
C	29023	29035	29043			
D	29024	29036	29046			
E	29025	29037	29047			
F	29030	29038	29048			
G	29031	29039	29049			
H	29032	29040	29050			
	JO1	JO1	JO1			
	JO2					

APPENDIX XX

RAW DATA OD OF VIRAL MARKERS AND AUTOANTIBODIES

sampleno	OD_ANA	OD AMA	OD_pANCA	OD_LKM	OD_SLA	OD_HbsAg	OD_antiHBctotal	OD_antiHBe	OD_HBeAg	OD_antiHCVIgG
29,001		1,429.00	241.00	273.00	320.00	0.10	0.14	0.13	0.09	0.14
29,002	221.00	785.00	206.00	219.00	222.00	4.00	0.14	0.08	1.42	0.53
29,003	1,438.00	624.00	177.00	179.00	195.00	0.15	0.14	1.20	0.18	0.49
29,004	311.00	1,508.00	256.00	321.00	195.00	0.11	0.17	0.11	0.09	0.69
29,005	172.00	807.00	162.00	208.00	182.00	0.16	1.32	1.77	0.58	0.44
29,006	-	1,547.00	128.00	238.00	299.00	4.00	0.10	2.65	1.66	0.83
29,007	457.00	1,483.00	196.00	240.00	187.00	0.52	1.13	0.16	0.11	0.14
29,008	391.00	1,382.00	209.00	293.00	266.00	4.00	0.11	0.11	0.20	0.38
29,009	1,326.00	2,236.00	238.00	99.00	239.00	4.00	0.10	0.12	0.18	0.47
29,010	398.00	1,543.00	317.00	205.00	231.00	0.19	0.10	1.13	0.17	0.61
29,011	757.00	1,671.00	355.00	261.00	425.00	4.00	0.12	3.55	3.22	1.03
29,012	636.00	1,094.00	176.00	288.00	185.00	4.00	0.13	0.09	0.10	0.98
29,013	314.00	502.00	150.00	199.00	161.00	4.00	0.09	0.92	0.11	1.21
29,014	602.00	1,153.00	274.00	110.00	158.00	0.22	0.11	0.51	0.13	3.06
29,015	171.00	580.00	184.00	165.00	152.00	4.00	0.09	0.08	0.09	0.42
29,016	-	1,313.00	218.00	150.00	179.00	4.00	0.10	0.12	0.09	0.24
29,017	240.00	1,122.00	216.00	209.00	236.00	4.00	0.10	0.09	0.10	0.09
29,018	-	993.00	192.00	199.00	182.00	4.00	0.12	0.14	0.09	0.66
29,019	-	1,970.00	180.00	266.00	195.00	4.00	0.12	0.10	0.10	0.59
29,020	390.00	1,205.00	214.00	369.00	317.00	4.00	0.12	3.12	2.57	0.54
29,021	323.00	360.00	122.00	136.00	150.00	4.00	0.10	0.09	0.11	0.28
29,022	855.00	1,128.00	183.00	239.00	215.00	4.00	0.09	0.00	0.00	0.30
29,023	418.00	605.00	195.00	178.00	147.00	4.00	0.09	0.10	0.12	0.19
29,024	717.00	220.00	194.00	450.00	491.00	0.30	0.93	1.43	0.11	0.94
29,025	1,065.00	1,538.00	275.00	342.00	386.00	4.00	0.10	0.47	0.39	0.64
29,026	654.00	520.00	175.00	203.00	190.00	0.78	0.14	0.71	0.09	0.48
29,027	703.00	571.00	161.00	163.00	147.00	0.18	0.12	1.48	0.11	0.55
29,028	254.00	669.00	198.00	231.00	226.00	4.00	0.14	0.10	0.11	0.54
29,029	327.00	603.00	176.00	178.00	150.00	4.00	0.14	0.97	0.10	-0.04
29,030	966.00	1,090.00	262.00	254.00	356.00	4.00	0.11	3.72	3.03	0.61

