

EVALUATION OF TH1 AND TH2 CYTOKINE PATTERNS AND THEIR DIAGNOSTIC ACCURACIES IN HEPATITIS B INFECTION

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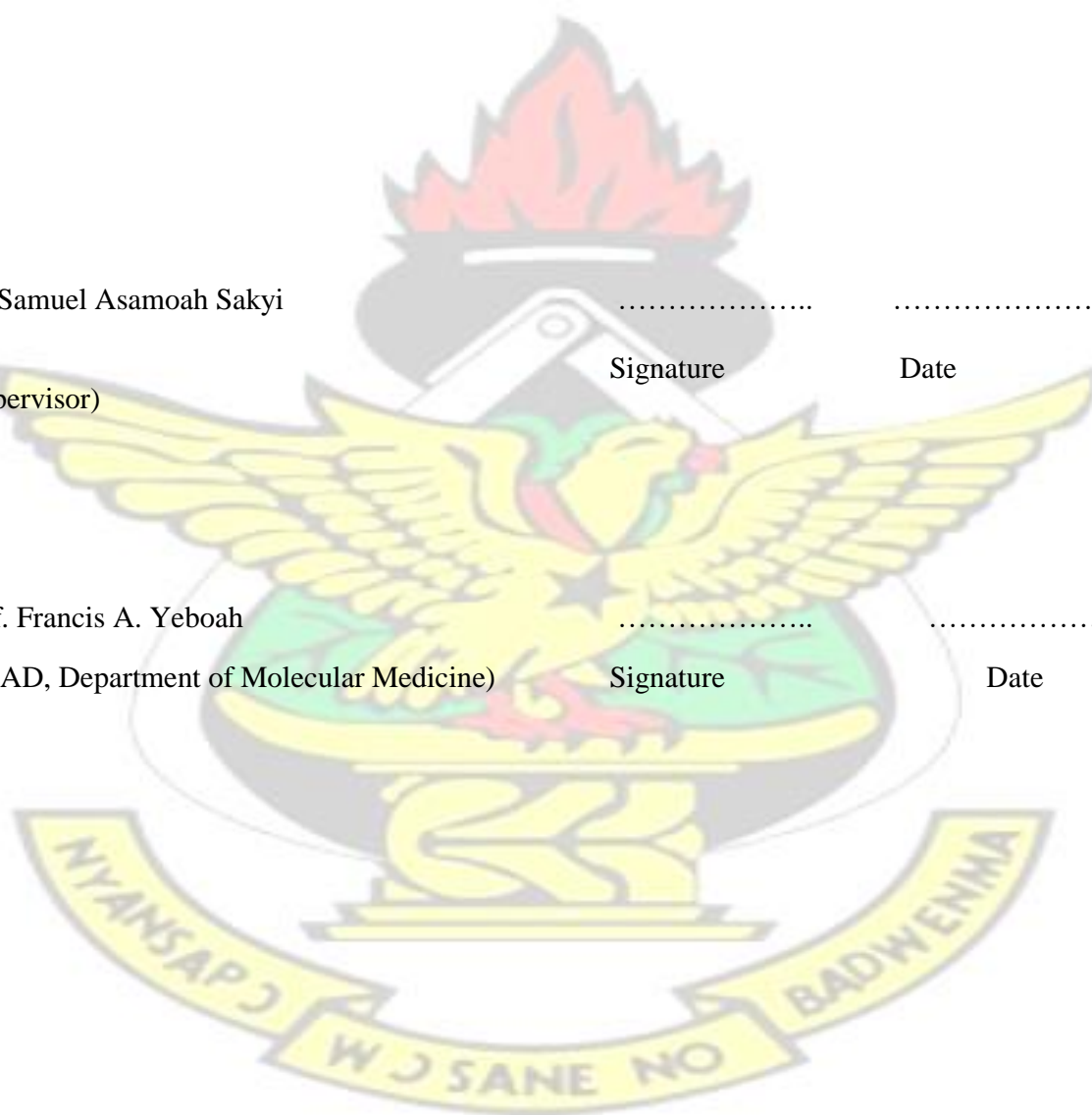
DECLARATION

The experimental work described in this thesis was carried out at the Department of Molecular Medicine, KNUST. This work has not been submitted for any other degree and the contents of thesis are mine.

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ABSTRACT

Background: The host immune response to hepatitis B viral (HBV) infection affects the clinical outcome of HBV infection and cytokines have been shown to directly inhibit HBV replication. The type of T helper (Th) cytokine which predominates at the onset of HBV infection is likely to determine the clinical outcome of HBV infection. Various liver diseases are associated with HBV infection but the percentage of HBV patients with HBV associated liver disease is not well known among Ghanaians. This study sought to define the disease pattern of Th1, Th2 and immunosuppressive cytokines, their diagnostic accuracy in classifying various stage of HBV infection as well as describing its associated liver disease.

Method: In a case-control study, patients with HBV infections and healthy blood donors were screened for HBV serological markers, HCV and HIV. Those positive with HCV and HIV were excluded. Finally, 120 HBV infected patients and 105 healthy blood donors were recruited as cases and control respectively. Th1 cytokines (IL-12p70, IFN- γ and TNF- α), Th2 cytokines (IL-4, IL-6) and immune suppressive cytokine (IL-10) were assayed by sandwich enzyme-linked immunosorbent assay. Their diagnostic performance were established by using receiver operating characteristics (ROC) curve and the area under the curve (AUC). Serum liver function tests were also performed. Data was analysed using GraphPad Prism version 6.01 and a P value <0.05 was considered significant for all statistical comparisons. **Results:** The HBV serological profile results gave 3 acute infections, 12 HBV recovery, 5 HBeAg positive chronic hepatitis B (CHB) and 100 HBeAg negative CHB. Median levels of IL-12p70, TNF- α and IFN- γ were elevated in HBV infections compared to controls ($p>0.05$). Median concentration of IL-4 was significantly elevated in HBeAg negative CHB and HBV recovery compared to controls ($p=0.0196$) whilst IL-10 was significantly elevated in HBeAg negative CHB infection ($p=0.0253$). At 95% confidence interval, the best diagnostic markers with AUC, sensitivity and specificity were IL-10 (0.72, 66.67% and 86.67%) for acute infection and 0.66, 67.0% and 60.0% for HBeAg negative CHB respectively, IL-4 (0.72, 66.67% and 84.44%) respectively for HBV recovery and IL-6 (0.75, 80.0% and 71.1%) respectively for HBeAg positive CHB respectively. Median alanine aminotransferase concentration were significantly elevated in HBeAg positive CHB compared to controls; median albumin concentrations were significantly reduced while median globulin and median direct bilirubin concentrations were significantly elevated in HBeAg negative compared to controls. Median globulin concentrations were also significantly elevated in HBV recovery compared to controls. 30% of cases had elevated aspartate aminotransferase (AST), 23.3% had elevated ALT whilst 5.8% had combined elevated AST, ALT, alkaline phosphatase (ALP) and γ -glutamyl transferase (GGT). Nine cases of non-alcoholic fatty liver disease (NAFLD), two intrahepatic cholestasis and one case of extrahepatic cholestasis were identified.

