# THE PREVALENCE OF HEPATITIS B, C AND, HIV MARKERS AND α-FETOPROTEIN LEVELS IN PATIENTS WITH PRIMARY HEPATOCELLULAR CARCINOMA AT KENYATTA NATIONAL HOSPITAL

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BY

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# DECLARATION

This dissertation is original work and has not been presented for a degree in any other university.

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# DEDICATION

This work is dedicated to my beloved children Michael and William.

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## LIST OF ABBREVIATIONS

**AIDS**: Acquired Immune Deficiency Syndrome

**ALP**: Alkaline phosphatase

**ALT**: Alanine aminotransferase

**Anti-HCV**: Anti-Hepatitis C virus antibodies

**AST**: Aspartate aminotransferase

**CAH**: Chronic active hepatitis

**DNA**: Deoxyribose Nucleic Acid

**ELISA**: Enzyme linked immunosorbent assay

**HAART**: highly active antiretroviral therapy

**HBsAg**: Hepatitis B surface antigen

**HBcAb**: Hepatitis core antibody

**HBeAg**: Hepatitis B e antigen

**HBxAg**: Hepatitis B x antigen

**HBV**: Hepatitis B virus

**HCC**: Hepatocellular carcinoma

**PHC**: Primary hepatocellular carcinoma

**HCV**: Hepatitis C virus

**HIV**: Human immunodeficiency virus

**IgG anti-HBc**: Immunoglobulin G antibodies against hepatitis B core antigen

**AFP ($\alpha$-FP)**: Alpha-fetoprotein

**KNH**: Kenyatta National Hospital

**LFTs**: Liver function tests

**PFC**: Paediatric filter clinic

**PCR**: polymerase chain reaction

**p53 gene**: tumour suppressor gene

**RNA**: Ribose Nucleic Acid

**RAR-$\alpha$**: retinoic acid receptor $\alpha$-gene

**SPSS**: Statistical Package for Social Sciences

**U.O.N.**: University of Nairobi

**USA**: United States of America

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## ACKNOWLEDGEMENTS

# ABSTRACT

**Objective:** The aim of the study was to determine the prevalence of alpha-fetoprotein levels, HBsAg, IgG anti-HBc, anti-HCV and HIV antibodies among patients with primary hepatocellular carcinoma at KNH.

**Study design & setting:** The study design was a cross-sectional descriptive survey, done at the Kenyatta National Hospital, Nairobi, Kenya.

**Methods:** Patients with a histological diagnosis of primary hepatocellular carcinoma were recruited. History was obtained, physical examination done, and blood taken for determination of alpha-fetoprotein level, hepatitis B, C and HIV seropositivity.

**Results:** 62 patients were studied, 39 males and 23 females, with an age range of 21 to 79 years and a mean of 49.7 years (median 50.0,SD 13.4). The prevalence of various viral markers was as follows: HBsAg 56.5%, IgG Anti-HBc 57.9%, HIV 17.7% and Anti-HCV 9.7%. 43.5% had associated liver cirrhosis. HCV positivity was significantly associated with cirrhosis (p= .039). HBsAg and anti-HBc positivity were significantly associated with higher AFP levels (p= .0008). Higher AFP levels were also significantly associated with age below 50 years (p= .021).

**Conclusions:** The prevalence of hepatitis B virus markers among PHC patients was (71%). All patients that had positive alpha-fetoprotein levels were positive for hepatitis B markers, and in this same group of patients, age played a role in that those below 50 years had higher levels. Hepatitis C accounted for 9.7% of patients with primary hepatocellular carcinoma. 17.7% of patients with primary hepatocellular carcinoma were positive for HIV (ELISA I & II). This figure was slightly lower than that expected for KNH in-patients (20%) but significantly higher than that of the general population (12%). 43.5% of the patients with primary hepatocellular carcinoma had associated liver cirrhosis.

# LITERATURE REVIEW

## I. INTRODUCTION

Primary hepatocellular carcinoma (PHC) is the most common malignant neoplasm arising within the liver in Africa, China, and in much of Asia [1-5]. Many possible aetiological factors have been implicated, but most of these (for instance androgenic steroids or hepatic parasites) do not occur widely enough or don't show the same geographical distribution as PHC and can explain only a small proportion of cases. Two implicated factors, aflatoxins and hepatitis B virus (HBV), occur widely; the two have major aetiological roles in PHC and macronodular cirrhosis, in which PHC arises [1,6,7]. Epidemiological studies have revealed a very strong correlation between the geographical frequency of the HBsAg carrier state and prevalence of PHC [1].

In Africa primary hepatocellular carcinoma often afflicts individuals in their fourth and fifth decades of life, when they are at their most productive period economically. Patients often present at an advanced stage when treatment options are limited, prognosis is poor, and median survival time is measured in months or even weeks.

Some preventable factors such as hepatitis B and C viruses present unique opportunities for early detection and even prevention of PHC [8].

In those geographic regions where hepatocellular carcinoma is common, the most important screening and diagnostic tests are measurement of the concentration of the serum alpha-fetoprotein and liver ultrasound scans. Approximately 90% of patients with PHC in these regions have raised levels, and 75% have levels in the diagnostic range ($\geq 500$ ng/ml) [9]. The performance characteristics of ultrasonography as a screening test for HCC have been defined in a study on healthy HBsAg carriers [10]. The sensitivity and specificity were 78% and

2

93% respectively. The positive predictive value was 14%. These performance characteristics make serum AFP and ultrasonography efficient, economical tests for HCC surveillance.

A study in Africa showed that more than 70% of PHC patients have HBsAg positive mothers in contrast to 14% positivity in the mothers of controls, which partly explains the familial clustering of PHC patients and HBsAg carriers [9].

PHC occurs consistently more frequently in men than women, with reported male-to-female ratios ranging between 8:1 and 3:1 [8].

The burden of PHC is irregularly distributed in the world, for the most part following the prevalence of the hepatitis B virus [11]. The incidence of PHC varies widely with the highest incidences in, sub-Saharan Africa, South-East Asia and China [9] and it is much higher among HbsAg carriers, with a relative risk of more than 100 compared to non-carriers [9]. Infact there is a familial clustering of PHC patients and HBsAg carriers [9].

Chronic hepatitis C virus infection, in patients with chronic active hepatitis (CAH) with cirrhosis, has been directly linked to the development of PHC [12]. Treating chronic type C hepatitis with interferon-alpha probably decreases the risk of PHC, if the patient responds to this therapy [13]. In Japan death rates from HCV-related PHC more than tripled between 1975 and 1992 while those due to HBV-related PHC remained constant pointing to the importance of HCV in the pathogenesis of PHC [14].

In USA about 15% of PHC cases occur in patients presumed to have alcohol-related cirrhosis only [15]. A high percentage of individuals with cirrhosis and a history of alcohol abuse

are also positive for HCV [16]. The combination of heavy alcohol use and chronic infection with HCV increases the risk of HCC beyond that due to either risk factor alone [17].

More than 80% of patients with PHC have cirrhosis. Cirrhosis per se seems to predispose to PHC, the risk varying according to the aetiology of the cirrhosis. PHC is unusual in patients with primary biliary cirrhosis or Wilson's disease but common when the cirrhosis is secondary to chronic viral hepatitis [11]. The risk of death from PHC in patients with haemochromatosis has been as high as 45% in some series [18].

## II. PREVIOUS LOCAL STUDIES

There have been very few studies carried out looking at the prevalences of HBsAg, HBcAb, HCV and HIV markers and, AFP levels among PHC patients in Kenya. Most of these studies were carried out in the eighties and early nineties. This section gives a summary of the studies involving both PHC patients and those involving patients with other medical conditions, especially chronic liver disease.

Mwangi in 1989 studied the role of HBV in the causation of primary hepatocellular carcinoma (PHC) and found that 33% of the specimens had HbsAg in contrast to only 2.5% positivity in normal livers. The HBcAg was detected in 11.5% of PHC. In this study she took liver biopsy specimens of patients with PHC processed at KNH department of human pathology in the period 1980-1984 and stained them by the orcein histochemical method, and using peroxidase-anti-peroxidase immunohistochemical method checked for the presence of HBsAg and HBcAg. The study included 108 cases of PHC, 35 cases of liver cirrhosis and 40 normal liver biopsies [19].

Bowry and Shah in 1980 studied HbsAg titres and alpha-fetoprotein levels in PHC in Kenya and found that HBsAg was positive in 51.3% of PHC cases. AFP was positive in 57.2% of the histologically or cytological confirmed cases of PHC. In this study 76 cases of PHC attending KNH from January 1976 to April 1979 and 33 matched controls were studied. HBsAg was detected and titrated by reverse passive haemagglutination test (Hepatest), while AFP was detected by countercurrent immunoelectrophoresis (CIEP) and positive cases quantified using radial immunodiffusion (M Partigen plates) [20].

In a controlled study of hepatitis B core antibody (HBcAb) in PHC and liver cirrhosis, Bowry and colleagues found seropositivity rates of 80% among PHC patients and 44% among patients with liver cirrhosis. In this study a total of 75 patients with liver disease were evaluated. Fifteen had confirmed liver cirrhosis (LC), 13 had PHC+LC and 47 had PHC [21].

In 1985 Greenfield and colleagues studied the molecular relationship between HBV infection and hepatoma in Kenya. They found that 8 out of 20 biopsies from hepatoma patients revealed integrated HBV-DNA sequences in the hepatic genome compared to only one case out of 29 controls, which supports the implication of HBV in the causation of PHC [22].

A study of hepatitis B markers in patients with acquired immunodeficiency syndrome (AIDS), in 1990, by Ogutu and colleagues found that 12.2% were HBsAg positive, 24.4% for HBsAb and 75.6% for HBcAb. Forty consecutive patients were included in this study [23].

Mwangi in a 1993 study of HBV, HCC and liver cirrhosis in Kenya found a HBsAg positivity of 33% in HCC, 25% in cirrhosis and 2.5% of normals. HbcAg positivity was 0% in normals, 11.5% in HCC and 14% in cirrhosis [24].

In another study by Lule and colleagues anti-HCV antibodies were found in 2.6% of patients with chronic hepatitis and in none with liver cirrhosis or hepatocellular carcinoma. This low prevalence in these patient groups was not in keeping with findings in studies done elsewhere and hence further studies are necessary to evaluate the infectivity of anti-HCV positive patients and the potential for cross infection [25].

A controlled study of three cases of chronic aggressive hepatitis, thirty-one of cryptogenic cirrhosis and seventy-six of PHC, by Bowry and Shah, found a 50% seroprevalence of HBsAg[26].

In their study of the aetiology of liver cirrhosis in Kenya, Okoth and colleagues recruited 40 patients with histologically confirmed liver cirrhosis, attending KNH. 61% of these were found to be HBsAg positive, compared with 10% in hospital controls and 17% in volunteer blood donors. The mean age was 46.1 years and the male to female ratio was 2:1 [27].

Kibuka in 1990 conducted a descriptive study of chronic hepatitis as seen at KNH and found HBsAg in 25% of the patients. A total of 16 patients with chronic hepatitis (9 males and 7 females) were studied between February and December 1989. The patients had both clinical and a histological diagnosis of chronic hepatitis. The age ranged from 11-59 years with a mean of 33 years [28].

Mwangi and colleagues in a 1998 study of viral markers in a blood donor population in Nairobi found HBV and HCV rates of 3.9% and 1.8% respectively [29].