29.031	741.00	1,875.00	400.00	501.00	483.00	3.90	0.10	0.00	3.52	1.26
29,032	-	1,627.00	208.00	366.00	369.00	0.19	0.12	1.35	0.14	3.11
29,033	935.00	1,472.00	218.00	410.00	357.00	0.13	0.11	0.81	0.10	0.73
29,034	-	1,223.00	211.00	232.00	235.00	0.12	0.97	1.55	0.11	0.58
29,035	761.00	1,200.00	274.00	310.00	338.00	0.27	0.11	0.89	0.12	2.09
29,036	-	1,138.00	144.00	170.00	367.00	0.14	0.19	1.01	0.11	0.22
29,037	653.00	1,061.00	201.00	279.00	326.00	4.00	0.12	0.84	0.16	0.26
29,038	1,054.00	1,364.00	332.00	546.00	510.00	3.56	0.09	0.17	0.10	2.10
29,039	900.00	1,467.00	220.00	230.00	293.00	0.20	0.14	1.30	0.11	0.26
29,040	910.00	1,815.00	331.00	287.00	491.00	3.95	0.13	0.00	0.00	0.13
29,041	269.00	660.00	125.00	209.00	180.00	0.63	0.16	0.61	0.13	0.14
29,042	-	1,525.00	150.00	201.00	142.00	0.12	0.10	1.03	0.09	0.34
29,043	436.00	419.00	138.00	196.00	158.00	0.12	0.12	1.42	0.10	-0.03
29,046	173.00	2,044.00	139.00	210.00	205.00	4.00	0.15	1.41	0.61	-0.01
29,047	1,130.00	1,089.00	193.00	232.00	339.00	4.00	0.10	3.53	4.00	0.12
29,048	-	950.00	194.00	269.00	288.00	0.25	0.10	2.67	0.09	0.23
29,049	377.00	765.00	150.00	188.00	186.00	0.34	0.20	1.37	0.10	0.40
29,050	393.00	490.00	192.00	314.00	255.00	0.22	0.10	1.00	0.08	0.12
29,051	-	1,186.00	217.00	381.00	288.00	0.64	0.10	0.66	0.13	0.25
29,052	-	796.00	177.00	171.00	264.00	4.00	0.10	0.09	0.09	0.25
29,053	-	827.00	156.00	179.00	490.00	0.38	0.10	0.97	0.09	0.11
29,054	403.00	661.00	137.00	285.00	184.00	0.15	0.10	0.92	0.09	-0.07
29,055	313.00	622.00	122.00	217.00	197.00	0.47	0.11	1.12	0.11	0.01
29,056	1,287.00	1,421.00	255.00	269.00	341.00	3.68	0.15	3.15	3.08	0.14
29,057	-	1,006.00	174.00	269.00	300.00	0.14	0.68	1.39	0.10	-0.03
29,058	-	445.00	145.00	154.00	193.00	0.15	0.12	0.74	0.09	0.24
29,059	-	709.00	168.00	79.00	285.00	0.22	0.11	0.12	0.10	0.02
29,060	862.00	1,888.00	234.00	193.00	382.00	4.00	0.10	0.09	0.10	0.68
29,067	126.00	423.00	131.00	125.00	359.00	4.00	0.10	0.09	0.10	0.18
29,068	696.00	1,381.00	203.00	223.00	216.00	4.00	0.12	0.09	0.09	0.58
29,069	991.00	987.00	211.00	270.00	192.00	4.00	0.11	1.20	0.20	0.40
29,070	650.00	816.00	204.00	247.00	364.00	1.55	0.09	0.09	0.09	0.00
29,071	255.00	800.00	153.00	152.00	300.00	4.00	0.11	1.31	0.89	0.07

29,072	84.00	478.00	158.00	274.00	179.00	3.84	0.11	0.28	0.16	-0.07
29,073	690.00	1,179.00	238.00	358.00	300.00	0.14	0.11	1.35	0.11	2.68
29,074	196.00	1,454.00	186.00	215.00	274.00	4.00	0.09	1.02	0.45	0.30
29,075	545.00	723.00	188.00	245.00	425.00	0.22	0.26	1.33	0.10	1.42
29,076	393.00	989.00	179.00	212.00	264.00	3.84	0.11	0.10	0.11	0.17
29,077	887.00	814.00	182.00	198.00	246.00	0.12	0.11	0.85	0.20	0.11
29,078	-	1,791.00	159.00	230.00	205.00	0.33	0.10	0.34	0.09	-0.01
29,079	-	2,315.00	743.00	332.00	631.00	4.00	0.10	3.10	2.71	0.06
29,081	786.00	1,075.00	162.00	234.00	240.00	4.00	0.11	0.10	0.10	0.32
29,084	814.00	1,366.00	237.00	295.00	314.00	4.00	0.13	0.11	0.10	0.26
29,085	-	742.00	150.00	166.00	233.00	4.00	0.12	0.11	0.09	0.41
29,086	-	948.00	207.00	297.00	283.00	3.32	0.14	0.11	0.09	0.13
29,087	-	601.00	162.00	214.00	261.00	3.87	0.11	3.35	4.00	0.15
29,088	201.00	619.00	128.00	169.00	166.00	0.10	0.11	1.42	0.09	0.03
29,089	-	538.00	130.00	132.00	137.00	0.13	0.11	1.19	0.09	1.82
29,090	267.00	790.00	127.00	224.00	207.00	4.00	0.20	1.36	0.11	0.68
29,091	-	1,780.00	237.00	97.00	225.00	0.09	0.63	1.25	0.10	0.30
29,092	-	1,423.00	139.00	151.00	161.00	0.11	0.14	0.19	0.09	0.24
29,093	368.00	1,260.00	136.00	130.00	194.00	0.16	0.09	0.16	0.10	1.20
29,094	384.00	655.00	130.00	81.00	275.00	4.00	0.15	2.76	2.47	0.26
29,095	621.00	1,110.00	173.00	129.00	291.00	4.00	0.11	0.82	0.13	0.35
29,096	262.00	1,402.00	191.00	143.00	257.00	0.10	0.68	1.22	0.18	0.15
29,097	657.00	1,339.00	162.00	61.00	217.00	3.94	0.09	0.16	0.09	0.25
29,098	-	1,707.00	175.00	80.00	192.00	0.12	0.13	0.80	0.10	0.25
29,102	-	1,876.00	281.00	288.00	263.00	0.87	0.59	0.98	0.09	0.12
29,103	-	192.00	497.00	80.00	351.00	0.10	0.14	1.21	0.13	0.69
29,104	607.00	2,174.00	179.00	211.00	357.00	4.00	0.10	0.09	0.10	0.99
29,105	200.00	1,125.00	145.00	277.00	209.00	4.00	0.10	0.81	0.25	0.05
29,106	-	701.00	127.00	141.00	156.00	4.00	0.11	0.10	0.09	0.09
29,107	396.00	1,431.00	199.00	226.00	292.00	4.00	0.16	0.10	0.12	0.03
29,109	177.00	676.00	141.00	174.00	192.00	4.00	0.14	1.79	0.29	0.00
29,110	-	1,654.00	151.00	150.00	220.00	4.00	0.16	1.11	0.09	1.01
29,111	383.00	1,007.00	115.00	77.00	168.00	1.19	0.49	1.29	0.09	0.03