Conclusion: HBeAg negative was the most common form of HBV infection among the adult population in Ghana. Hepatic injury was common among HBV patients although no severe form of liver disease was found among them. The Th2 cytokines (IL-4, IL-6) and immune suppressive cytokine (IL-10) are better diagnostic markers for classifying the various stages of HBV infections. IL-4 is associated with both HBV recovery and HBeAg negative CHB whilst increased IL-10 is associated with HBeAg negative CHB. This could provide useful information to improve the clinical management of HBV infected patients based on the diagnostic value of well-defined cytokine profiles.

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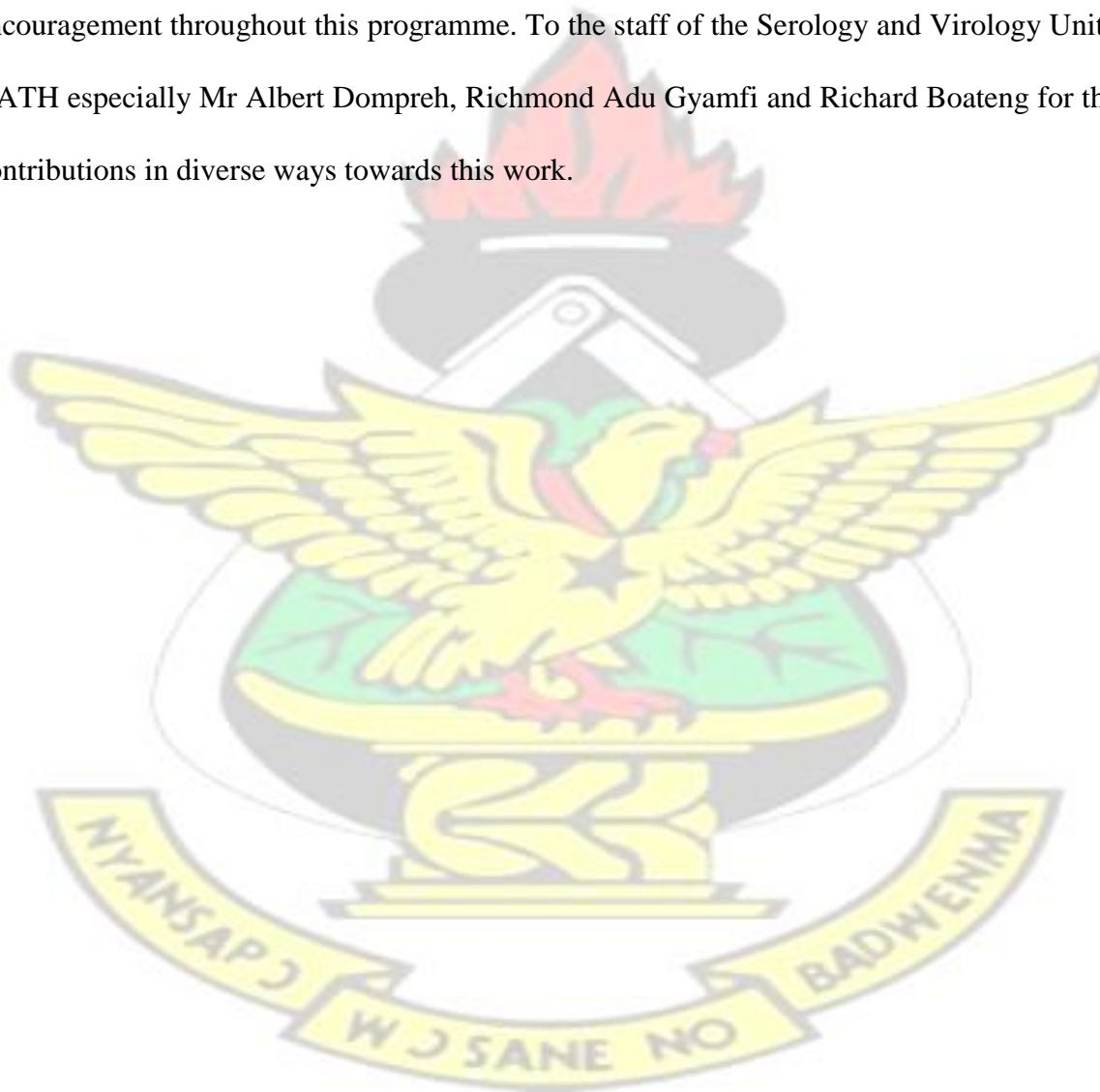
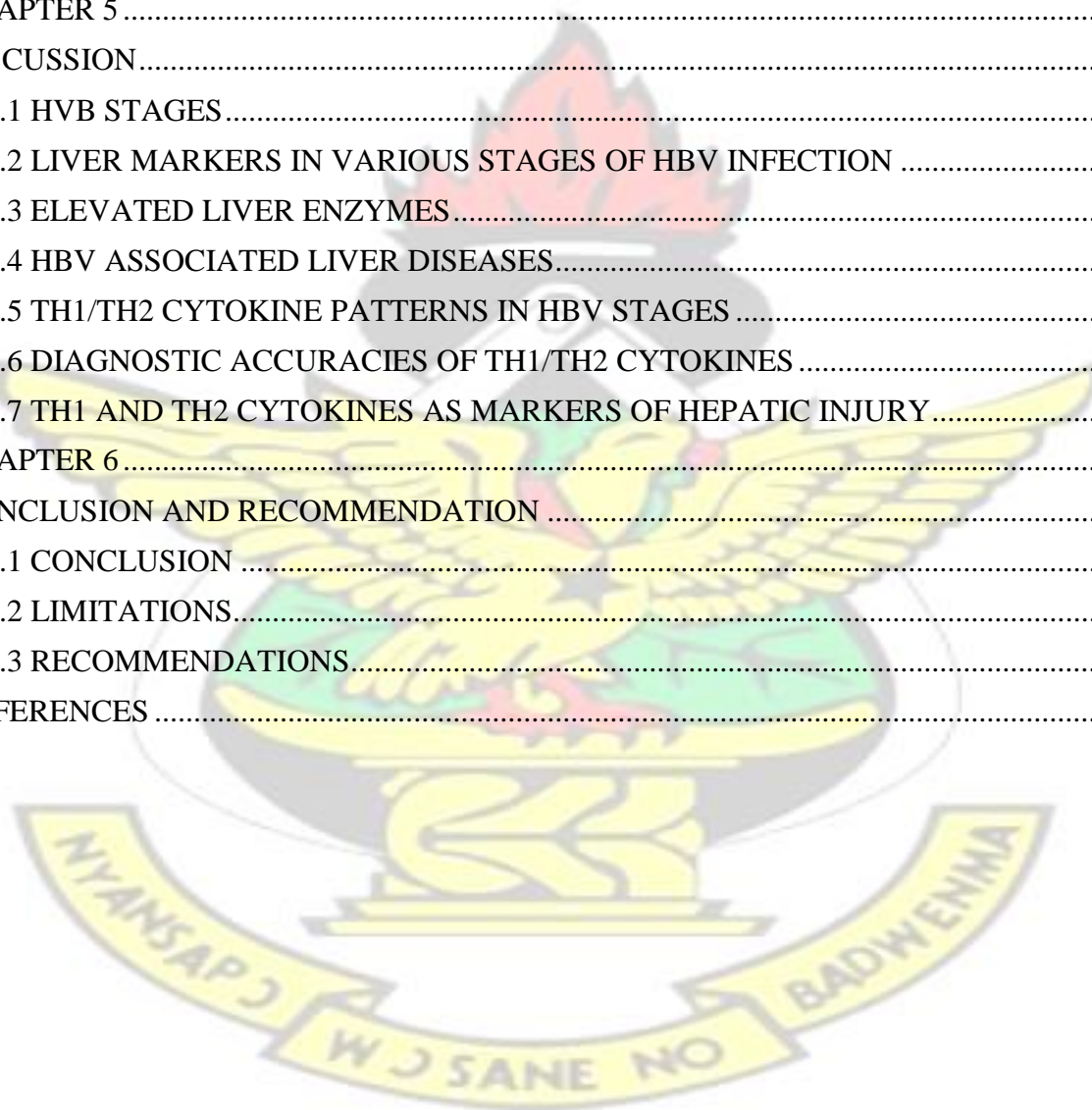