In Ethiopia, a 1977 study of Hepatocellular carcinoma by Tsega, found that 50% of the patients and 7% of controls were positive for HBsAg. 65% of the patients and none of the

controls were positive for alpha-fetoprotein. Alpha-fetoprotein and HBsAg were determined using the radioimmunoassay technique in the sera of 46 patients with hepatoma and 90 control subjects [30].

Tables 1 and 2 below summarize the prevalence rates of HBsAg and anti-HCV antibodies in other parts of the world.

**Table 1. Prevalence of hepatitis B surface antigen, among patients with HCC in various parts of the world.**

| Study population | Number of HCC patients | HBsAg positive (%) |
|---|---|---|
| USA | 86 | 17.9 |
| Greece | 194 | 45.9 |
| Senegal | 165 | 61.2 |
| Hong Kong | 289 | 61.6 |
| Philippines | 104 | 70.0 |

Data from Munoz N. et al [31]

**Table 2. Prevalence of Anti-HCV antibodies in patients with HCC in various parts of the world**

| Country | Number of patients | Anti-HCV positivity in HCC (%) |
|---|---|---|
| Japan | 83 | 73.5 |
| Italy | 200 | 76 |
| Spain | 96 | 75 |
| USA | 59 | 52.5 |
| Taiwan | 66 | 33.3 |

Data from Okuda K. et al [32]

It is evident from tables 1 and 2 above that the prevalence of hepatitis B is higher in underdeveloped nations while that of hepatitis C is higher in developed ones.

# VIROLOGY

## I. HEPATITIS B

The hepatitis B virus is a DNA virus in the family of animal viruses called hepadnaviruses and is classified as hepadnavirus type 1. It has a partially double- stranded and partially single-stranded genome [33].

HBV has three morphologic forms; 22 nm particles which appear as spheres or long filaments which are excess envelope proteins, 42 nm double shelled spheres representing the intact virions, and the 27 nm nucleocapsid core which is only found within hepatocytes. The intact virion and the envelope protein express HBsAg. The nucleocapsid core protein expresses HBcAg on its surface. A third HBV antigen is the HBeAg, which is a soluble, non-particulate, nucleocapsid protein [33].

During replication, which occurs in the liver, the HBcAg particles remain in the hepatocyte, and only leave the cell after encapsulation with HBsAg. In contrast HBeAg is secreted into the circulation where it provides a convenient, readily detectable, qualitative marker of HBV replication and relative infectivity [33].

HBV is mainly transmitted percutaneously via tattooing, ceremonial scarification, blood transfusion, and injections. Significant transmission of the virus also occurs during sexual intercourse [34]. Perinatal transmission is another important mode of spread with 50% of children born of infected mothers getting infected and 90% of these becoming chronic carriers. However, horizontal transmission is responsible for most spread in Africa. Studies in Kenya have shown that, unlike South East Asia, vertical transmission is rare here [35,36].

Soon after infection HBsAg appears in the circulation preceding elevation of aminotransferases in serum and clinical symptoms. It disappears during convalescence at about the 24th week from the time of exposure when anti-HBs antibodies become detectable. HBsAg persists beyond 6 months if chronic disease or carriage follows the acute infection [37].

Anti-HBc antibodies are also elaborated by the host; initially of IgM type but later replaced by IgG, which last indefinitely. HBeAg appears concurrently with or soon after HBsAg and disappears when · peak transaminase levels are reached before HBsAg becomes undetectable. Anti-HBe becomes detectable from then onward [33].

Effective vaccines are available for protection against hepatitis B [38].

## II. HEPATITIS C

The hepatitis C virus (HCV) is a linear, single-stranded RNA virus that constitutes its own genus in the family Flaviviridae. Direct visualization of the virus has been difficult because it circulates in very low titre. Similarly in vitro culture has not been convincingly achieved [33].

No fewer than six genotypes have been demonstrated by nucleotide sequencing. This high genotypic diversity, which is due to the high mutation rate of the virus, interferes with the development of effective humoral immunity after acute infection. Variation in pathogenicity and response to antiviral therapy has been shown among genotypes [39,40,41].

HCV transmission occurs by blood and blood products, and illicit drug use with sharing of needles. Occupational exposure to blood and its products can transmit the virus. The probability of infection is high in haemodialysis units [42]. The risk of HCV infection is increased in organ transplant recipients and in patients with AIDS [33].

HCV replicates in hepatocytes though the virus has been demonstrated in peripheral blood lymphocytes as well [33].

There are no effective vaccines available for protection against HCV infection so far [33].

## III. HIV AND HEPATITIS B AND C

HIV, HBV and HCV may infect a person simultaneously (*co-infection*) or may superinfect a person already infected with either of them (*superinfection*) [3].

The HIV and Hepatitis viruses may interact by paracrine means or by co-infection/ superinfection of the same cell. HIV, HBV and HCV are all capable of infecting peripheral blood lymphocytes as well as hepatocytes [43].

## A). HEPATITIS B AND HIV

The soluble HBV X protein has been shown to stimulate HIV replication in vivo, which may accelerate the progression of HIV infection. HIV infected people are more likely to become chronically infected with HBV. Co-infected patients who are also intravenous drug users have a higher frequency of liver cirrhosis than patients without co-infection [44].

HBV infected people who get HIV infection may demonstrate reduction in HBs antibody and the HBsAg may re-appear [45]. Co-infected patients have high levels of HBV DNA and HBeAg because of enhanced HBV replication; hence their body fluids are more infectious [46]. In addition response to the hepatitis B vaccine, which is T-cell dependent, is generally impaired and short-lived in HIV infected people although there may be adequate protection if immunization occurs before significant immunosuppression occurs [47].

## B). HEPATITIS C AND HIV

HCV/HIV co-infected patients have higher HCV titers and are therefore more infectious. Women are more likely to transmit the infection to their offspring compared to the low rates with HCV infection alone [48,49].

HIV co-infection leads to severer and more rapidly progressive disease to cirrhosis, with the duration to cirrhosis being as low as 3 years [50].

HIV co-infected chronic hepatitis C patients respond poorly to α-interferon therapy [51]. HCV loads also appear to be affected by anti-retroviral therapy. Co-infected hemophiliacs put on HAART have suffered an increase in HCV viral loads during treatment [52,53].

## IV. LABORATORY FEATURES

Detecting HBsAg in serum indicates infection with HBV. Demonstration of IgM, and IgG, anti-HBc antibodies is used to establish whether the infection is acute or chronic.

HCV infection is diagnosed by demonstrating anti-HCV antibodies in serum. First generation assays detect antibodies to the non-structural protein C100-3, between 1-3 months after onset of acute hepatitis. Second generation assays detect antibody to C100-3, C22 and C33 and become positive from the fourth week [33]. Third generation assays also detect anti-HCV from the fourth week but lack specificity for genotypes other than type 1 [54]. Fourth generation assays are able to detect type 2 and 3a as well [55].

Supplementary tests such as the recombinant immunoblot assay (RIBA) and HCV RNA PCR are used to confirm the diagnosis [56]. Detection of HCV RNA by PCR is the most sensitive method of detecting HCV infection. HCV RNA can be detected even before aminotransferase elevation and before the appearance of anti-HCV in acute hepatitis [33].

The diagnosis of HIV infection depends on the demonstration of antibodies to HIV by ELISA and or the direct detection of HIV or one of its components by polymerase chain reaction. Antibodies to HIV generally appear 4-8 weeks after infection. A positive ELISA test is most commonly confirmed by Western blot [33].

# III. PATHOGENESIS

Primary hepatocellular carcnoma (PHC) is among the most common cancers in the world and one of the rarer human cancers showing seroepidemiological association with viral infection. The role of HBV as a major aetiological agent of PHC has been firmly established, and the increased risk of developing PHC been estimated to be100-fold for chronic HBV carriers as compared with non-infected populations, placing HBV in the first rank among known human carcinogens [57,58].

Apart from epidemiological evidence, HBV has been shown to have both direct and indirect oncogenic effects on liver cells [59,60,61,62,63,64,65].

## A. EPIDEMIOLOGICAL ASSOCIATION BETWEEN HBV AND PHC

Primary liver cancer (PHC) ranks among the most frequent cancers of males in many countries. A recent estimate [66] indicates that PHC represents the eighth most common cancer, with about 250,000 new cases each year, 70% of which occur in Asia.

Several lines of evidence associate chronic HBV infection with the development of PHC:

1. The incidence of PHC and the prevalence of HBV serological markers follow the same general geographical pattern of distribution. HBV is common in regions where HBV is endemic, but comes far behind other types of cancer in regions where HBV infection is uncommon [58,67,68].

2. Serological evidence of HBV infection is detected in about 70% of PHC patients in Africa and more than 90% in Mainland China, as compared with 10% to 20% of the total population residing in the same areas [68].

3. A marked increase in the risk of PHC has been shown among hepatitis B surface antigen (HBsAg) carriers compared with non-carriers (up to a 200 times risk factor has been reported in some ethnic or social groups using different methodologies of investigations) [57,69,70,71].

A gender discrepancy (males have a two to eightfold elevated risk of developing PHC compared to females) and familial tendency (familial clustering of PHC are common in Asia) have also been documented as factors involved in the frequency of tumour development [60,72,73].

## B. ONCOGENIC PROPERTIES OF HBV VIRAL PROTEINS

The mechanism of hepatocarcinogenesis is not clearly understood [9].

In carriers of HBV, expression of the tumour suppressor gene, p53, is regulated by the viral X gene. The protein product of this gene binds to the p53 gene itself, inhibits p53-stimulated transcription, and suppresses entry of p53 into the nucleus, hence inactivating it [74,75,76,77,78,79]. This leads to uncontrolled hepatocyte proliferation.

Interaction of HBx with XAP-1, a cellular DNA-binding protein, also affects hepatocyte ability to repair mutated DNA sequences, and allows the accumulation of genetic changes [80]. Overexpression of HBx in chronically infected livers and in HCCs has been shown to partly result from HBV DNA integration in a hepatoma cell line [81].

## HBV SURFACE GLYCOPROTEINS AND PHC

It has been shown that the number and rate of appearance of preneoplastic nodules and primary tumour following carcinogen administration are slightly increased in HBsAg-positive transgenic mouse livers, as compared with negative littermates, suggesting that HBsAg expression might enhance the effects of hepatocarcinogenesis [82].

Among HBsAg carriers infected at an early age, an additional PHC risk has been associated with HbeAg carriage, with significant liver damage and high level of anti-HbcAg antibodies in chronic active hepatitis, and with the presence of cirrhosis.

Inappropriate expression of the large S protein (overexpression or overproduction) is directly cytotoxic to the hepatocyte and initiates a cascade of events that ultimately progress to malignant transformation [83,84,85].

## HEPATITIS B VIRUS DNA INTEGRATION IN PHC

Hepadnaviruses share with other retroelements of common evolutionary origin [86] the ability to integrate their DNA into cellular chromosomes [87]. Integrated HBV sequences have been observed in established hepatoma cell lines and in about 80% of human PHCs [88,89,90,91,92,93]. HBV DNA integration enhances chromosomal instability: in many tumours, larger inverted duplications, deletions, amplifications, or chromosome translocations are associated with HBV insertions suggesting that this process functions as a random mutagen, promoting chromosomal defects in hepatocytes [94,95,96,97,98,99,100]

HBV insertion in PHC has been noted to occur in two regions:

1) retinoic acid receptor $\alpha$-gene (RAR-$\alpha$) [63]. This insertion leads to inappropriate activation of RAR-$\alpha$ resulting in expression of a chimeric HBV/RAR-$\alpha$ protein at greater levels than that of native protein, and participates in the tumorigenic process. Retinoic acid and retinoids are vitamin A-derived substances that have striking effects on differentiation and proliferation in a large number of systems [101].