APPENDIX XX

RAW DATA OD OF VIRAL MARKERS AND AUTOANTIBODIES

samplano	OD_ANA	OD AMA	OD_pANCA	OD_LKM	OD_SLA	OD_HbsAg	OD_antiHBctotal	OD_antiHBc	OD_HBeAg	OD_antiHCVIgG
29,001		1,429 00	241 00	27300	320.00	0 10	0 14	0 13	0 09	0 14
29,002	221 00	785.00	206 00	2 19 00	222.00	4 00	0 14	0 08	1 42	0 53
29,003	1,438 00	624.00	177 00	179.00	195.00	0.15	0 14	1.20	0. 18	0.49
29,004	311.00	1,508 00	256 00	321 00	195 00	0 11	0.17	0 11	0 09	0 69
29,005	172 00	807.00	162 00	208.00	182 00	0 16	1 32	1 77	0 58	0.44
29.006	-	1,547 00	128 00	238.00	299 00	4 00	0. 10	2.65	1 66	0 83
29,007	457 00	1,483 00	196.00	24000	187.00	0 52	1. 13	0. 16	0 11	0 14
29,008	391 00	1,382 00	209 00	293 00	266 00	4 00	0 11	0 11	0 20	0.38
29,009	1,326.00	2,236 00	23800	99.00	239.00	4 00	0 10	0 12	0 19	0.47
29,0 10	398 00	1,543 00	317.00	20500	231 00	0.19	0 10	1.13	0.17	0 61
29,0 11	757 00	1,671.00	35500	261 00	42500	4 00	0 12	3.55	3 22	1 03
29,012	636.00	1,094.00	176.00	288.00	185.00	4 00	0 13	0 09	0.10	0 99
29,013	314 00	50200	150.00	199 00	161 00	4.00	0 09	0.92	0 11	1 21
29,0 14	602 00	1,153 00	274 00	110 00	15800	0 22	0 11	0 51	0 13	3 06
29,0 15	17 1.00	580.00	184.00	165 00	152.00	4 00	0 09	0.08	0.09	0 42
29,0 16	-	1,3 13 00	2 18 00	150.00	179.00	4.00	0 10	0 12	0.09	0.24
29,0 17	240.00	1, 122.00	216.00	209.00	236.00	4.00	0 10	0 09	0 10	0.09
29,0 18	-	99300	192.00	199.00	182.00	4 00	0. 12	0 14	0.09	0 66
29,0 19	-	1,970 00	180 00	266 00	195 00	4.00	0 12	0 10	0 10	0 59
29,020	390 00	1,205.00	2 14 00	368.00	317.00	4.00	0. 12	3 12	2.57	0 54
29,021	323.00	360 00	122 00	136.00	150 00	4 00	0 10	0.09	0 11	0 26
29,022	855 00	1,128 00	183 00	23900	215.00	4 00	0.09	0.00	0.00	0 30
29,023	418.00	605.00	195.00	178.00	147.00	4.00	0.09	0 10	0 12	0 19
29,024	717.00	220.00	194.00	45000	491.00	0.30	0.93	1.43	0 11	0 94
29,025	1,065 00	1,538.00	275.00	342.00	386 00	4.00	0 10	0 47	0.39	0 64
29,026	654 00	520 00	175.00	203.00	19000	0 78	0.14	0.7 1	0.09	0 46
29,027	703.00	571.00	161.00	163.00	147.00	0 18	0 12	1.46	0 11	0 55
29,028	254 00	669.00	19800	231.00	226 00	4 00	0 14	0 10	0 11	0.54
29,029	327.00	60300	176 00	178.00	15000	4 00	0 14	0 97	0 10	-0 04
29,030	966 00	1,090.00	262.00	254 00	35600	4 00	0 11	3 72	3 03	0 61