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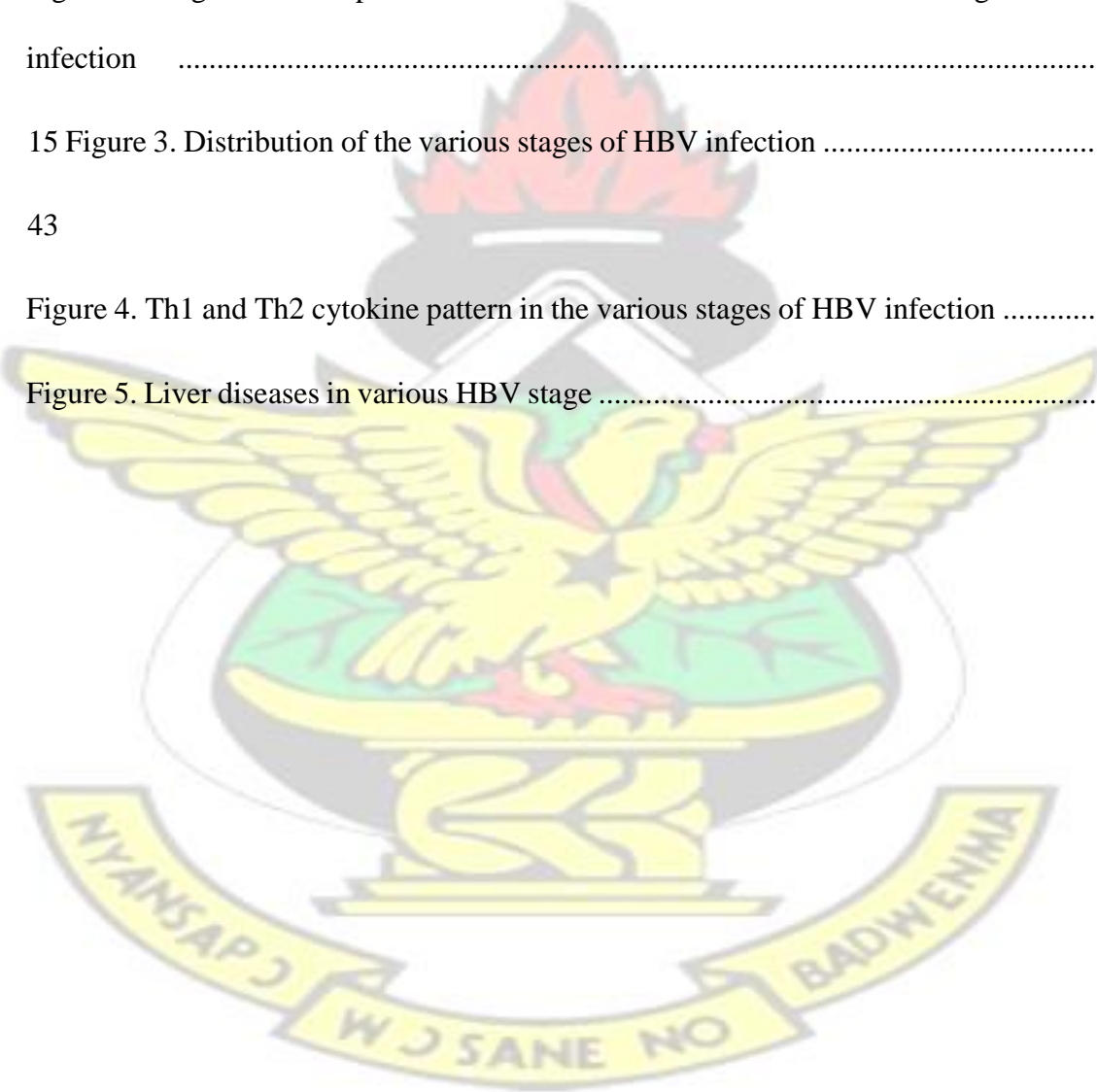
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ABBREVIATIONS

| | |
|-----------|--------------------------------------------------|
| ALP | Alkaline phosphatase |
| ALT | Alanine aminotransferase |
| APCs | Antigen presenting cells |
| AST | Aspartate aminotransferase |
| AUC | Area under the curve |
| CHB | Chronic hepatitis B |
| CI | Confidence interval |
| CSIF | Cytokine synthesis inhibitory factor |
| CTL | Cytotoxic T lymphocyte |
| DC | Dendritic cell |
| ESRD | End-stage renal disease |
| GGT | γ -glutamyl transferase |
| GM-CSF | Granulocyte–macrophage colony-stimulating factor |
| Gp130 | Glycoprotein 130 |
| HBcAb | Antibody to core antigen |
| HBcAb-IgM | Immunoglobulin M of HBcAb |
| HBcAg | Hepatitis B core antigen |
| HBeAb | Antibody to e antigen |
| HBeAg | Hepatitis B e antigen |
| HBIG | Hepatitis B immune globulin |
| HBsAb | Antibody to surface antigen |

| | |
|----------------|----------------------------------------------------------------|
| HBsAg | Hepatitis B surface antigen |
| HBV | Hepatitis B virus |
| HCC | Hepatocellular carcinoma |
| HCV | Hepatitis C virus |
| HIV | Human immunodeficiency virus |
| IFNGR | Interferon gamma receptor |
| IFN- γ | Interferon gamma |
| IL-10 | Interleukin-10 |
| IL-12p70 | Interleukin-12p70 |
| IL-4 | Interleukin-4 |
| IL-6 | Interleukin-6 |
| JAK | Janus associated kinase |
| LFT | Liver function test |
| M-CSF | Macrophage colony stimulating factor |
| MHC | Major Histocompatibility Complex |
| NAFLD | Non-alcoholic fatty liver disease |
| NF- κ B | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NK | Natural killer |
| NKSF | Natural killer cell stimulating factor |
| NO | Nitric oxide |
| PCR | Polymerase chain reaction |
| PD1 | Programme cell death 1 |
| ROC | Receiver operating characteristics |
| RT-PCR | Real time polymerase chain reaction |
| SOCS | Suppressor of cytokine signalling |
| STAT | Signal transducer and activator of transcription |