2) Intron of the human cyclin A gene, resulting in a strong expression of hybrid HBV/cyclin transcripts [65,102]. Cyclin A plays an important role in both G2/M and G1/S checkpoints of the cell cycle [103]. HBV/cyclin A is not degradable as occurs in the normal cell [102], and this leads to unregulated, and premature DNA synthesis and thus cell proliferation.

## HCV AND PHC

HCV is an important aetiological factor for PHC and chronic hepatitis plays a major role in the liver carcinogenesis related to this virus.

It is presently not clear whether HCV might also exert direct effects in liver cell transformation, although some evidence supports this hypothesis. Infact rare but well characterized PHCs have been reported in livers with minimal histological lesions and that, despite detailed analysis, showed persistence of HCV RNA in the tumours as the only identifiable risk factor [104], i.e. in some cases, PHC might be associated with HCV in the absence of chronic active hepatitis (CAH).

It is thought that the viral core, especially carboxy-terminal deleted forms, might modulate expression of cellular genes, such as *fos,myc,*and *jun* oncogenes, the *retinoblastoma* suppressor gene, or the gene encoding for the interferon-$\beta$ [105,106].

HCV genotypes have an important impact in the risk of developing PHC. A number of recent studies point to the severity of HCV-1 associated liver lesions, including PHC. There is higher relative prevalence of HCV-1 in patients with cirrhosis than those with moderate CAH [107,108,109]

In recent prospective studies on the risk of developing PHC in patients with HCV-related cirrhosis, infection by genotype 1 has emerged as an independent risk factor [109]. Along the same line, HCV-1b was the most prevalent type among patients with HCV-associated PHC in the absence of cirrhosis [104].

## CHRONIC ALCOHOL CONSUMPTION AND HBV, AND HCV

HBV and HCV can interact with chronic alcohol consumption, and there is circumstantial evidence for a high prevalence of HBV and HCV infections in alcoholics.

The increased prevalence of anti-HCV in alcoholics with cirrhosis (approximately 40-50%) as compared with those with minimal liver damage (approximately 10%) suggests that HCV infection might be implicated in the development of the cirrhosis in some of these patients. This observation may also account for the high prevalence of anti-HCV (50%) in alcoholics with HCC [110,111,112,113].

In contrast, there is no evidence for a role of HBV in the development of alcoholic cirrhosis, as the prevalence of anti-HBs and anti-HBc, although higher than in the general population, does not significantly differ whether or not cirrhosis is diagnosed (approximately 20%); however, there is evidence for the role of HBV in the liver cancers occurring in alcoholics, since the prevalence of HBV serological markers is significantly increased in these patients (approximately 50%), and the tumours frequently contain HBV DNA sequences [114,115,116,117].

The relationship between cirrhosis and PHC appears to be complex, and the degree of correlation varies with aetiology of cirrhosis, macronodular cirrhosis, precedes or accompanies a majority of HBV (more than 80% in Asia and 40% to 60% in Africa) and HCV associated PHCs in children, as well as at older ages. The risk of PHC has been considered to be lower in HBsAg-negative micronodular cirrhosis observed in alcoholics [118,119,120].

## HISTOLOGICAL DIAGNOSIS OF HCC AND LIVER CIRRHOSIS

The most important criterion in histological diagnosis is similarity of cancer cells to normal hepatocytes, namely, relatively large acidophilic cytoplasm. Cancer cells are arranged in thick cell cords or plates, in a trabecular arrangement lined by endothelial cells, which form blood sinuses.

In extremely well differentiated HCC, cancer cells are distinguished from normal liver cells by subtle structural changes such as the strong cell-to-cell cohesiveness and the tendency to grow in an expanding fashion, not infiltrating into the parenchyma.

In moderately/poorly differentiated HCC, large multinucleated cells are often present and the cells have least cohesiveness with a tendency to grow in an infiltrating fashion.

In anaplastic HCC, the trabecular arrangement is lost and the cancer cells may resemble sarcoma cells growing in a solid or compact arrangement [9].

**Note**

HCC cells often contain cytoplasmic inclusion bodies such as globules, Mallory hyaline bodies, spherical inclusions and ground glass cytoplasm. Occasionally cancer cells are arranged in an acinar fashion assuming a picture that may be called "pseudoglandular" [9].

The presence of cirrhosis is established histologically when well-demarcated nodules are seen entirely surrounded by fibrous septa. Based on this criterion alone the diagnostic yield is very low especially when the specimen is small. Hence to avoid missing substantial numbers of cirrhotic livers use is made of a combination of more subtle criteria to improve diagnosis [9]. These criteria include:

1. Fragmentation of the sample especially if a Menghini needle is used
2. Presence of fibrous septa
3. Presence of fibrous tissue at the edges of fragments of parenchyma
4. Abnormalities of vascular relationships
5. Changes in the appearance of the liver parenchyma e.g. thickening of the hepatocellular plates, normally one cell thick in adults but often two cells thick in regenerating liver.

## JUSTIFICATION FOR THE STUDY

Primary hepatocellular carcinoma (PHC) is the most common malignancy arising within the liver and is particularly frequent in many developing countries because of its association with chronic viral hepatitis. In Kenya for example PHC is the third commonest solid cancer in males after skin cancer and lymphoma [121].

The major causes of PHC worldwide are known and preventable. Hepatitis B and C are human diseases; the viruses have no known non-human reservoirs. Transmission of these viruses can be interrupted by: (i) vaccination against HBV infection and treatment of chronic HCV infection with, alpha interferon and ribavirin combination therapy, which is 47% effective[122], and (ii) educating the public about the modes of transmission.

In present day Kenya, hepatitis B vaccine is not universally available through the Expanded Programme on Immunization (EPI) except to children aged six months and below. The data from the present study could therefore be forwarded to the Ministry of health and thus help reinforce the need to offer universal vaccination against hepatitis B, and also encourage education of the public on modes of transmission. There is also paucity of local data on the pattern of alpha-fetoprotein levels and HBV markers in PHC.

There has been no study of this kind over the last 10 years and the HIV serostatus in patients with PHC is not known. There is also no known data on HCV in relation to PHC. PHC is universally fatal within a short duration; hence it is an important disease entity to study. It is against this background that the present study was undertaken.

## OBJECTIVES

The broad objective of this study was to determine some serological markers (for HBV, HCV and HIV) and alpha-fetoprotein levels in patients with primary hepatocellular carcinoma (PHC) at KNH.

**The specific objectives were.**

1. To determine the prevalence of HBsAg in patients with PHC at KNH

2. To determine the prevalence of IgG anti-HBc antibodies in patients with PHC at KNH

3. To determine Alpha-fetoprotein levels in patients with PHC at KNH

4. To determine the HIV serostatus in patients with PHC at KNH

5. To determine the prevalence of anti-HCV antibodies in patients with PHC at KNH

6. To determine the prevalence of cirrhosis in patients with PHC.

## PATIENTS AND METHODS

### Study period

The study was carried out over a duration of five months, between the months of November

2001 and April 2002.

**Study design & setting:** The study design was a cross-sectional descriptive survey, done at the Kenyatta National Hospital (KNH), Nairobi, Kenya.

### Study population

This comprised all patients aged 13 years and above seen in KNH with a diagnosis of PHC.The catch points were casualty, liver, medical and surgical outpatient clinics, and the medical, and surgical wards.

### Sample size

The minimum sample size was 62 patients. This was calculated using the formula shown in appendix 1.

### Patient selection (sampling)

Consecutive patients seen at the casualty, liver, medical and surgical outpatient clinics, and those admitted in the medical and surgical wards, meeting the inclusion criteria were recruited.

### Patient recruitment

The investigator recruited patients from the liver, medical and surgical outpatient clinics. Posters were placed at the Casualty department asking clinicians there to refer all patients

with PHC/suspected PHC to the investigator at the liver clinic for evaluation and recruitment.

Ward patients were recruited by the investigator, a day after their admission.

**Case definition**

All patients with a histological diagnosis of primary hepatocellular carcinoma.

**Inclusion criteria**

Patients of both sexes aged over 13years were included in the study if they had a histological diagnosis of primary hepatocellular carcinoma and they (or their parents or guardians in case of those under 18 years of age) gave informed written consent to participate in the study.

**Exclusion criteria**

Patients who had any form of testicular abnormality, those on treatment for primary hepatocellular carcinoma and those who declined to give consent were excluded from the study.

**History**

Each recruited patient was allocated a study number and his/her hospital number, age, sex, occupation, education, marital status and residence recorded on an already prepared data sheet (appendix 2). History of chronic excessive alcohol abuse, chronic liver disease, jaundice, right upper quadrant abdominal pain, liver mass, weight loss and ascites was asked for.

**Physical Examination**

Each recruited patient had a general examination done in which the general condition, jaundice, pallor, oedema, wasting, lymphadenopathy, and scratch marks were noted.
The abdomen was examined for hepatomegally, liver mass, right upper quadrant tenderness, liver span, splenomegaly, other masses and ascites. The examination findings were recorded in the already prepared data sheet.

## LABORATORY METHODS

5millitres of venous blood was drawn from each patient into a biochemically clean bottle and taken to the immunology laboratory at KNH, where the serum was separated from the cells by centrifugation and the various serological markers and α-fetoprotein levels determined as detailed in appendices 4 to 8.

### Serological tests

All serological tests were done at the Immunology Lab KNH using the ELISA based kits [123]. HBsAg was assayed using the Enzygnost HBsAg 5.0 kit manufactured by Dade Behring Marburg GmbH, which has a sensitivity of 100% and a specificity of 99.8% (appendix 4). IgG anti-HBc was tested using the ImmunoComb II kit manufactured by Orgenics, which has a sensitivity of 100% and a specificity of 99% (appendix 5) and HCV antibodies using the 4th generation kit HCV AB IV from Innogenetics, which has a sensitivity of 100% and a specificity of 99.8% (appendix 6).

After pretest counseling, assays for antibodies against HIV-1 and HIV-2 were done using the innotest kit manufactured by Innogenetics, which has a sensitivity of 100% and a specificity of 99.7% (appendix 7). Thereafter post test counseling was done. Finally alpha-fetoprotein levels were assayed using the DRG AFP MTPL kit from DRG Instruments-Germany, which has a sensitivity of 100% and a specificity of 99.8%(appendix 8).

### HISTOLOGICAL DIAGNOSIS OF HCC AND LIVER CIRRHOSIS

All liver biopsies for the study patients were performed using the Tru-Cut needle (Hepafix kit gauge 17). The specimens were put in 10% buffered formalin and transported to the histopathology laboratory at KNH, where they were processed and paraffin wax blocks made in the standard manner [124]. Sections were then made and stained by haematoxylin and eosin (appendix 9), and Massons trichrome (appendix 10). The slides were read by two independent pathologists using the criteria devised by Edmondson and Steiner [125] for the diagnosis of HCC, and that by World Health Organization [126] for diagnosis of cirrhosis.