29,031	741.00	1,875.00	400.00	501.00	483.00	3.90	0.10	0.00	3.52	1.26
29,032	-	1,627.00	208.00	366.00	369.00	0.19	0.12	1.35	0.14	3.11
29,033	935.00	1,472.00	218.00	410.00	357.00	0.13	0.11	0.81	0.10	0.73
29,034	-	1,223.00	211.00	232.00	235.00	0.12	0.97	1.55	0.11	0.58
29,035	761.00	1,200.00	274.00	310.00	338.00	0.27	0.11	0.89	0.12	2.09
29,036	-	1,138.00	144.00	170.00	367.00	0.14	0.19	1.01	0.11	0.22
29,037	653.00	1,061.00	201.00	279.00	326.00	4.00	0.12	0.84	0.16	0.26
29,038	1,054.00	1,364.00	332.00	546.00	510.00	3.56	0.09	0.17	0.10	2.10
29,039	900.00	1,467.00	220.00	230.00	293.00	0.20	0.14	1.30	0.11	0.26
29,040	910.00	1,815.00	331.00	287.00	491.00	3.95	0.13	0.00	0.00	0.13
29,041	269.00	660.00	125.00	209.00	180.00	0.63	0.16	0.61	0.13	0.14
29,042	-	1,525.00	150.00	201.00	142.00	0.12	0.10	1.03	0.09	0.34
29,043	436.00	419.00	138.00	196.00	158.00	0.12	0.12	1.42	0.10	-0.03
29,046	173.00	2,044.00	139.00	210.00	205.00	4.00	0.15	1.41	0.61	-0.01
29,047	1,130.00	1,089.00	193.00	232.00	339.00	4.00	0.10	3.53	4.00	0.12
29,048	-	950.00	194.00	269.00	288.00	0.25	0.10	2.67	0.09	0.23
29,049	377.00	765.00	150.00	188.00	186.00	0.34	0.20	1.37	0.10	0.40
29,050	393.00	490.00	192.00	314.00	255.00	0.22	0.10	1.00	0.08	0.12
29,051	-	1,186.00	217.00	381.00	288.00	0.64	0.10	0.66	0.13	0.25
29,052	-	798.00	177.00	171.00	264.00	4.00	0.10	0.09	0.09	0.25
29,053	-	827.00	156.00	179.00	490.00	0.38	0.10	0.97	0.09	0.11
29,054	403.00	661.00	137.00	285.00	184.00	0.15	0.10	0.92	0.09	-0.07
29,055	313.00	622.00	122.00	217.00	197.00	0.47	0.11	1.12	0.11	0.01
29,056	1,287.00	1,421.00	255.00	269.00	341.00	3.68	0.15	3.15	3.08	0.14
29,057	-	1,006.00	174.00	269.00	300.00	0.14	0.68	1.39	0.10	-0.03
29,058	-	445.00	145.00	154.00	193.00	0.15	0.12	0.74	0.09	0.24
29,059	-	709.00	168.00	79.00	285.00	0.22	0.11	0.12	0.10	0.02
29,060	862.00	1,888.00	234.00	193.00	382.00	4.00	0.10	0.09	0.10	0.68
29,067	126.00	423.00	131.00	125.00	359.00	4.00	0.10	0.09	0.10	0.18
29,068	696.00	1,381.00	203.00	223.00	216.00	4.00	0.12	0.09	0.09	0.58
29,069	991.00	987.00	211.00	270.00	192.00	4.00	0.11	1.20	0.20	0.40
29,070	650.00	816.00	204.00	247.00	364.00	1.55	0.09	0.09	0.09	0.00
29,071	255.00	800.00	153.00	152.00	300.00	4.00	0.11	1.31	0.89	0.07