TACE

TNF-converting factor

Th

T helper

TLR2

Toll-like receptor 2

TNF- α

Tumour necrosis factor-alpha

Tyk2

Tyrosine

kinase

KNUST



CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Hepatitis B Viral (HBV) infection is a public health problem due to its high morbidity and mortality. Globally, about two billion people are infected with HBV of which 350 million develop chronic HBV infection (Merrill and Hunter, 2011). An estimated one million people die yearly from HBV associated complication such as cirrhosis and hepatocellular carcinoma (Lin and Kirchner, 2004). Moreover, 73% of all liver cancers and liver cirrhosis have been linked with HBV infection (Shepard *et al.*, 2006). The highest prevalence of HBV is found in Sub-Saharan Africa and East Asia regions with 5 to 10% of the adult population being infected (Ott *et al.*, 2012) while the prevalence in Ghana ranges from 8.68% to 13.8% (Nkrumah *et al.*, 2011; Amidu *et al.*, 2012).

The clinical outcome of HBV infection is strongly linked with the immune response the host mounts against the virus (Khan *et al.*, 2011). It is usually self-limiting in immunocompetent adults whilst it results in chronic infection in neonates (Rehermann and Nascimbeni, 2005; Bertolotti and Gehring, 2006).

Cytokines, soluble short lived proteins secreted by T lymphocytes and other immune and nonimmune cells play essential roles in HBV pathogenesis and the host response pattern (Koziel, 1999). Th1 cytokines such as interleukin-12 (IL-12), tumour necrosis factor-alpha (TNF- α) and interferon-gamma (IFN- γ) have been directly or indirectly shown to inhibit HBV replication and subsequent viral clearance while Th2 cytokines like interleukin-4 (IL-4) and immune suppressive cytokine, interleukin-10 (IL-10) are thought to facilitate viral persistence and development of chronic infection (Falasca *et al.*, 2006).

The diagnosis and staging of HBV in clinical practice is made by the detection of hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (HBsAb), hepatitis B e antigen (HBeAg), hepatitis B e antibody (HBeAb), hepatitis B core antibody (HBcAb), immunoglobulin M isotype of core antibody (HBcAb –IgM) and HBV DNA in the serum of patients (Chevaliez and Pawlotsky, 2008; Bessone, 2014). These markers together with liver biochemical tests as well as liver histology, a patient can be classified to be in an acute or chronic infection state or has resolved HBV infection (Lok and McMahon, 2001; D'Souza and Foster, 2004). Mutations may occur in the HBV genome which may lead to the non-secretion of HBsAg and HBeAg. The use of these viral markers as diagnostic markers may therefore result in discordant results at certain times (Pan and Zhang, 2005; Chevaliez and Pawlotsky, 2008). Measuring HBV DNA would be difficult in resource deprived settings coupled with the fact that obtaining sufficient liver biopsy for histological diagnosis is difficult and an invasive procedure. There is therefore the need for additional diagnostic markers in the work up for classifying the various stages of HBV infections. Cytokines have proven to play key role in HBV associated liver damage (Yan *et al.*, 1999), cancers (Wong *et al.*, 2009) and the treatment response to various anti-viral agents (Hall and Cash, 2012). The disease pattern of Th1 and Th2 cytokines and their ability to identify an acute HBV infection, HBV recovery, HBeAg positive CHB and HBeAg negative CHB has however not been studied in Ghana. In addition, variations in host genetic immune response make it imperative to study the balance between immune suppressive, Th1 and Th2 cytokines among the Ghanaian populace. It is against this background that this study for the first time was conducted to determine the ability of Th1 and Th2 cytokines to identify the various stages of HBV infection and disease pattern of these cytokines in HBV infection as well as its liver associated diseases among the Ghanaian population.

1.2 PROBLEM STATEMENT

HBsAg which is used as a diagnostic marker for HBV is encoded by the preS gene. Mutations may occur in this gene as a result of antiviral therapy, low replicating HBV carriers as well as inhibition of replication by hepatitis delta. This may subsequently lead to the secretion of HBsAg which cannot be detected by HBV kits. Again, HBeAg which is also encoded by the core promoter region in the HBV genome is affected due to mutations which may occur in this region of the HBV genome. Discordant results may be obtained when these viral markers are used in classifying the various stages of HBV infection. Furthermore, the measurement of HBV DNA may not be possible in resource deprived settings. Th1 and Th2 cytokines which are released at various stages of HBV infection may be helpful in identifying the various stages of HBV infection.

Moreover, serum aspartate aminotransferases (AST) and alanine aminotransferase (ALT) which are used to evaluate liver damage in HBV infection are also affected by a number of other conditions including myocardial infarction, skeletal muscle disease, pancreatitis and severe tissue hypoxaemia. Elevation of these enzymes alone do not give a true reflection of liver damage and function in HBV. The release of these liver enzymes is also delayed compared to the release of Th1 and Th2 cytokines. Again, obtaining sufficient liver biopsy for liver histology is difficult and an invasive procedure and hence the quest for alternative noninvasive markers for liver damage in HBV infection.

Furthermore, the already existing medication for the management of HBV have begun to develop resistance and this requires the development of new therapeutic targets as there is still no cure for chronic hepatitis B. Immunotherapy to this end has great potential for the treatment of HBV. Evaluating cytokine production by T helper cells therefore provides a great initial step in developing immune therapies for the treatment of HBV.

1.3 JUSTIFICATION

There is paucity of information on the involvement of Th1 and Th2 cytokines in the pathogenesis of HBV in the Ghanaian population. The use of these cytokines as diagnostic biomarkers have also not been studied in HBV infection in the Ghanaian population. This study seeks to provide such information and also determine their potential diagnostic performance in identifying acute HBV infection, HBV recovery, HBeAg positive and HBeAg negative chronic hepatitis B infections in the Ghanaian population.

Again, although HBV is linked with varying degrees of liver damage, the percentage of HBV patients with HBV associated liver disease is not well known among Ghanaians.

In the management of HBV infection, it is important to monitor the liver disease burden. To this end, early detection of liver injury and damage is of great essence. This study seeks to explore the use of Th1 and Th2 cytokines as surrogate non-invasive markers for early detection of liver damage.

1.4 HYPOTHESIS

Th1 and Th2 cytokines patterns can be used classify various stages of HBV infection

1.5 OBJECTIVE

To evaluate the expression of Th1 and Th2 cytokine patterns and their diagnostic accuracies in acute HBV infection, HBV recovery, HBeAg-positive chronic hepatitis B (CHB) and HBeAgnegative CHB well as its liver associated disease.

1.5.1 Specific Objectives

1. To classify HBV infections into acute, HBV recovery, HBeAg-positive CHB and HBeAg-negative CHB using serological markers.
2. To measure serum levels of Th1 cytokines (TNF- α , IFN- γ and IL-12) and Th2 cytokines (IL-4, IL-6 and IL-10) in the various stages of HBV infection and healthy controls so as to establish the disease pattern and their diagnostic accuracy for identifying the various stages of HBV infection.
3. To determine the burden of HBV infection on the liver by performing liver function test (LFT) among HBV infected individuals and healthy controls. .
4. To evaluate Th1 and Th2 cytokines as markers of hepatic injury or inflammation by comparing with alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) levels.