## DATA MANAGEMENT

Data was gathered using a questionnaire (appendix 2). It was verified and entered into a computer spreadsheet. Analysis was done using SPSS 10.0 software. Frequencies, percentages, means, ranges and standard deviations were calculated. The results were presented in tables, pie charts and bar charts. The 95% confidence level was used to assess significance. The Pearson chi-square test was utilized in assessing the statistical significance of association.

# RESULTS

The study was carried out between November 2001 and April 2002. 62 patients with histologically proven primary hepatocellular carcinoma (PHC) were included in the study.

## I. AGE AND SEX DISTRIBUTION OF THE 62 PATIENTS WITH HCC.

The mean age of all patients was 49.74 ± 13.04 years. The median age was 50 years (range 21-79 years). There were 39 males (62.9%) and 23 females (37.1%) giving a male-to-female ratio of 1.7:1 (Figure 1). The mean age for male patients was 48.77 years with a standard deviation of 12.26 years, whereas that of female patients was 51.39 years with a standard deviation of 14.39 years.

# Figure 1. Age And Sex Distribution of The 62 Patients with HCC



Age distribution
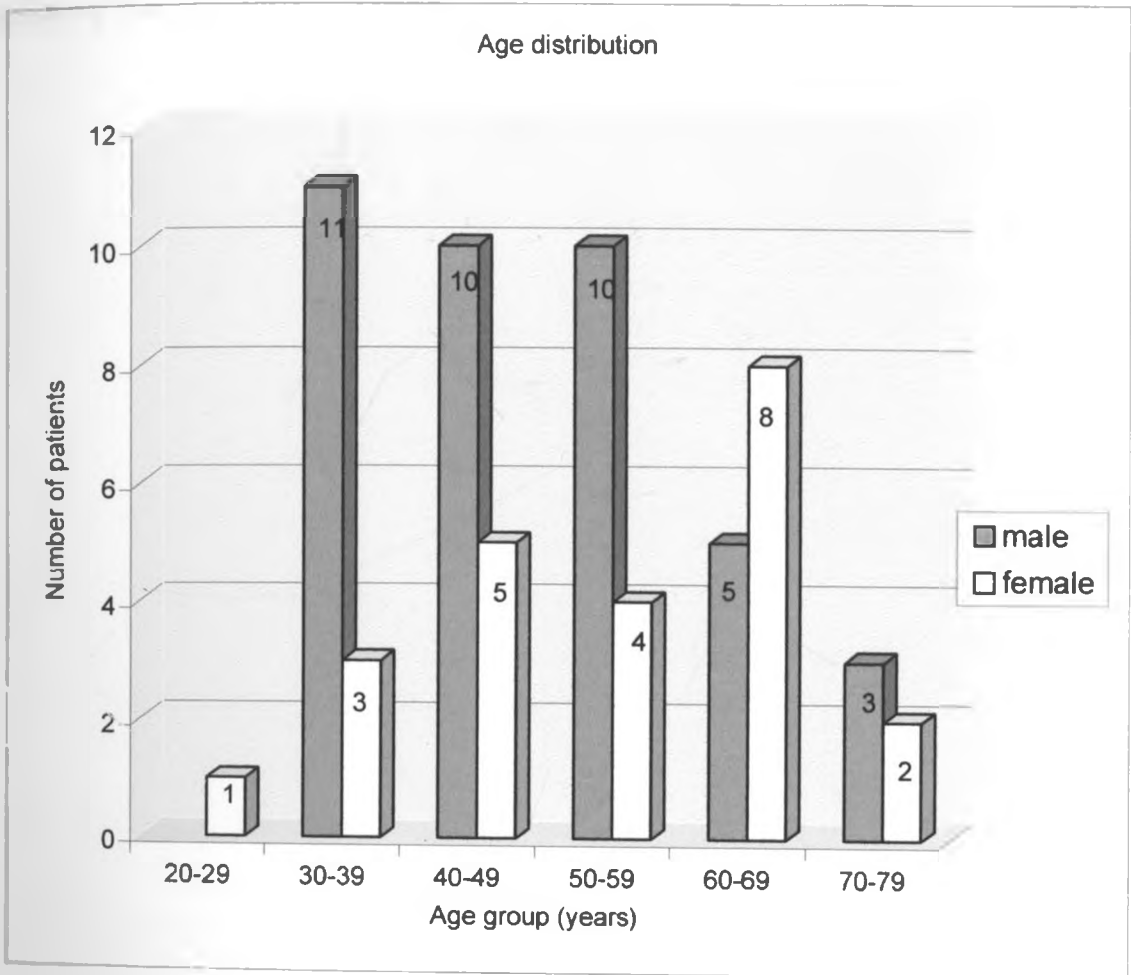
The mean age was 49.74years with a standard deviation of 13.04 years. Most patients were young with an age range of 30-50 years (47% of patients).

# PATHOLOGY FINDINGS

**Figure 2. Photograph of a postmortem liver specimen of one of the study patients with primary hepatocellular carcinoma.**

This is a postmortem specimen from a 44-year-old male patient, who had primary hepatocellular carcinoma, showing a multinodular tumour. The liver weighed 5025g and as seen here more than a third of the organ has been replaced by a large mass of yellowish tumour (right). Numerous deposits of tumour also infiltrate the rest of the liver. There is no evidence of pre-existing cirrhosis.

**Figure 3.** Photograph of a histology specimen of the same patient above (fig. 2)

As seen in this photograph the tumour was a clear cell (hypernephroid) variant of primary

hepatocellular carcinoma. Note the sheets of closely packed cells with abundant cytoplasm,

which is extensively vacuolated and clear. HE X 370.

# II.SEROLOGY.

## PREVALENCE OF SEROLOGICAL VIRAL MARKERS

The prevalence of hepatitis B and C markers in the 62 patients with HCC is shown in table 3 below.

Table 3: Prevalence of viral markers among the 62 patients with HCC

| Marker | Number | Percent (%) |
|---|---|---|
| HbsAg and HBcAb | 39 | 62.9 |
| HCV-Ab alone | 1 | 1.6 |
| HbsAg, HBcAb & HCV-Ab | 5 | 8.1 |
| No HbsAg, HBcAb & HCV-Ab | 17 | 27.4 |
| Total | 62 | 100 |

Out of the 62 patients 71% were seropositive for Hepatitis B whereas only 9.7% were seropositive for hepatitis C.

**Table 4. Prevalence of HBV markers in the 62 patients with HCC**

| Marker | Number | Percent (%) |
|---|---|---|
| HBsAg alone | 7 | 11.3 |
| HBcAb alone | 9 | 14.5 |
| HBsAg + HBcAb | 28 | 45.2 |
| None | 18 | 29 |
| Total | 62 | 100 |

As shown in table 4 above, using IgG-anti HBc antibodies, 9 (HBsAg negative) patients were identified who had had previous exposure to HBV.

## A. HBsAg

Hepatitis B surface antigen was detected in 35 patients representing 56.5% of all patients studied. Among these 28 were males and 7 females. Figure 4 below shows the prevalence of HBsAg according to age group.

**Figure 4. Prevalence of HBsAg among the 62 patients with HCC By Age Group.**
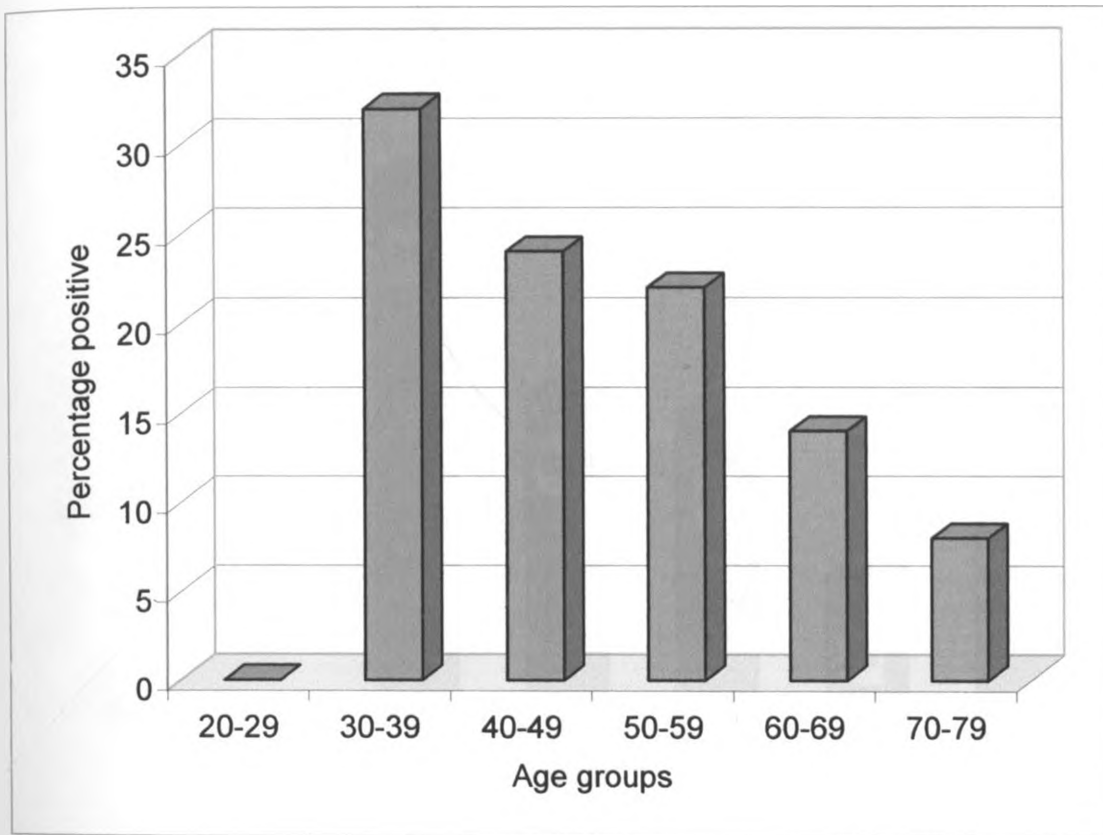


The majority of the patients were young especially between 30-50 years of age (60%). The association between age and HBsAg positivity was statistically significant p=0.037.

## B. HBcAb

IgG anti-HBc antibody was detected in 37 patients representing 59.7% of all patients studied. Of these 27 were males and 10 females. Nine (14.5%) were IgG-anti-HBc positive with a negative HBsAg. Figure 5 below shows the prevalence of HBcAb among the 62 patients with HCC according to age group.