29,072	84.00	478.00	158.00	274.00	179.00	3.84	0.11	0.28	0.16	-0.07
29,073	690.00	1,179.00	238.00	358.00	300.00	0.14	0.11	1.35	0.11	2.68
29,074	196.00	1,454.00	186.00	215.00	274.00	4.00	0.09	1.02	0.45	0.30
29,075	645.00	723.00	186.00	245.00	425.00	0.22	0.26	1.33	0.10	1.42
29,076	393.00	989.00	179.00	212.00	264.00	3.84	0.11	0.10	0.11	0.17
29,077	887.00	814.00	182.00	198.00	246.00	0.12	0.11	0.85	0.20	0.11
29,078	-	1,791.00	159.00	230.00	205.00	0.33	0.10	0.34	0.09	-0.01
29,079	-	2,315.00	743.00	332.00	631.00	4.00	0.10	3.10	2.71	0.06
29,081	786.00	1,075.00	162.00	234.00	240.00	4.00	0.11	0.10	0.10	0.32
29,084	814.00	1,386.00	237.00	295.00	314.00	4.00	0.13	0.11	0.10	0.26
29,085	-	742.00	150.00	166.00	233.00	4.00	0.12	0.11	0.09	0.41
29,086	-	948.00	207.00	297.00	283.00	3.32	0.14	0.11	0.09	0.13
29,087	-	601.00	162.00	214.00	261.00	3.67	0.11	3.35	4.00	0.15
29,088	201.00	619.00	128.00	169.00	166.00	0.10	0.11	1.42	0.09	0.03
29,089	-	538.00	130.00	132.00	137.00	0.13	0.11	1.19	0.09	1.82
29,090	267.00	790.00	127.00	224.00	207.00	4.00	0.20	1.36	0.11	0.68
29,091	-	1,780.00	237.00	97.00	225.00	0.09	0.63	1.25	0.10	0.30
29,092	-	1,423.00	139.00	151.00	161.00	0.11	0.14	0.19	0.09	0.24
29,093	368.00	1,260.00	136.00	130.00	194.00	0.16	0.09	0.16	0.10	1.20
29,094	384.00	655.00	130.00	81.00	275.00	4.00	0.15	2.76	2.47	0.26
29,095	621.00	1,110.00	173.00	129.00	291.00	4.00	0.11	0.92	0.13	0.35
29,096	262.00	1,402.00	191.00	143.00	257.00	0.10	0.68	1.22	0.18	0.15
29,097	657.00	1,339.00	162.00	61.00	217.00	3.94	0.09	0.16	0.09	0.25
29,098	-	1,707.00	175.00	8000	192.00	0.12	0.13	0.80	0.10	0.25
29,102	-	1,876.00	281.00	288.00	263.00	0.87	0.59	0.98	0.09	0.12
29,103	-	192.00	497.00	80.00	351.00	0.10	0.14	1.21	0.13	0.69
29,104	607.00	2,174.00	179.00	211.00	357.00	4.00	0.10	0.09	0.10	0.99
29,105	200.00	1,125.00	145.00	277.00	209.00	4.00	0.10	0.81	0.25	0.05
29,106	-	701.00	127.00	141.00	156.00	4.00	0.11	0.10	0.09	0.09
29,107	396.00	1,431.00	199.00	226.00	292.00	4.00	0.16	0.10	0.12	0.03
29,109	177.00	676.00	141.00	174.00	192.00	4.00	0.14	1.79	0.29	0.00
29,110	-	1,654.00	151.00	150.00	220.00	4.00	0.18	1.11	0.09	1.01
29,111	38300	1,007.00	115.00	7700	168.00	1.19	0.49	1.29	0.09	0.03

29.072	84.00	478.00	158.00	274.00	179.00	3.84	0.11	028	0.16	-0.07
29,073	690.00	1,179.00	238.00	358.00	300.00	014	0.11	1.35	0.11	2.68
29.074	196.00	1.454.00	186.00	215.00	274.00	4.00	0.09	1.02	0.45	0.30
29,075	645.00	723.00	186.00	245.00	425.00	0.22	0.26	1.33	0.10	1.42
29,076	393.00	989.00	179.00	212.00	264.00	3.84	0.11	0.10	0.11	0.17
29.077	887.00	814.00	182.00	198.00	24600	0.12	0.11	085	020	0.11
29,078	-	1,791.00	159.00	230.00	205.00	0.33	0.10	0.34	009	-0.01
29.079	-	2,3 15.00	743.00	332.00	631.00	4.00	0.10	3.10	2.71	0.06
29.081	786.00	1,075.00	162.00	234.00	240.00	4.00	011	0.10	0.10	0.32
29,084	814.00	1,386.00	237.00	295.00	314.00	4.00	0.13	0.11	0.10	0.26
29.085	-	742.00	150.00	166.00	233.00	4.00	0.12	0.11	009	0.41
29,086	-	948.00	207.00	297.00	283.00	3.32	0.14	0.11	0.09	0.13
29,087	-	601.00	162.00	214.00	261.00	3.67	0.11	335	4.00	0.15
29.088	201.00	619.00	128.00	169.00	166.00	0.10	0.11	1.42	0.09	0.03
29,089	-	538.00	130.00	132.00	137.00	0.13	0.11	1.19	0.09	1.82
29.090	287.00	790.00	127.00	224.00	207.00	4.00	020	1.36	0.11	068
29,091	-	1.780.00	237.00	97.00	225.00	0.09	063	1.25	010	0.30
29.092	-	1.423.00	139.00	151.00	161.00	0.11	0.14	0.19	0.09	0.24
29.093	368.00	1,260.00	136.00	130.00	194.00	0.16	0.09	0.16	0.10	1.20
29,094	384.00	655.00	13000	81.00	275.00	4.00	0.15	2.76	2.47	0.26
29.095	621.00	1,110.00	173.00	129.00	291.00	4.00	0.11	092	0.13	0.35
29.096	262.00	1,402.00	19 1.00	143.00	257.00	0.10	068	1.22	0.18	0.15
29,097	65700	1,339.00	162.00	81.00	217.00	3.94	0.09	0.16	0.09	025
29,098	-	1,707.00	175.00	80.00	192.00	0.12	0.13	080	0.10	025
29,102	-	1,876.00	281.00	288.00	283.00	0.87	0.59	0.98	0.09	0.12
29,103	-	192.00	497.00	80.00	351.00	0.10	0.14	1.21	0.13	0.69
29,104	607.00	2,174.00	179.00	211.00	357.00	4.00	0.10	0.09	0.10	0.99
29,105	200.00	1,125.00	145.00	277.00	209.00	4.00	0.10	0.81	0.25	0.05
29,108	-	701.00	127.00	141.00	156.00	4.00	0.11	0.10	0.09	009
29,107	396.00	1,431.00	199.00	228.00	292.00	4.00	0.16	0.10	0.12	0.03
29,109	177.00	676.00	141.00	174.00	192.00	4.00	0.14	1.79	029	0.00
29,1 10	-	1.654.00	151.00	150.00	220.00	4.00	0.16	1.11	0.09	1.01
29.111	383.00	1,007.00	115.00	77.00	168.00	1.19	0.49	1.29	0.09	0.03