CHAPTER 2

LITERATURE REVIEW

2.1 GENERAL INTRODUCTION

Hepatitis B virus is a DNA virus in the family of *Hepadnaviridae* viruses (Bertoletti and Gehring, 2006). *Hepadnaviruses* have a high predilection for infecting hepatocytes, although some quantities of HBV DNA can be found in kidney, pancreas and mononuclear cells (Ganem and Prince, 2004). The virus does not cause a direct destruction to liver cells but rather the immune response that the body mounts against the virus causes liver pathology (Ocama *et al.*, 2005).

2.2 TRANSMISSION OF HBV

Transmission of HBV occurs through three main routes; perinatal/vertical, sexual and parenteral routes. The prevalence of the disease within a location greatly influences the route of transmission. In high endemic areas, transmission occurs mainly through perinatal route while in low endemic areas, sexual contact and parenteral are the main routes of transmission (Hou *et al.*, 2005; Pan and Zhang, 2005).

Perinatal transmission occurs mainly from an infected mother to a baby and this can occur through three main ways; through the placenta, during child birth and during child care after birth including breast feeding. Ninety percent (90%) of children who acquire HBV perinatally develop chronic infection (Hou *et al.*, 2005). In Asia, Africa, Pacific Island and the Arctic, this is a major route of transmission (Pan and Zhang, 2005)

Sexual interaction is a global route of transmission but more common in the low endemic areas such as Western countries. Though homosexual men are at high risk of acquiring HBV, heterosexuals with multiple sex partners, those who patronise prostitutes or those with partners

who inject drugs are also at high risk of acquiring HBV infection (Hou *et al.*, 2005; Pan and Zhang, 2005)

The parenteral route of transmission occurs through needle sharing, acupuncture, blood transfusions and dialysis, occupational or health care related, tattooing and household contact. Spread of HBV through sharing contaminated needles accounts for 23% of HBV infection in the United States and Western Europe (Hou *et al.*, 2005; Shepard *et al.*, 2006). Infection by these routes normally occurs in adults and adolescents with less than 5% of these cases resulting in chronic infection (Hou *et al.*, 2005).

HBV is known to be stable in the environment for even up to 7 days and hence can be spread by contaminated surfaces. Although blood and serum have the highest concentration of HBV and possess the highest risk of infection, other body fluids such as semen and saliva can also spread HBV (Shepard *et al.*, 2006; Mkandawire *et al.*, 2013).

2.3 CLINICAL SIGNS AND SYMPTOMS OF HBV INFECTION

The clinical presentation of infected patients differs depending on the stage of the disease and the age of the patient. For example new-borns are normally asymptomatic while a majority of older children and adults may develop symptoms. Among the signs of acute HBV are fever, malaise, vomiting, anorexia, nausea, jaundice, dark urine, pale stools and abdominal pain (Mahoney, 1999). In few situations, affected persons may develop skin rashes, arthralgias, and arthritis. Persons with chronic infections are often asymptomatic until later in the course of the disease where fibrosis, cirrhosis and ultimately hepatocellular carcinoma may develop (Mahoney, 1999).

2.4 DIAGNOSIS AND MONITORING OF HBV INFECTION

The diagnosis of HBV in clinical practice is mostly made by the detection of HBsAg, HBsAb, HBeAg, HBeAb and HBcAb, HBcAb -IgM and HBV DNA in the serum of patients (Chevaliez and Pawlotsky, 2008; Bessone, 2014). With these markers together with biochemical test as well as liver histology, a patient can be classified to be in an acute or chronic infection state or has resolved HBV infection (Lok and McMahon, 2001; D'Souza and Foster, 2004).

A person is considered unexposed and susceptible if they test HBsAg-negative, anti-HBs negative, and HBcAb-negative while a person is said to be protected as a result of vaccination, if they test HBsAg-negative, HBsAb-positive, and HBcAb-negative. One is said to be acutely infected if they tested HBsAg-positive, HBsAb-negative, HBcAb-positive and IgM-HBcAb positive. Chronic infection is characterized by the persistence of a positive HBsAg for more than 6 months, serum HBV DNA greater than 10^5 copies/mL and persistent or sporadic elevation in ALT or AST concentrations. Resolution of HBV infection is characterized by HBsAg-negative, HBsAb-positive, and HBcAb-positive, undetectable serum HBV DNA and normal ALT levels (Mahoney, 1999; Lok and McMahon, 2001; Stadler *et al.*, 2008).

Sandwich enzyme immunoassays (EIAs) and automated chemiluminiscent microparticle immunoassays have made it possible for the detection and quantification of HBV antigens and antibodies (Chevaliez and Pawlotsky, 2008).

HBsAg can be detected as early as 6 to 10 weeks after a person has been exposed to the virus even before the person shows symptoms of the disease. Though HBsAg is a very sensitive marker for HBV, it may not be detected in a person who has resolved or responded to antiviral therapy, in low replicating HBV carriers as well as inhibition of replication by hepatitis delta and in mutations in the S gene of HBV leading to the secretion of HBsAg which cannot be detected by HBV kits (Chevaliez and Pawlotsky, 2008).

HBsAb is present in persons who have recovered from HBV infection or have been vaccinated with hepatitis B vaccine. HBcAb comes in two types, the IgG and the IgM types and it is present even in low replicating and asymptomatic patients. HBeAg can be detected at 6 to 12 weeks post exposure to the virus. Its presence is an indication of high replication and infectivity. Seroconversion of HBeAg is an indication of good prognosis and it is normally followed with the appearance of HBeAb (Chevaliez and Pawlotsky, 2008).

HBV DNA levels in the serum can also be measured by the use conventional polymerase chain reaction (PCR) although real-time polymerase chain reaction (RT-PCR) is gradually replacing the classical technique. These assays are used in determining HBV viral load to help determine patients in need of therapy and in the monitoring of treatment. DNA hybridization assays may also be employed in detecting drug resistant variants and mutations that may result from treatment (Sablon and Shapiro, 2005; Chevaliez and Pawlotsky, 2008).

There are various assays available for the detection of these markers including immunoassays, polymerase chain reaction (PCR) amplification techniques, *in situ* hybridization and immunohistochemical techniques. Liver disease markers used in the monitoring of liver disease burden in HBV infection include ALT and AST.

2.4 PREVENTION OF HBV INFECTION

Three main strategies have been adopted to prevent the spread of HBV and they include; behavioural change, passive immunoprophylaxis and active immunization (Hou *et al.*, 2005).

Among the behavioural preventive measures for HBV include screening of blood donors, plasma, organ tissue and semen as well as changes in sexual practices. Inactivating viruses in plasma-

derived products, implementation of infection control measures especially at health care facilities (D'Souza and Foster, 2004; Hou *et al.*, 2005; Shepard *et al.*, 2006).