**Figure 5. Prevalence of Hepatitis B Core Antibody Among the 62 Patients with HCC According to Age Group.**
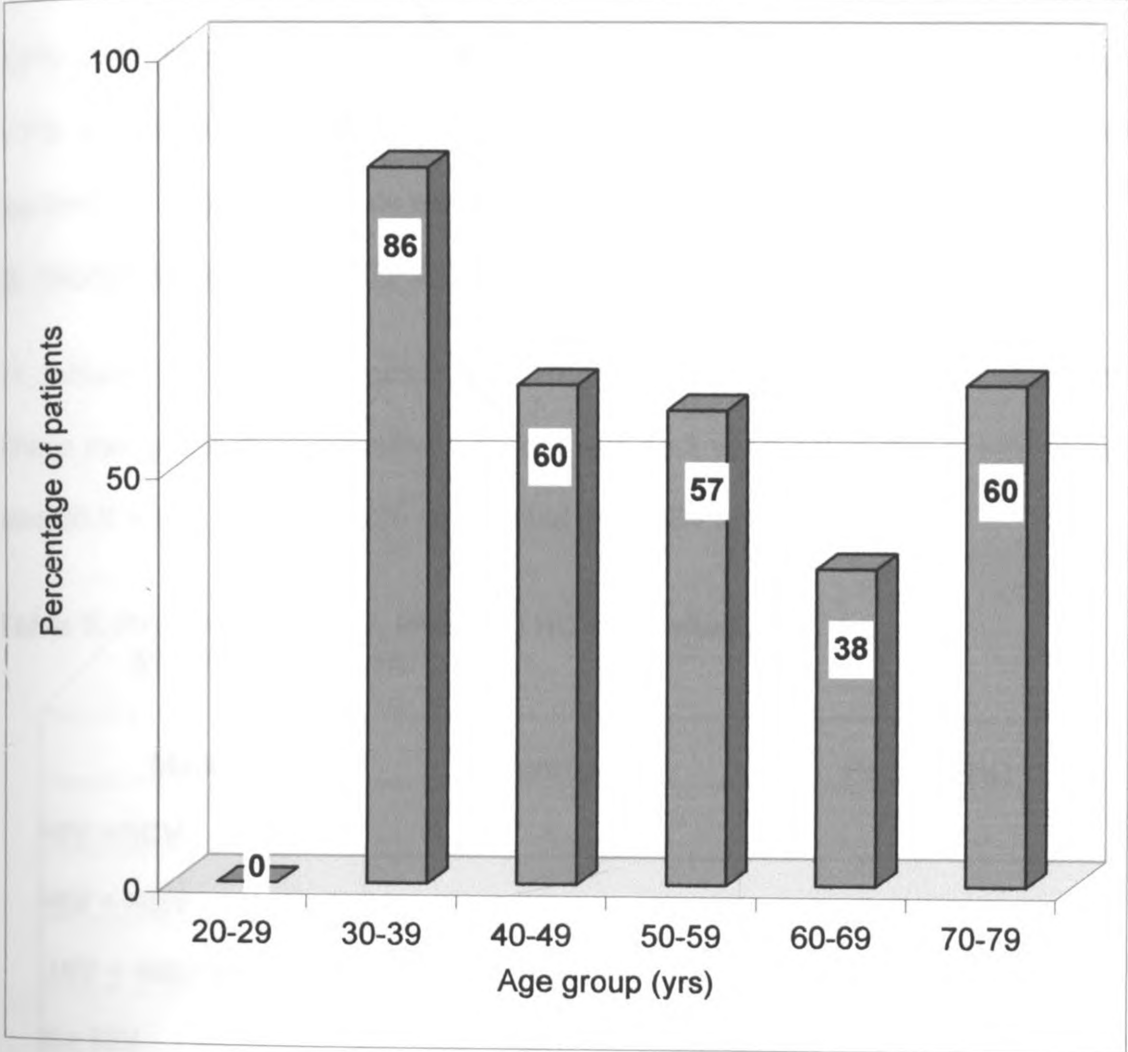


The majority of the patients were young especially between 30-50 years of age 56.8%.

## C. HEPATITIS C VIRUS (HCV)

Anti-hepatitis C antibodies (anti-HCV-antibodies) were detected in 6 patients representing 9.7% of all patients studied. Of these 4 were males and 2 females representing 10.3% and 8.7% of patients within the male and female sex respectively. The mean ages of HCV positive and negative patients were 56.2 and 49.1 years respectively.

## D. HUMAN IMMUNODEFICEINCY VIRUS (HIV)

11 patients (17.7%) were positive for HIV antibodies. There were 8 males and 3 females. There mean age of HIV positive patients was 44.2 years while that of HIV negative patients was 50.9 years. Ten (10) were co-infected with HBV and 1 with HCV.

**Table 5. Prevalence of HIV, HBV and HCV co-infection in the 62 patients with HCC**

| Marker | Number | Percent (%) |
|---|---|---|
| HIV +HCV | 1 | 1.6 |
| HIV + HBV | 9 | 14.5 |
| HIV + HBV +HCV | 1 | 1.6 |
| No HIV | 51 | 82.3 |
| Total | 62 | 100 |

Figure 6. The prevalence of liver cirrhosis in the 62 patients with HCC



44%

■ Cirrhosis
No cirrhosis
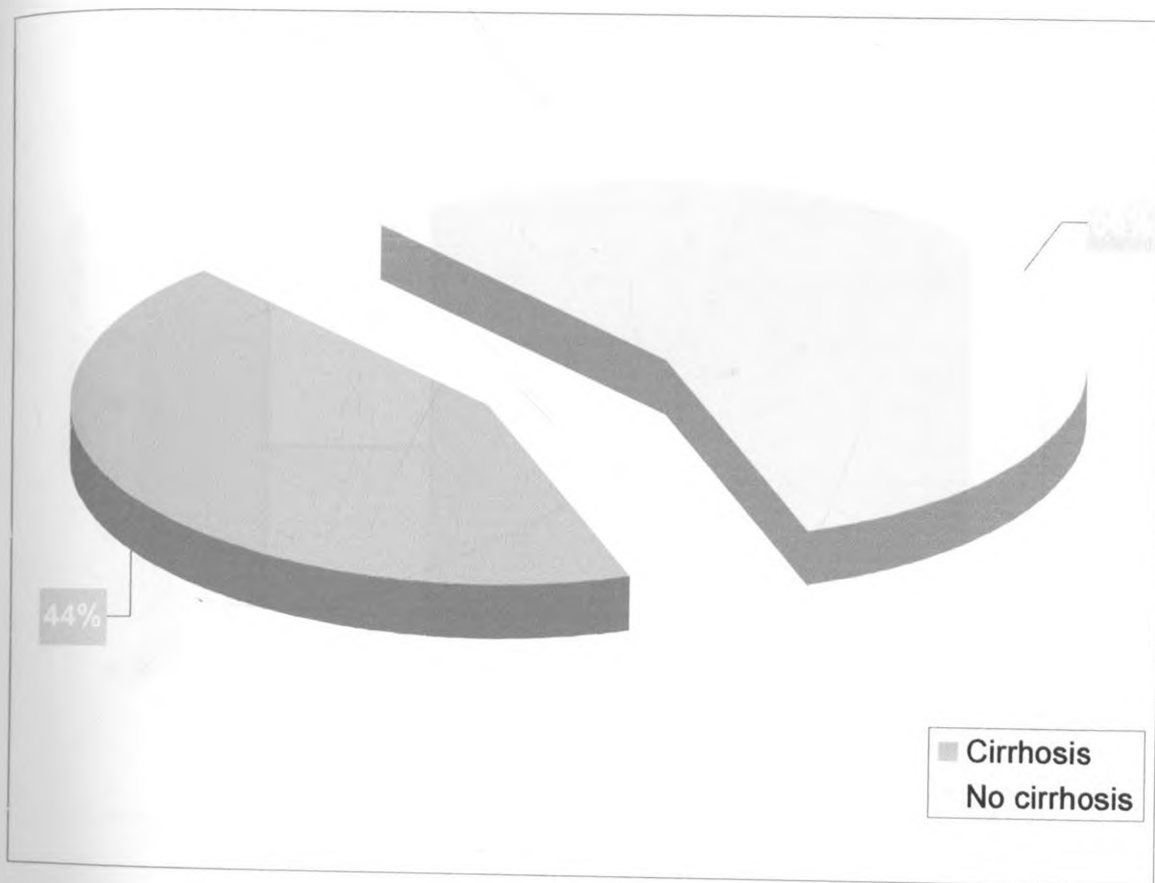
Liver cirrhosis was detected in 27 (44%) of the 62 patients with HCC by histology. There were 22 males and 5 females.

**Figure 7. Prevalence of hepatitis markers in cirrhosis**
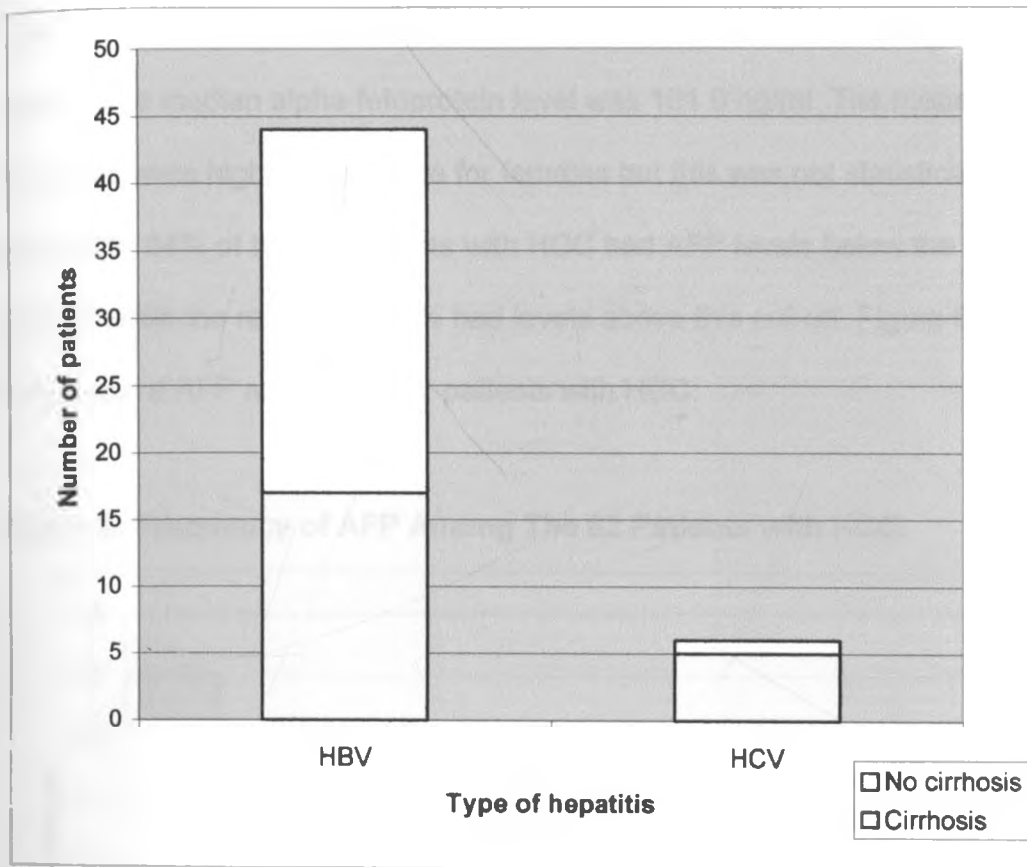


All the five (5) patients with HCV who had cirrhosis were also co-infected with HBV. The association between HCV and cirrhosis was statistically significant (p=0.039) whereas that between HBV and cirrhosis was not (p=0.352).

## G. ALPHA-FETOPROTEINS

The mean alpha-fetoprotein level was 46,913.2ng/ml ± 164,175.9 ng/ml (range 1-1,092,610 ng/ml). The median alpha-fetoprotein level was 101.0 ng/ml. The mean and median values for males were higher than those for females but this was not statistically significant (p=0.333). 64% of the 62 patients with HCC had AFP levels below the diagnostic cut-off of 500ng/l while the remaining 36% had levels above this cut-off. Figure 6 below shows the frequency of AFP among the 62 patients with HCC.