29,112	379.00	1,751.00	242.00	98.00	356.00	0.19	0.17	1.34	0.09	0.43
29,113	-	1,777.00	206.00	115.00	233.00	3.53	0.16	0.09	0.08	0.05
29,114	-	2,569.00	179.00	118.00	155.00	0.16	0.15	1.16	0.10	0.04
29,117	-	1,512.00	180.00	191.00	312.00	4.00	0.17	0.51	0.10	0.15
29,120	-	1,515.00	175.00	224.00	258.00	0.24	0.15	1.20	0.10	0.01
29,121	749.00	1,064.00	229.00	156.00	249.00	1.74	0.13	1.01	0.09	0.08
29,122	343.00	1,325.00	170.00	139.00	202.00	1.44	1.54	1.48	0.15	0.05
29,123	391.00	952.00	135.00	127.00	183.00	4.00	0.21	0.75	0.12	0.11
29,124	818.00	1,586.00	254.00	68.00	300.00	4.00	0.16	0.12	0.10	0.01
29,125	1,125.00	217.00	162.00	233.00	295.00	2.76	0.17	0.09	0.09	1.16
29,126	1,682.00	180.00	157.00	255.00	217.00	0.21	0.18	1.41	0.10	0.53
29,127	371.00	137.00	130.00	106.00	136.00	4.00	0.15	1.08	0.10	0.08
29,128	887.00	134.00	125.00	198.00	148.00	1.22	0.16	3.76	3.22	0.43
29,129	401.00	142.00	139.00	141.00	290.00	4.00	0.34	3.78	3.56	0.13
29,130	863.00	144.00	178.00	216.00	151.00	4.00	0.17	0.95	0.10	0.29
29,131	200.00	154.00	144.00	122.00	242.00	4.00	0.15	4.00	3.57	0.01
29,132	-	151.00	173.00	242.00	330.00	4.00	0.23	3.23	2.72	0.14
29,133	792.00	1,418.00	168.00	83.00	288.00	2.85	0.17	0.08	0.09	0.07
29,134	851.00	1,064.00	172.00	258.00	269.00	4.00	0.19	2.64	2.06	0.62
29,135	-	698.00	142.00	356.00	433.00	4.00	0.16	0.13	0.11	0.24
29,136	-	469.00	112.00	168.00	152.00	4.00	0.16	0.10	0.10	0.07
29,137	1,901.00	1,038.00	139.00	781.00	147.00	4.00	0.19	0.10	0.28	0.11
29,138	-	1,768.00	142.00	142.00	185.00	0.30	0.16	1.20	0.10	0.20
29,139	-	1,262.00	149.00	143.00	186.00	1.15	0.17	1.03	0.11	0.12
29,140	-	1,884.00	182.00	387.00	309.00	3.59	0.22	1.44	0.12	0.08
29,141	560.00	1,194.00	243.00	170.00	248.00	2.10	0.22	0.16	0.12	0.00
29,142	-	1,826.00	127.00	172.00	258.00	3.45	0.16	1.25	0.11	0.07
29,143	244.00	424.00	110.00	103.00	124.00	0.78	0.19	1.15	0.10	0.07
29,144	647.00	496.00	108.00	126.00	182.00	4.00	0.18	1.07	0.27	0.05
29,145	298.00	569.00	134.00	145.00	152.00	1.50	0.16	0.79	0.11	0.17
29,201	332.00	598.00	547.00	185.00	149.00	0.59	0.58	1.70	0.10	0.40
29,202	-	772.00	179.00	177.00	303.00	0.24	0.25	0.47	0.10	0.05
29,203	1,018.00	1,781.00	200.00	167.00	221.00	0.73	0.26	0.36	0.10	0.08