Passive immunoprophylaxis involves the administering of Hepatitis B Immune Globulin (HBIG). This is normally given to new-borns to HBsAg-positive mothers, after exposure to contaminated needles, after sexual exposure and after liver transplant (Hou *et al.*, 2005).

However, the most effective mode of prevention remains active immunization with hepatitis B vaccine (Hou *et al.*, 2005; Shepard *et al.*, 2006). In fact, there has been a significant drop in the prevalence of HBV especially in countries that have adopted the universal vaccination with HBV (Hahné *et al.*, 2013).

Plasma derived vaccines were the first to be produced but vaccine production using DNA recombinant technology are now available (Hou *et al.*, 2005). Three doses are required to be taken although other doses may be available for certain groups of people by some manufacturers. About 90% of healthy infants, children and adults are able to produce HBsAb titre of greater than 10 mIU/ml and are considered as protected after vaccination (Shepard *et al.*, 2006).

Hepatitis B vaccine is very immunogenic and in countries like the United States, it is not even recommended to test for HBsAb after vaccination (Shepard *et al.*, 2006). Though vaccination induced HBsAb wanes over time, most immunocompetent people remain protected after completing the vaccination schedule for up to ten and even more years (Hou *et al.*, 2005; Shepard *et al.*, 2006). A key challenge to the development of HBV vaccine has been the emergence of strains of HBV that are resistant to HBsAb neutralization as well as mutations in the small surface protein gene (S mutants) (Shepard *et al.*, 2006).

2.5 NATURAL PROGRESSION AND PATHOGENESIS OF HBV INFECTION

Primary HBV infection may lead to either acute or chronic infection and may be associated with clinical symptoms or may be asymptomatic. This outcome is influenced by a number of factors including the age of acquisition of the virus, the immune status, gender and genetic factors. Infections acquired during childhood or during perinatal period normally become chronic while infections in young adults are often acute with subsequent resolution of the infection (Ganem and Prince, 2004; Pan and Zhang, 2005; Rehermann and Nascimbeni, 2005). It is estimated that about 90% to 95% of adults with acute infection resolve or recover from the infection and develop life-long immunity to HBV with the remaining 10% progressing to chronic infection (Lok *et al.*, 2001; Rehermann and Nascimbeni, 2005). Chronic infection may however ensue in about 80% to 90% if the infection occurs during perinatal period or in infants less than a year (Lok *et al.*, 2001).

The pathogenesis of CHB infection may be classified into immune tolerant phase, immune active/clearance phase and inactive carrier phase which could be low replication or high replication based on the viral-host immune system interactions (Pan and Zhang, 2005; Rehermann and Nascimbeni, 2005).

2.5.1 Acute Infection

Annually, approximately 5 million cases of acute hepatitis infection are recorded globally, this incidence however varies from one geographical area to the other (Lok *et al.*, 2001). About 30% of adults with acute HBV present with severe jaundice or icteric hepatitis while about 0.1% to 0.5% develop fulminant hepatitis. A greater majority, up to 95% of adult infections with acute

hepatitis B are self-limiting conferring life-long immunity to such people (Lok *et al.*, 2001; Pan and Zhang, 2005).

HBsAg, a serological marker for HBV infection is detectable in an infected person at about 4 to 10 weeks after exposure while viral DNA can even be detected as early as 1 month when using sensitive polymerase chain reaction assays (Pan and Zhang, 2005; Rehmann and Nascimbeni, 2005). In this stage of infection, HBeAg and IgM isotype of the HBcAb are both positive. Liver injury does not occur at the onset of HBV infection but may take about 10 to 15 weeks before being detected by the rise in alanine aminotransferase (ALT). This represents the time the body takes to initiate an effective T-cell-mediated immune response which in turn causes harm to hepatocytes. So during the early stages of the infection when there is high viremia, there are no signs of liver damage; conversely, there is low viremia during the onset of clinical signs of the disease (Pan and Zhang, 2005; Rehmann and Nascimbeni, 2005).

In a successful viral clearance, HBsAb is produced to neutralize the HBsAg which at this stage become undetectable. Very little HBV DNA may still persist in a person even after complete resolution of the disease and this is kept under check by the body's immune system (Rehmann and Nascimbeni, 2005). Failure to clear the virus result in chronic infection. A diagrammatic representation of the course of acute HBV infection is shown in Figure 1.