**Figure 8: Frequency of AFP Among The 62 Patients with HCC.**
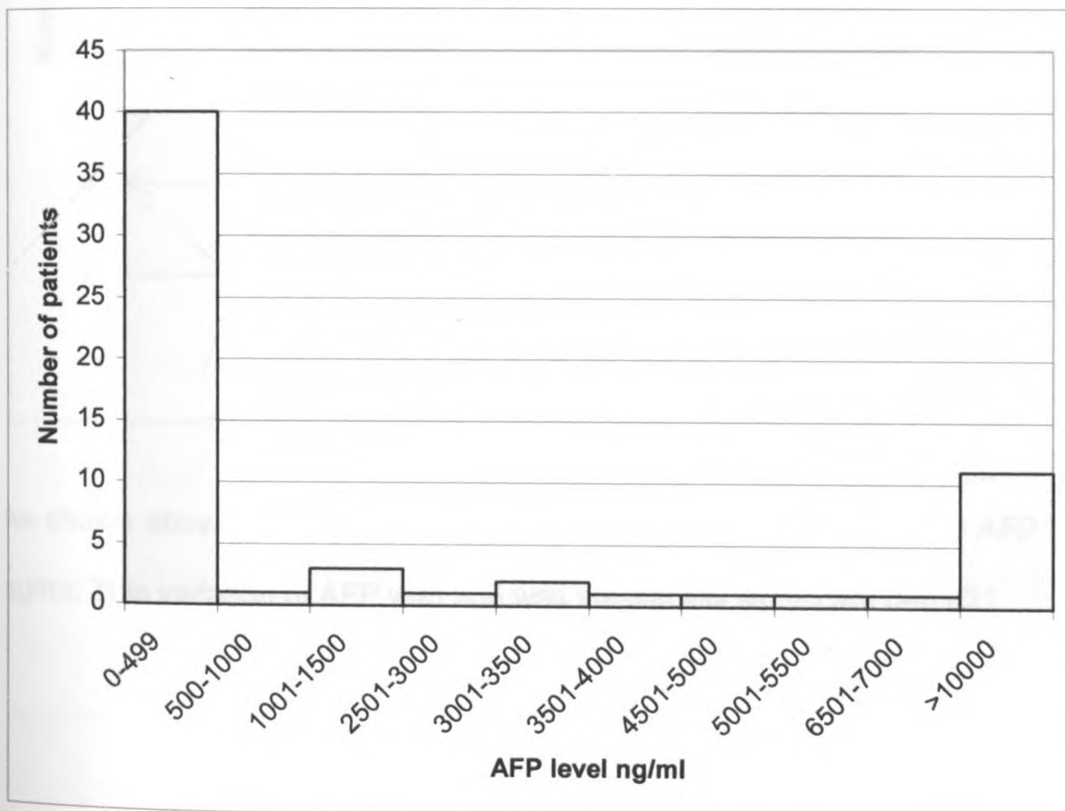


As shown above the majority of patients (58.1%) had levels below the diagnostic cut-off of 500 ng/ml.

**Figure 9: Comparison of AFP Levels Between Patients Aged Below 50 and those Above 50 Years**
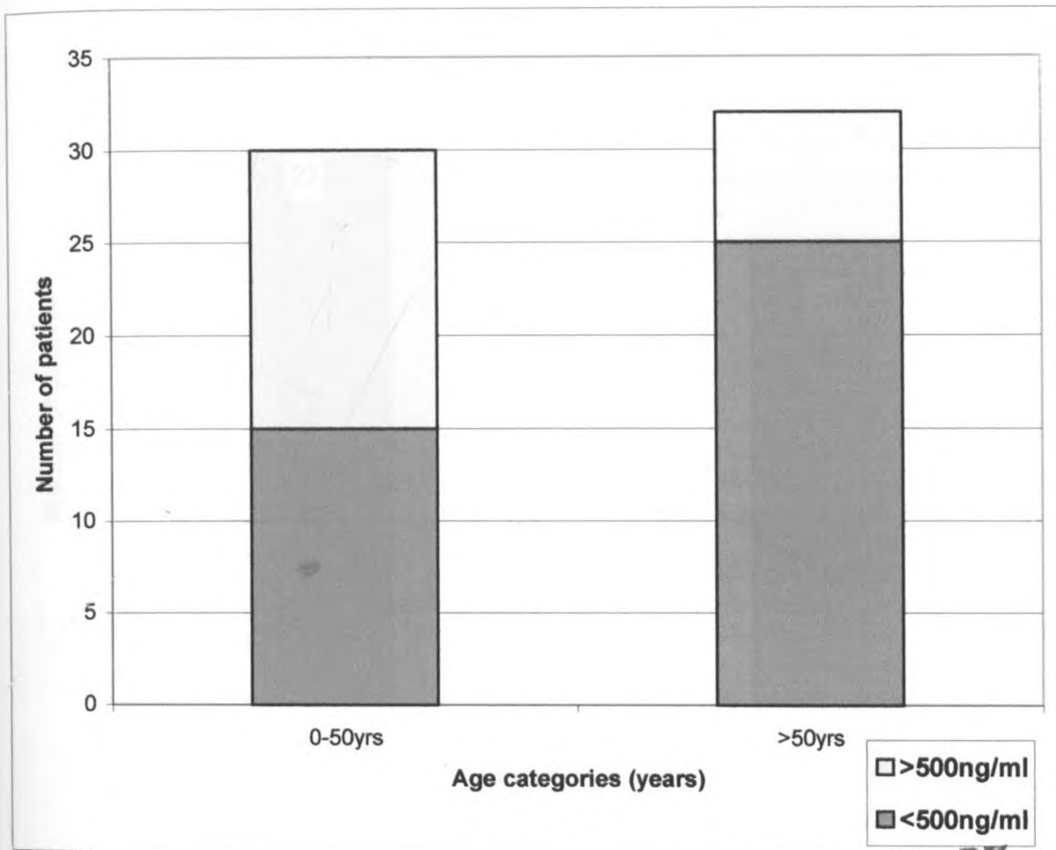


As shown above the majority of patients aged >50 years (25/32) had AFP levels below 500 ng/ml. This variation of AFP with age was statistically significant p=0.021.

**Figure 10: Comparison of AFP levels between hepatitis B positive and hepatitis B negative patients.**
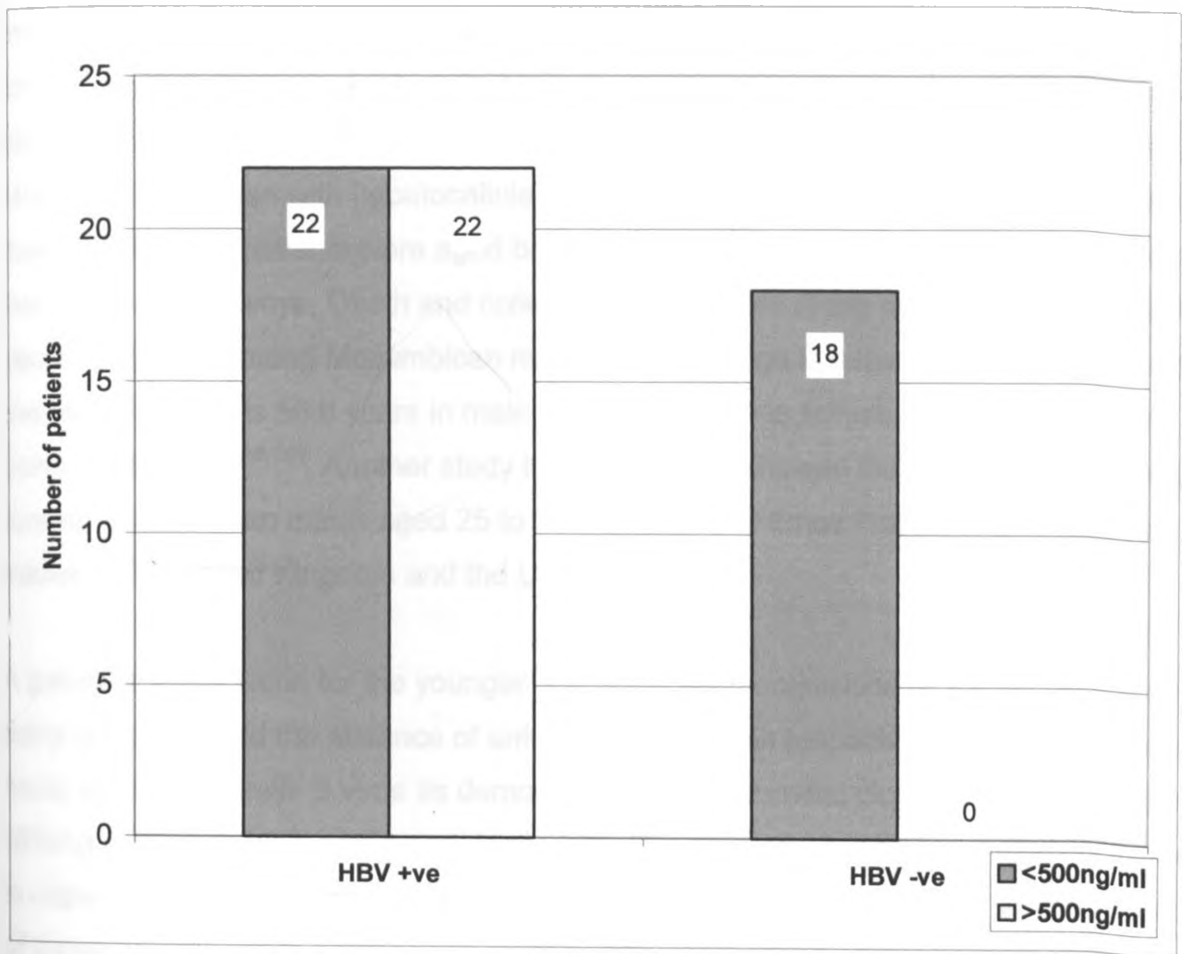


All the 22 patients that were hepatitis B positive had AFP levels above 500 ng/ml (Positive). Those that were negative had AFP levels below 500 ng/ml (Negative). This relationship is statistically significant (p=0.007).

# DISCUSSION

The mean age of all patients was 49.7 years. 48.4% of the studied patients were below 50 years old. This is in agreement with previous studies, which have shown that in high incidence areas such as sub-Saharan Africa, the incidence of PHC begins to rise in childhood, and reaches very high levels in the third and fourth decades of life. This is because of its close association with chronic hepatitis B virus infection [8]. In a prospective study of 22,707 men with hepatocellular carcinoma in Taiwan, Beasley and colleagues found that 47.5% of the patients were aged below 50 years [127]. In another study of the aetiology of liver cirrhosis in Kenya, Okoth and colleagues found 55% of the patients to be below 50 years of age [27]. Among Mozambican males, the peak age is between 25 and 34 years; the average in Japan is 56.8 years in males and 59.9 years in females, and it is higher in northern Europe [128,129]. Another study in Mozambique showed that the crude incidence rate among Mozambican males aged 25 to 34 was over 500 times that among comparable white males in the United Kingdom and the United States [130].

A possible explanation for the younger age is the early acquisition of the hepatitis B virus in early childhood and the absence of universal vaccination (especially in Africa and South-East Asia) against hepatitis B virus as demonstrated by the familial clustering of HCC patients and HBsAg carriers in both Africa and Japan [9,131]. For example, in a national study of liver cancer in Japan (1968-1977), 150 families in whom there was a patient with HCC were recruited; 48 of these were found to have one or more family members who were carriers of HbsAg [132].