29,204	278.00	1,226.00	243.00	208.00	265.00	1.04	0.21	1.23	0.10	0.56
29,205	-	638.00	149.00	346.00	153.00	3.49	0.22	0.26	0.10	0.40
29,206	314.00	564.00	181.00	98.00	174.00	0.45	0.25	1.63	0.11	0.07
29,207	576.00	408.00	133.00	109.00	127.00	0.30	0.16	1.50	0.12	0.00
29,208	666.00	858.00	157.00	197.00	156.00	4.00	0.16	0.09	0.11	0.02
29,209	1,899.00	1,466.00	201.00	162.00	191.00	0.40	0.18	0.16	0.11	0.13
29,210	-	1,070.00	460.00	210.00	297.00	0.58	0.23	1.11	0.11	0.03
29,211	-	50.00	149.00	152.00	178.00	0.23	0.17	1.16	0.09	0.05
29,212	-	1,194.00	140.00	90.00	227.00	1.18	0.16	0.20	0.09	0.14
29,213	-	964.00	124.00	80.00	217.00	0.30	0.16	1.14	0.09	0.12
29,214	660.00	1,023.00	224.00	108.00	227.00	0.22	0.38	1.42	0.09	0.06
29,215	276.00	921.00	139.00	107.00	205.00	0.29	0.15	1.27	0.10	0.35
29,216	660.00	934.00	192.00	175.00	367.00	0.72	0.17	1.32	0.08	0.11
29,217	609.00	1,582.00	204.00	133.00	247.00	0.22	0.45	1.53	0.10	0.01
29,218	1,591.00	1,660.00	198.00	247.00	286.00	0.15	0.17	0.46	0.11	0.77
29,219	1,154.00	1,345.00	168.00	193.00	283.00	0.22	0.17	0.11	0.09	0.29
29,220	387.00	645.00	136.00	142.00	201.00	0.13	0.18	0.14	0.09	0.12
29,221	-	589.00	128.00	112.00	147.00	0.22	0.20	0.51	0.11	0.02
29,222	-	833.00	141.00	93.00	166.00	0.25	0.20	0.64	0.10	0.28
29,223	125.00	643.00	129.00	87.00	151.00	0.31	0.21	0.95	0.13	0.01
29,224	173.00	611.00	117.00	96.00	144.00	0.15	0.22	0.56	0.10	0.01
29,225	398.00	549.00	131.00	119.00	144.00	0.38	0.16	1.44	0.10	-0.01
29,226	-	648.00	138.00	105.00	173.00	0.33	0.89	1.05	0.10	-0.01
29,227	-	1,530.00	138.00	99.00	248.00	0.14	0.74	1.54	0.12	0.04
29,228	888.00	839.00	134.00	161.00	127.00	0.18	0.15	1.37	0.12	0.10
29,229	169.00	598.00	114.00	104.00	111.00	0.36	0.60	1.51	0.09	0.04
29,230	167.00	424.00	121.00	110.00	94.00	0.13	0.15	0.13	0.10	0.02
29,231	-	704.00	127.00	129.00	130.00	0.14	0.28	1.43	0.11	0.02
29,232	183.00	399.00	111.00	103.00	85.00	0.18	1.43	1.56	0.11	0.05
29,233	644.00	531.00	117.00	118.00	78.00	0.25	0.15	1.16	0.12	0.09
29,234	-	632.00	149.00	122.00	106.00	0.74	0.15	1.29	0.12	0.01
29,235	779.00	1,483.00	191.00	349.00	199.00	1.85	0.21	0.78	0.11	0.07
29,236	240.00	629.00	228.00	105.00	120.00	0.26	0.87	1.15	0.10	0.06