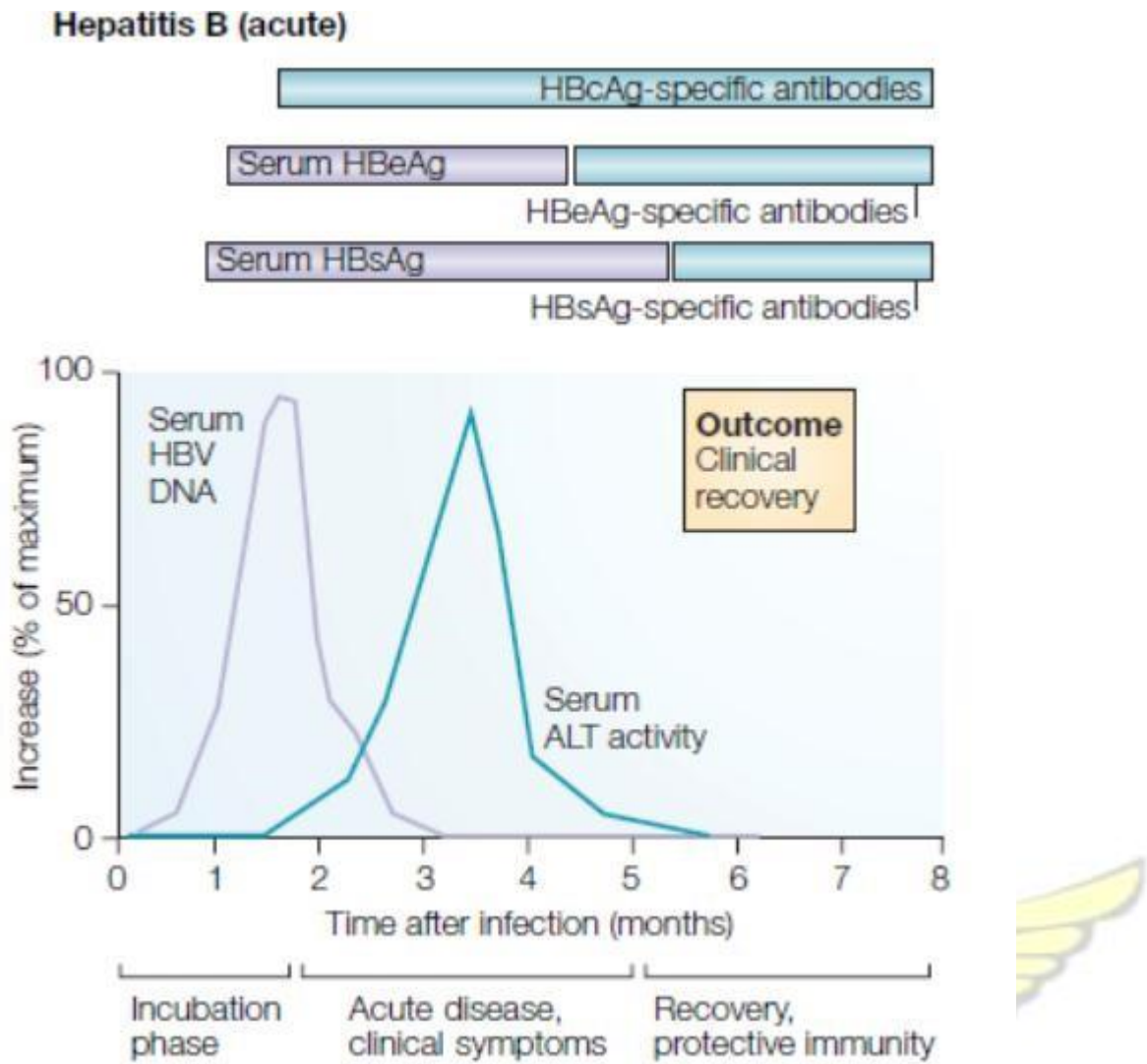


Figure 1. A diagrammatic representation of the course of HBV during the acute stage of the infection.

Adopted from (Rehermann and Nascimbeni, 2005)

2.5.2 Chronic Infection

In chronic HBV infection, HBsAg, HBeAg and high viral DNA are present for more than six months (Pan and Zhang, 2005). Globally, about 350 million people have developed chronic hepatitis B infection (Ott *et al.*, 2012). People who are exposed to the virus at an early age are more likely to develop chronic infection as compared to those who get exposed at a later age.

Ninety five percent (95%) of children do progress to chronic infection while about 3 to 5% of adults progress to chronic infection after primary exposure (Pan and Zhang, 2005).

Chronic infection often evolves through an immune tolerant phase, immune active/clearance phase and inactive carrier phase which could be associated with low or high replication. This is based on the interaction between hepatitis B virus and the host immune system (Pan and Zhang, 2005; Rehermann and Nascimbeni, 2005). To begin, the immune tolerant phase presents with HBeAg positive, a high viral DNA titre and ALT values which are normal. ALT levels are normal at this early stage of the infection due to the time taken by the body to elicit an effective T-cell mediated response.

The immune tolerant phase may then progress to the immune active phase which is characterised by HBeAg positivity, low viral DNA titre and high ALT values indicating a more severe liver disease. By this phase of the infection, the body would have elicited a more effective T-cell mediated immune response to clear the virus. It is this immune response to the virus which results in liver damage. By the end of this phase, there is a seroclearance of HBeAg and production of HBeAb. The immune active phase may progress to liver cirrhosis which is a more severe form of liver damage or to the inactive carrier phase.

The inactive phase is characterized by HBeAg negativity and HBeAb positivity and could be associated either with low or high replication. In the low replication phase, viral DNA titres are normally very low and ALT levels are normal. In immune-competent patients, this phase may last for life but occasionally, reactivation may occur in immunosuppressed patients and patients on cytotoxic chemotherapy. This results in high viral replication, high ALT levels and occasionally HBeAg positivity (Pan and Zhang, 2005; Rehermann and Nascimbeni, 2005). Thus, HBeAg positive chronic hepatitis B infection coincides with the immune tolerant phase and early immune active phase of the natural progression of the disease while HBeAg negative hepatitis B

infection coincides with the final outcome of immune active phase and the inactive carrier phase of the disease.

It must be said that in some population of HBeAg negative chronic hepatitis B patients, there is a mutation in the precore or core promoter region and this subsequently leads to the loss of production of HBeAg (Pan and Zhang, 2005). CHB patients have an increased risk of developing liver cirrhosis and HCC (Mahoney, 1999). The progression of chronic HBV infection is depicted in Figure 2 below;

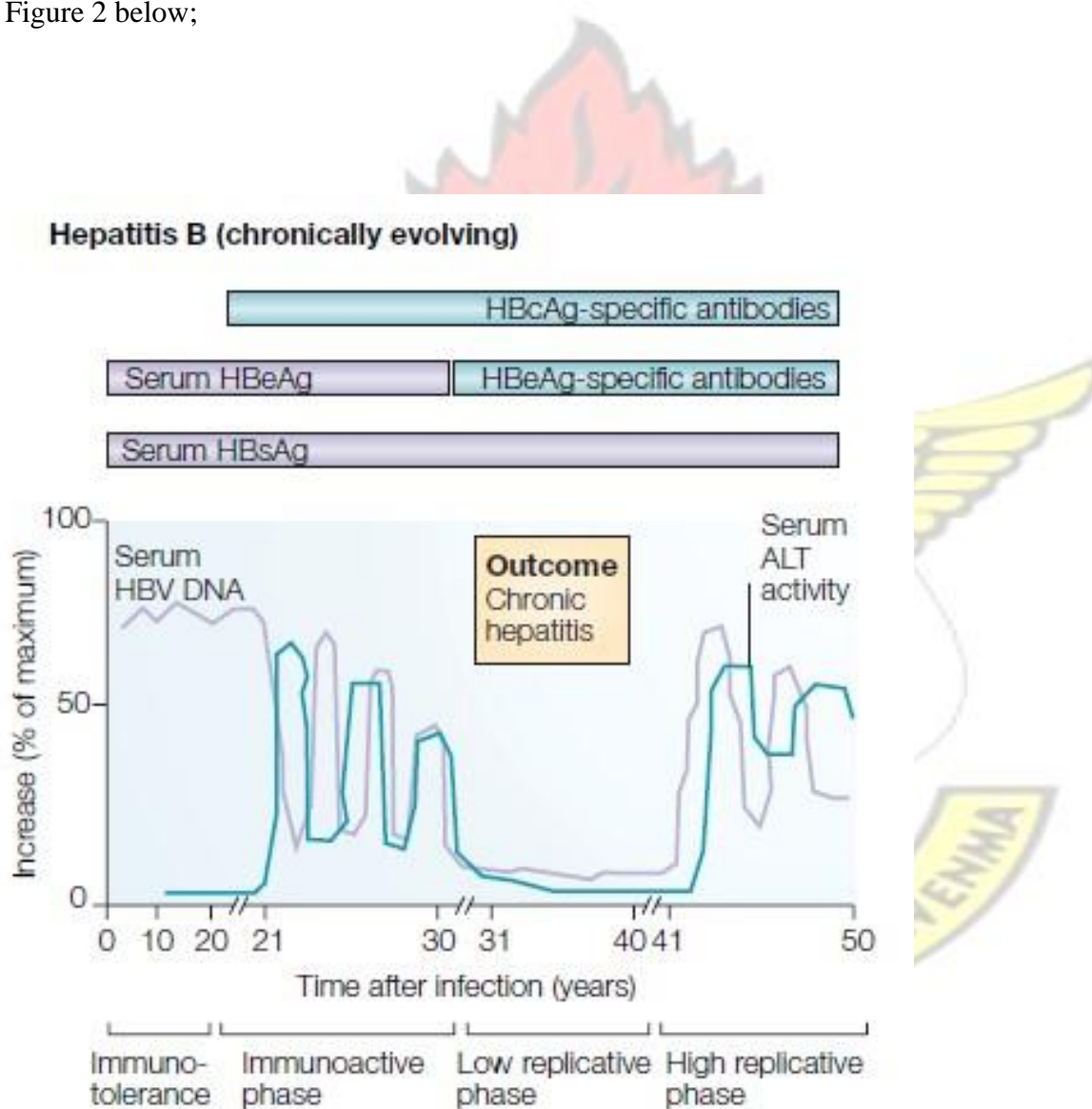


Figure 2. Diagrammatic representation of the course of HBV infection during chronic infection