62.9% (39/62) of the studied patients were males, giving a male to female ratio of 1.7:1 (~2:1). Bosch and colleagues reported that men are afflicted twice as often as women and that although PHC ranks eighth in frequency among cancers worldwide, it is sixth among men and eleventh among women [128]. Adrian and colleagues reported male to female ratios ranging between 8:1 and 3:1 [8]. It is evident that the incidence is lower in females than in males, regardless of population, perhaps because chronic liver disease is less common in the former [133].

HBsAg was detected in 35 (56.5%) of the studied patients. This is comparable to what has been reported by other workers based both in Kenya and other parts of the world (50% to 80%)[31]. A marked increased risk of PHC has been shown among hepatitis B surface antigen (HBsAg) carriers compared with non-carriers (up to 200 risk factors have been reported in different ethnic or social groups using different methodologies of investigations)[57,69,70,71]. This is because inappropriate expression of the large S protein (overexpression or overproduction), is directly cytotoxic to the hepatocyte and initiates a cascade of events that ultimately progress to malignant transformation [83,84,85].

HBcAb was detected in 59.7% of the 62 patients studied, which compares well with previous findings of 80% by Bowry from Kenya [134], Tswan 56.5% from South Africa [135]. The high rate of HBsAg and HBcAb in PHC is in agreement with previous findings that the virus is involved in the pathogenesis of PHC [74,75,76].

HCV-antibody was detected in 9.7% of all cases. Although no previous local study has looked at the prevalence of HCV in PHC patients, studies looking at HCV-Ab in other groups of patients have shown figures ranging from 0% to 7.1% [29, 136,137,138]. Ilako and colleagues in 1995 found a hepatitis C antibody prevalence of 6.3% in renal transplant patients, 2.8% in chronic liver disease patients at Kenyatta National Hospital. None of the patients with hepatocellular carcinoma or liver cirrhosis carried the antibodies in their bloodstream. They found a seroprevalence of 0.9% among blood donors [25].

A possible explanation for differences in prevalence rates is the differences in the sensitivity of the techniques used. Earlier studies by Mwangi and colleagues and, Ilako and colleagues utilized 1st and 2nd generation ELISA based kits, which are less sensitive. Those from Ethiopia, South Africa and china utilized the more sensitive Polymerase chain reaction (PCR) based HCV RNA test, which is the gold standard especially in HIV infected patients. Our present study utilized a fourth generation ELISA based HCV-antibody test whose sensitivity is 99.7%.

All the previous local studies were carried out at Kenyatta National Hospital. The prevalence in our present study is much higher than the global prevalence of chronic hepatitis C that is estimated to average 3% (ranging from 0.1 to 5% in different countries) [139]. A possible reason for this disparity is the difference in the populations studied. Global screening

programmes have studied normal healthy individuals without liver disease while the above studies have been carried out among individuals with chronic posthepatitic liver disease.

Hepatitis virus co-infection was present in five patients. Tsega and colleagues reported 55% from Ethiopia [140], Soriano 50% from spain [141],Kupper reported 3% from Greece [134] and Tradati 7.5% from Italy [142]. Co-infection with hepatitis B and C and HIV may occur because of the shared modes of transmission. Interaction of the HIV and Hepatitis viruses may occur via co-infection of the same cell or by release of soluble factors from infected cells. The immunodeficiency due to HIV infection is known to augment replication of many viruses. The HIV, HBV and HCV are all capable of infecting peripheral blood lymphocytes as well as hepatocytes as demonstrated by William Sievert [143].

The soluble hepatitis B virus X protein has been demonstrated to stimulate HIV replication in vivo, which is likely to accelerate the progression of HIV infection.

HIV infected persons with chronic hepatitis B have been shown to have poor response to interferon-alpha therapy [144]. Similarly zidovudine has not been shown to decrease HBV replication in HIV patients [145].

A recent report from France showed that co-infected HBV and HIV patients who are also intravenous drug users have a higher frequency of liver cirrhosis [146].

HCV patients with HIV co-infection have higher blood HCV titers [147].

HIV co-infection has been shown to lead to severer and more rapidly progressive disease to cirrhosis. The duration to cirrhosis has been shown to be as low as 3 years [148].

11 patients (17.7%) were positive for HIV antibodies. This is similar to the 19% seroprevalence rate for in-patients in the medical wards at the KNH reported by Gilks and colleagues [149]. Weinig reported 28.8% (17/59) from Zimbabwe [150] and Tswan 7.7% (14/182) from South Africa [135]. Tanaka and colleagues reported a case of PHC with HIV infection with no evidence of hepatitis B or C virus (HBV or HCV infection [151]. This is the first report of HCC in HIV-infected patient with no evidence of hepatitis virus infection. A possible explanation for this is provided in a study by Bierhoff and colleagues in which centrilobular fibrosis was significantly more marked in patients with HIV/HCV co-infection than in HCV monoinfection (p <0.05), suggesting an HIV-associated fibrogenic effect [152].

In the present study, 43.5% of PHC was associated with cirrhosis. Mwangi and colleagues reported 52% locally [153]. It is postulated that in areas with a high incidence of PHC the HBV is acquired at birth and PHC develops without preceding cirrhosis in many cases, hence the lower value compared to that reported from the western countries of 80-90% [9,154]. In our study there was a statistically significant correlation between HCV and liver cirrhosis (p=. 013).

Although this is the first local study of the prevalence of HCV in PHC with liver cirrhosis, in Western countries HCV accounts for 40% of cases of end-stage cirrhosis and 60% of cases of PHC [154]. In our study 3 patients (4.8%) with cirrhosis were co-infected with both HBV and HCV. One (1.6%) was infected with HBV, HCV, and HIV. A recent report from France showed that co-infected HBV and HIV patients have a higher frequency of liver cirrhosis [146]. HIV co-infection has been shown to lead to severer and more rapidly progressive disease to cirrhosis. The duration to cirrhosis has been shown to be as low as 3 years [155]. Garcia-Samaniego and colleagues in their study on hepatocellular carcinoma in HIV-infected patients with chronic hepatitis C found that among co-infected patients, PHC was typically diagnosed at a younger age and relatively soon after infection with HCV [156]. Soto and colleagues reported that HIV infection modifies the natural history of chronic parenterally acquired hepatitis C leading to an unusually rapid progression to cirrhosis [157].

In our study 36% of all studied patients had AFP level above the conventional cut off of 500ng/ml. Tangkijvanich and colleagues reported 50.2% [158]. Higher levels tended to be in younger patients and those who were HBV positive (p-value =0.021 and 0.007 respectively). Tangkijvanich and colleagues [155], Bagshawe and colleagues [159], and Bowry and colleagues [20] have reported similar results. Younger patients tend to have higher AFP levels because they tend to have diffuse (i.e. infiltrating) tumour compared to the old who have nodular types. They also tend to be hepatitis B positive. These two factors (younger age & HBV positivity) have been shown to be associated with higher AFP secreting HCC [160,161]. AFP is synthesized more frequently and in larger quantities by rapidly growing undifferentiated tumour and cancer cell lines [162,163]. It could be postulated that HBsAg induced chronic liver

disease with malignant transformation in African patients commonly leads to poorly differentiated, fast growing AFP producing tumours.

Other factors associated with high AFP levels in HCC include larger tumour size, bilobar involvement and portal vein thrombosis [161].

As a diagnostic test the determination of alpha-fetoprotein levels when used with the conventional cut-off point of 500 ng/ml has a sensitivity of about 50% and a specificity of more than 90% in detecting the presences of HCC in a patient with coexisting liver disease [164]. Lopez and colleagues showed a sensitivity of 58.8% and a specificity of 97.4% hence confirming AFP to be the most efficient marker for HCC [165].

## STUDY LIMITATIONS

1. Due to financial constraints it was not logistically possible to rule out all other markers of HBV, hence there was a possibility of under-reporting hepatitis B virus prevalence.

2. The same could have been the case for hepatitis C virus as only antibodies were screened for whereas HCV-RNA is the test of choice especially in HIV infected people.

# CONCLUSIONS

1. The prevalence of hepatitis B virus markers among patients with primary hepatocellular carcinoma was high and stood at 71% in this study.

2. All patients that had positive alpha-fetoprotein levels were positive for the tested hepatitis B markers (HBsAg & IgG anti-HBcAb).

3. In those who were positive for alpha-fetoprotein, age played a role in that those below 50 years had higher values.

4. Hepatitis C accounted for 9.7% of patients with primary hepatocellular carcinoma.

5. 17.7% of the patients were positive for HIV antibodies (ELISA I & II). This figure was slightly lower than that expected for KNH in-patients (20%), but significantly higher than that of the general population (12%).

6. 43.5% of the patients with primary hepatocellular carcinoma had associated liver cirrhosis.

## RECOMENDATIONS

1. Routine screening of Hepatitis B markers should be undertaken in patients

   with hepatocellular carcinoma.


2. In view of the high association of hepatitis B in primary hepatocellular

   carcinoma, routine vaccination should be encouraged.


3. In those patients that are hepatitis B positive routine monitoring of the alpha-

   fetoprotein levels should be encouraged during follow-up.


4. Routine screening of hepatitis C and HIV markers should be undertaken in patients with

   cirrhosis and/or hepatocellular carcinoma in view of the rapid progression of HCV-related

   cirrhosis to hepatocellular carcinoma in the presence of HIV, and the high association of

   HCV and cirrhosis.

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## APPENDIX 1: SAMPLE SIZE CALCULATION

$$n = \frac{(Z_{1-\alpha/2})^2 \, P \, (1-P)}{d^2}$$

Where    n= 62 ( minimum sample size)

        Z= standard normal deviation

        P= estimated prevalence of the characteristic (80% for HBsAg)

        d= The desired degree of precision or accuracy(10%)

        $\alpha$= significance level

  $(Z_{1-\alpha/2})^2$= 1.96, corresponding to a significance level of 0.05

# APPENDDIX 2

## QUESTIONAIRRE

Hospital No..........................      Height (m)........................

Study No...............................      Weight (kg).....................

Age (years) ........................      Ethnicity.......................

Sex....................................Male/Female

Address................................................

Residence:..........................rural / urban............................

Marital status:..............single / married / monogamous / polygamous

Occupation....................................................................

Education:.................primary / secondary / college.....................

Alcohol: amount (>80g daily) / duration (>5years) ...........................

Date of diagnosis (if already diagnosed).........................................

## HISTORY

Chronic liver disease...........................Yes/No

Chronic hepatitis................................Yes/No

Liver cirrhosis...................................Yes/No

Blood transfusion...............................Yes/No

Jaundice .......................................Yes/No

Fever..............................................Yes/No

Pruritus..........................................Yes/No

Tattooing / Ear piercing.......................Yes/No

Sharing needles / toothbrushes..............Yes/No

Sexual habits: homosexual / heterosexual / bisexual / no of sexual partners in the last three months.

## SYMPTOMS

General malaise.......................................Yes/No

Abdominal pain.........................................Yes/No

Full sensation in abdomen............................Yes/No

Anorexia.................................................Yes/No

Weight loss..............................................Yes/No

Ascites...................................................Yes/No

Palpable mass.......................................Yes/No

Pedal oedema.......................................Yes/No

Jaundice................................................Yes/No

Fever.....................................................Yes/No

Nausea / Vomiting................................Yes/No

Haematemesis / Malaena......................Yes/No

Dyspnoea..............................................Yes/No

**PHYSICAL FINDINGS (SIGNS) AT RECRUITMENT**

General.