29.237	118.00	579.00	254.00	108.00	117.00	0.14	0.14	1.21	0.10	0.11
29.238	142.00	551.00	283.00	91.00	108.00	0.11	0.13	0.34	0.10	0.13
29.239	-	1,525.00	215.00	145.00	92.00	0.12	1.25	1.49	0.11	0.08
29.240	186.00	742.00	289.00	110.00	136.00	0.15	0.20	0.39	0.10	0.05
29.241	101.00	489.00	261.00	124.00	118.00	0.31	0.17	1.52	0.11	-0.02
29.242	778.00	1,160.00	274.00	108.00	171.00	0.20	0.25	1.48	0.11	0.04
29.243	349.00	739.00	289.00	113.00	142.00	0.14	0.14	0.29	0.10	0.58
29.244	266.00	579.00	495.00	293.00	102.00	0.16	0.13	1.33	0.10	0.25
29.245	-	847.00	219.00	227.00	102.00	0.28	0.13	1.25	0.10	0.21
29.246	292.00	1.103.00	222.00	200.00	141.00	0.11	0.71	1.69	0.10	0.06
29.247	-	1.712.00	352.00	294.00	171.00	0.12	1.39	1.72	0.11	0.31
29.248	-	807.00	245.00	154.00	119.00	0.17	1.32	1.74	0.10	0.06
29.249	712.00	1.806.00	333.00	508.00	148.00	0.28	0.16	1.41	0.11	-0.01
29.250	198.00	549.00	264.00	149.00	104.00	0.18	1.47	1.68	1.25	0.09
29.251	-	1.692.00	410.00	304.00	274.00	0.20	0.23	0.88	0.14	0.00
29.252	1,097.00	1,422.00	314.00	285.00	180.00	0.46	1.34	1.34	0.14	0.03
29,253	186.00	453.00	210.00	167.00	89.00	022	0.14	0.46	0.14	-0.01
29,254	336.00	788.00	261.00	200.00	147.00	0.13	0.15	1.02	0.16	0.14
29.255	376.00	612.00	275.00	185.00	152.00	0.17	0.33	0.10	021	0.00
29.256	593.00	1,119.00	254.00	165.00	163.00	0.13	1.85	1.24	0.15	0.03
29.257	1,039.00	818.00	243.00	164.00	98.00	0.19	2.01	1.38	0.14	0.08
29,258	-	528.00	257.00	165.00	109.00	021	0.14	1.30	0.15	0.01
29,259	364.00	768.00	247.00	198.00	211.00	0.19	009	1.19	0.16	-0.01
29,260	209.00	674.00	249.00	234.00	120.00	0.19	1.51	1.45	0.14	-0.01
29,261	206.00	1,282.00	334.00	274.00	121.00	0.12	0.11	1.03	0.15	0.31
29,262	677.00	621.00	234.00	141.00	156.00	021	2.09	1.39	0.15	-002
29,263	1,294.00	1,000.00	280.00	181.00	193.00	0.16	0.09	1.17	1.17	0.00
29,264	-	751.00	258.00	139.00	135.00	0.12	0.11	1.00	1.22	0.04
29,265	727.00	755.00	250.00	179.00	137.00	0.33	0.18	1.29	1.25	0.03
29,266	-	808.00	243.00	161.00	133.00	0.17	0.11	1.18	1.19	-0.01
29,267	-	1,747.00	304.00	634.00	327.00	0.11	0.15	1.05	1.28	0.22
29,268		756.00	210.00	174.00	123.00	0.14	0.10	088	1.10	0.00
29,269	1,745.00	1,933.00	277.00	443.00	238.00	0.11	069	1.22	1.33	0.11

29.270	395.00	521.00	209.00	118.00	156.00	0.14	0.11	1.28	1.26	0.00
29.271	1.517.00	1,889.00	248.00	260.00	309.00	0.13	0.41	1.25	0.11	0.07
29,272	280.00	1.242.00	353.00	187.00	300.00	0.20	1.96	0.89	0.11	0.01
29,273	195.00	493.00	232.00	144.00	133.00	0.22	1.93	1.38	0.10	-0.01
29.274	-	1,317.00	258.00	550.00	167.00	0.13	1.78	1.40	0.10	0.01
29,275	2,135.00	1,879.00	246.00	205.00	171.00	0.13	1.24	1.32	0.10	1.17
29,276	1.065.00	1,527.00	206.00	178.00	331.00	0.29	1.90	1.21	0.09	0.43
29.277	1,071.00	1.212.00	206.00	187.00	120.00	0.15	0.17	0.50	0.10	-0.01
29.278	713.00	785.00	245.00	162.00	133.00	0.19	0.21	1.26	0.10	0.05
29.279	1.506.00	1,253.00	252.00	208.00	126.00	1.33	0.15	0.82	0.11	0.04
29.280	757.00	1.344.00	203.00	202.00	121.00	0.16	0.19	0.94	0.10	0.05
29,281	258.00	1,119.00	254.00	157.00	175.00	0.19	0.22	1.14	0.10	0.02
29,282	-	887.00	253.00	251.00	165.00	0.24	0.14	1.08	0.11	0.09

OD Range of markers

ANA	
Test	Control
560- 1901	576-2135
AMA	
948- 2569	858-1933
HBsAg	
0.18-4.00	0.16-4.00
Anti-HBc	
0.0896-0 .6286	0 .0916-0 .6892
Anti-HBc	
0.0004-0 .8917	0 .0884 -0 .7783
HBcAg	
0.1993-4	1.2491 - 1.2491

Anti-HCV		
0.403- 3.1126	0.3489- 1.1748	