Wasting...........................................Yes/No

Jaundice.........................................Yes/No

Hair changes: axillary/pubic...................Yes/No

Pallor..............................................Yes/No

Pedal oedema..................................Yes/No

Fever...............................................Yes/No

Lymphadenopathy.............................Yes/No

White nails.......................................Yes/No

Specific.

Distended abdominal veins....................Yes/No

Hepatomegally: Span...........cm          tender                    non-tender

Ascites:        present        absent        colour........................

Splenomegally...............................Yes/No          span.............cm

Right upper quadrant tenderness............Yes/No

Other.

Flapping tremors...............................Yes/No

Palmar erythema...............................Yes/No

duputyrens contractures......................Yes/No

 Scratch marks...................................Yes/No

Testicular atrophy..............................Yes/No

Gynaecomastia (males).......................Yes/No

## LABORATORY RESULTS

HBsAg……..positive/negative

HBcAb……..positive/negative

HCV-b……..positive/negative

HIV………..positive/negative

$\alpha$-FP………………………ng/L

HISTOLOGY:

      cirrhosis…present/absent

## APPENDIX 3

## INFORMED CONSENT

This is to confirm that I have agreed to participate in the research "The prevalence of

hepatitis B, C and HIV markers, and alpha-fetoprotein levels in patients with primary

hepatocellular carcinoma at Kenyatta national hospital, Nairobi Kenya".

.   As explained to me, this will involve undergoing tests for HIV, HBsAg, HBcAb, HCV-

antibody, alpha-fetoproteins, liver biopsy, liver function tests, full haemogram, PTI tests and

abdominal ultrasound.   I still have the right to decline participation before or after

commencement of the study, and in so doing my treatment will not be compromised.   While

the results remain a confidential property of the investigator, significant findings that may

influence further management of my condition may be made available to me.


Patient's Name..................................................................

Signed.............................   Patient / Guardian if under 18 years

Date ...............................

Witness ......................................................................

Name ........................................................................

Date............................................................................

ν

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# APPENDIX 4: HEPATITIS B SURFACE ANTIGEN TEST
## PRINCIPLE
Enzygnost HBsAg 5.0 is an enzyme immunoassay based on a two-step method. In the first step, the HBsAg contained in the sample reacts simultaneously with the polyclonal anti-HBs antibodies attached to the wells of the microtitration plate and with the Conjugate 1(Anti-HBs/Biotin, coloured blue). In the second step, after removing the unbound reactants, Conjugate 2 (Streptavidin/peroxidase (POD), coloured yellow) then reacts with Conjugate 1. After removing the unbound reactants, the enzyme activity of the bound Conjugate 2 is determined (blue colour reaction).

The enzymatic conversion of the chromogen is terminated by the addition of Stopping Solution POD (yellow coloured reaction). The colour intensity is proportional to the concentration of antigen in the sample.

## PROCEDURE
1. The number of wells required is ascertained (=number of samples to be tested plus 5wells for the controls)
2. 100 µl/well of the negative control is pipetted into 3 wells (A1 to C1) and 100 µl/well of the positive control into one well (D1). The test wells are then filled with 100 µl/well of undiluted sample and finally at the end of the series/test plate one well is again filled with 100 µl of positive control. Pipetting must be completed within 30 minutes per test plate.
3. Each well is filled with 25 µl of Conjugate 1(Anti-HBs/Biotin), sealed with foil and, immediately after completing the conjugate dispensing step, placed into the incubator.
4. The test plate is incubated at $37 \pm 1^0$C for $60 \pm 2$ min. and immediately afterwards washed (first washing step).
5. The foil is removed and all wells are aspirated, and then washed 4 times with approximately 0.3 ml of washing solution each time, with a 10-sec soak interval per wash. Immediately after completing the washing cycles Conjugate 2 dispensed so that the wells do not dry out.
6. Each well is filled with 100 µl of Conjugate 2 (Streptavidin/POD), sealed with foil and, immediately after dispensing the conjugate, placed into the incubator.
7. The test plate is incubated at $37 \pm 1^0$C for $30 \pm 2$ min. and then immediately afterwards washed (second washing step).
8. The foil is removed and all wells aspirated, and then washed 4 times with approximately 0.3 ml of wash solution each time, with a 10-sec soak interval per wash. Immediately after completing the washing cycles the substrate is dispensed so that the wells well do not dry out.
9. Each well is filled with 75 µl of the Working Chromogen Solution and the plate sealed with fresh foil.
10. The test plate is incubated at +15 to +25 $^0$C for 30 ±2 min protected from light.
11. The reaction is stopped by removing the foil and adding 75 µl of stopping solution (POD) to each well, keeping to the same timing as in step 9.
12. The test is read at 450 nm within 1 hour.

## APPENDIX 5: IgG ANTI-HBc ASSAY

PRINCIPLE

The immunocomb II IgG anti-HBc test is a solid-phase enzyme immunoassay,based on an immmunocapture principle. The solid phase is a comb with 12 projections (teeth). Each tooth is sensitized with goat antibodies to human IgG (lower spot), and rabbit anti-HBc (upper spot), which acts as the internal control. The developing plate has 6 rows (A-F) of 12 wells, each row containing a reagent solution ready for use at a different step in the assay. The test is performed stepwise by moving the comb from row to row, with incubation at each step.

PROCEDURE

1. Serum is pre-diluted 1:50, added to diluent in the wells of row A, mixed, and incubated for 1 hour at $37^0$C. IgG will be captured by the anti-IgG antibodies on the teeth.

2. The comb is inserted into the rows of row B that contains HbcAg that reacts with any bound IgG anti-HBc.

3. The comb is inserted in row C, mixed and incubated for 20 minutes at $37^0$C. The bound HBcAg will react with rabbit anti-HBc antibody labeled with alkaline phosphatase.

4. The comb is inserted into the wells of row D, and agitated for 2 minutes.

5. The comb is similarly washed for 2 minutes in the wells of row E.

6. The comb is inserted into row F wells, which contain chromogenic components, which react with the bound alkaline phosphatase, and left for 10 minutes.

7. The reaction is stopped by re-inserting the comb into row E, and incubating for 1 minute. The comb is then dried in air.

8. Results are read by comparing the intensity of the lower spot of each specimen tooth with that of the lower spot of the control tooth; a positive test produces a spot with an intensity equal to or higher than that of the positive control whereas negative tests will produce no spot or one with an intensity lower than that of the positive control.

## APPENDIX 7: HIV TEST

PRINCIPLE

The innotest HIV-1/HIV-2 test is an enzyme immunoassay. Wells coated with synthetic peptides representing envelope antigens of HIV-1 and HIV-2 are used. Test sera are incubated in the wells. Viral specific antibodies to HIV-1 and HIV-2 if present will bind to the solid phase antigens. Subsequently an affinity-purified rabbit anti-human IgG labeled with the enzyme horseradish peroxidase is added. Upon a positive reaction this labeled antibody becomes bound to any solid phase antigen-antibody complex previously formed. Incubation with enzyme substrate produces a blue colour, which turns yellow when the reaction is stopped with sulphuric acid.

PROCEDURE

1. A 1:20 dilution of test serum is made by adding diluent to serum and mixing.
2. The wells are incubated at $37^0$C for 30 minutes to (antibody capture)
3. Each well is washed 5 times with wash solution.
4. Conjugate (containing enzyme labeled anti-human IgG) is added to each well, mixed and incubated at $37^0$C for 30 minutes.
5. Each well is washed 5 times with wash solution.
6. Substrate is added and the wells incubated for 30 minutes at $20\text{-}25^0$C.
7. The reaction is stopped by adding 1-2 mol/L sulphuric acid and mixing thoroughly.
8. The absorbance of the solution in the wells is read at 450nm.
9. The cut-off value is calculated by adding the mean absorbance of the negative and positive controls, and then dividing by 6. a sample is positive or negative if its absorbance is greater or less than the cut-off value respectively.

## APPENDIX 8: ALPHA-FETOPROTEIN ASSAY (AFP)

PRINCIPLE

DRG AFP ELISA KIT is solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle. The microtiter wells are coated with a monoclonal antibody directed towards a unique antigenic site on a AFP molecule. An aliquot of patient sample containing endogenous AFP is incubated in the coated well with enzyme conjugate, which is an anti-AFP antiserum conjugated with horseradish peroxidase. After incubation the unbound conjugate is washed off with water. The amount of bound peroxidase is proportional to the concentration of AFP in the sample. Having added the substrate solution, the intensity of colour developed is proportional to the concentration of AFP in the patient sample.

PROCEDURE

1. The desired number of microtiter wells are secured in the holder.

2. 25 µl AFP standards (0; 10; 40; 80; 160 IU/ml), controls and serum specimen are dispensed with new disposable tips into appropriate wells.

3. 100 µl of anti-AFP enzyme conjugate is dispensed into each well.

4. The wells are thoroughly mixed for 10 seconds.

5. The wells are incubated for 30 minutes at room temperature.

6. The contents of the wells are then shaken out briskly.

7. The wells are then rinsed 5 times with running tap water.

8. The wells are then struck sharply on absorbent paper to remove residual water droplets.

9. 100 µl of the substrate solution is then added to each well.

10. The wells are now incubated for 10 minutes at room temperature.

11. The enzymatic reaction is stopped by adding 50 µl of stop solution to each well.

12. The optical density is read at 450±10 nm with a microtiterplate reader.

13. Using computer capability the corresponding concetration of AFP is determined in ng/ml.

## APPENDIX 9: STANDARD HAEMATOXYLIN AND EOSIN STAINING

**Method**

1. Sections are dewaxed and hydrated through graded alcohols to water

2. Staining is done in Ehrlich's haematoxylin for 20-45 min.

3. Sections are washed well in running tap water until they "blue" for 5 minutes or less

4. Differentiation is done in 1% acid alcohol (1% HCl in 70% alcohol) for 5-10 seconds

5. Sections are washed well in tap water until they are again "blue" for 5 min. or less

6. Staining is done in 1% eosin for 10min.

7. Sections are washed in running tap water for 1-5min.

8. Finally the sections are dehydrated through alcohol, cleared and mounted.

**Results**

Nuclei----------------------------------------------blue/black

Cytoplasm--------------------------------------varying shades of pink

Fibrin-----------------------------------------------deep pink

Muscle fibers-----------------------------------deep pink/red

Red blood cells---------------------------------orange/red

# APPENDIX 10: MASSONS TRICHROME STAINING TECHNIQUE

Solutions

      a. Acid fuschin

      b. Phosphomolybdic acid

      c. Methyl blue

Method

1. Sections are dewaxed and brought to water.
2. Mercury pigment is removed by iodine, thiosulphate sequence
3. Sections are washed in tap water.
4. Nuclei are stained by the celestin blue-haemalum method.
5. Sections are differentiated with 1% acid alcohol.
6. Washing is done in tap water.
7. Sections are stained in acid fuschin solution a, for 5 minutes.
8. Rinsing is done in distilled water.
9. Treatment is done with phosphomolybdic acid solution b, for 5 minutes.
10. The sections are then drained.
11. Staining with methylene blue solution c is done for 2-5 minutes.
12. The sections are then rinsed in distilled water.
13. Treatment is done with 1% acetic acid for 2 minutes.
14. Sections are dehydrated through alcohols.
15. Finally the sections are cleared in xylene and mounted in DPX.

## <u>Results</u>

Nuclei------------------------------------------------------------blue/black

Cytoplasm, muscle and erythrocytes------------------red

Collagen---------------------------------------------------------blue