Adopted from (Rehermann and Nascimbeni, 2005)

2.6 LIVER DISEASES ASSOCIATED WITH HBV INFECTION

2.6.1 Cirrhosis

One of the complications of chronic hepatitis B infection is cirrhosis. Cirrhosis is liver damage which is characterized by the replacement of the normal liver tissue with fibrous or scarred tissue and thus affecting the normal functioning of the liver. Cirrhosis can be grouped into two categories; compensated and decompensated cirrhosis. Compensated cirrhosis is marked by less severe scarring of liver and the liver is still able to maintain some of its functionalities. This is further sub divided into two based on the presence or absence of varice. Decompensated cirrhosis is marked by extensive scarring of the liver with loss of liver function leading to ascites, variceal hemorrhage, hepatic encephalopathy, jaundice and coagulopathy (Schuppan and Afdhal, 2008; Garcia-Tsao *et al.*, 2010). Five percent (5%) of HBV related deaths are as a result of cirrhosis. Though cirrhosis in HBeAg positive patients is high, much higher rates have been reported in HBeAg negative patients (Pan and Zhang, 2005). The genotype of HBV may also play a role in the risk of developing cirrhosis. As reported in an Asian study, genotype C was associated with much higher risk compare to genotype B (Pan and Zhang, 2005). Liver cirrhosis is an important risk factor for developing hepatocellular carcinoma.

2.6.2 Hepatocellular Carcinoma (HCC)

Hepatocellular carcinomas are a leading cause of death worldwide especially in persons with chronic hepatitis B and liver cirrhosis (Dunn *et al.*, 2007). It is responsible for one-third of all cancer related deaths and happens to be the fifth most diagnosed cancer worldwide (Sitia *et al.*, 2012). HBV infection has been linked with 73% of all liver cancers and liver cirrhosis (Ott *et al.*, 2012). There is a strong link between HBV infection and HCC. Indeed chronic carriers are 100 times more at risk of developing HCC compared to non-carriers while the presence of

HBsAg also puts an individual at 10 times increased risk of developing HCC. HBeAg positive individuals are 60 times more at risk of HCC. Though cirrhosis precedes most cases of HCC, HCC can also develop independent of cirrhosis (Pan and Zhang, 2005).

2.7 IMMUNE RESPONSE TO HBV INFECTION

2.7.1 Innate immune response

Generally, infection with viruses induce the production of cytokines consisting mainly of interferons (IFN's), which eventually inhibits the viral replication. Despite immune evasion, HBV does elicit some immune responses particularly innate immune responses during a primary HBV infection (Chisari *et al.*, 2010). For example, in primates like chimpanzees, HBV DNA clearance has been observed even before the induction of the adaptive immune system and thus supporting the important role the innate immune system may be playing in the fight against HBV infection (Rehermann and Nascimbeni, 2005). As part of the innate response, interferon- α (IFN- α) and interferon- β (IFN- β) are known to have the capacity to inhibit the formation of viral capsid and even to degrade the already formed ones. IFN- α/β are also known to mediate the recruitment of antigen presenting cells (APCs) such and Kupffer cells and dendritic cells (DC). These APCs are able to produce some cytokines including interleukin-18 (IL-18) and interferons which stimulate natural killer (NK) and natural killer T (NKT). The NK cells, NKT cells and Kupffer cells produce another cytokine, interferon- γ (IFN- γ) which is able to inhibit HBV replication in HBV transgenic mice independent of CD4⁺ and CD8⁺ T cell activity (Rehermann and Nascimbeni, 2005; Chang and Lewin, 2006). Kupffer cells play an essential role in linking the activities of the innate and adaptive immune systems by producing cytokines and chemokines including IFN- γ , CXCL9, CXCL10 all of which are involved in recruitment and growth of HBV specific T cells (Chang and Lewin, 2006).

2.7.2 Adaptive immune response

The cellular and humoral components of the adaptive immune response are crucial in the pathogenesis of HBV. APCs, mainly Kupffer cells and dendritic cells which are recruited during the innate response to HBV infection presents antigen (HBV) to CD4⁺ and CD8⁺ T cell which in turn secrete cytokines such as IL-12 and TNF- α . These cytokines subsequently stimulate the secretion of IFN- γ and expansion CD8⁺ T cells (Chang and Lewin, 2006).

2.7.2.1 Cellular response

Activated CD4⁺ T cells produce a vigorous, multi-specific response against HBcAg, HBeAg and other HBV gene products including envelope and polymerase following presentation with HBV viral particles. HBV-specific CD4⁺ T-cells activity is further linked with the fight against HBV as its numbers increase almost the same period as there is a rise in HBV DNA (Chang and Lewin, 2006).

Despite the important task played by CD4⁺ T cells in the clearance of HBV, CD8⁺ T cells are recognised as the main effector cells for the clearance of HBV infection (Thimme *et al.*, 2003). In an experiment to demonstrate the role CD4⁺ and CD8⁺ T cells in HBV clearance during an acute infection in chimpanzee, depletion of CD8⁺ T cells were found to lead to persistent infection unlike depletion of CD4⁺ T cells. Hence it was concluded that CD8⁺ T cells were the main T cell subset responsible for viral clearance during the course of acute HBV infection and it does that through cytolytic and non-cytolytic mechanism (Thimme *et al.*, 2003). The noncytolytic mode of viral clearance was mediated by IFN- γ and TNF- α as has been shown in human infections as well. In this situation, clearance of HBV after an acute episode was not associated with clinical symptoms and no elevation of alanine aminotransferase (ALT) (Chang and Lewin, 2006). The non-cytolytic activity of IFN- γ and TNF- α was carried out by destabilizing HBV capsid through the NF- κ B pathway, degrading various viral proteins through

the nitric oxide and proteasome activity and finally by post-transcriptional degradation of HBV mRNA (Chang and Lewin, 2006). The administration of anti-IFN- γ and anti-TNF- α terminated HBV clearance by CD8⁺ T cells thus confirming the role of IFN- γ and TNF- α in the clearance of HBV by non-cytolytic mechanism (Chang and Lewin, 2006). Cytotoxic T lymphocytes (CTLs) are able to recognise a wide range of epitopes on HBV due to the polyclonal and multi-specific nature of CTLs response during acute infections.

2.7.2.2 Humoral response

The humoral immune response play a key role in viral clearance especially with respect to free circulating viral particles in the body (Chisari *et al.*, 2010). The presence of HBV antibodies including HBsAb, HBcAb and HBeAb may give us an indication of the stage of the disease as well as the prognosis. For example, the presence of IgM to HBcAb gives an indication of an acute infection while the presence of HBsAb together with HbcAb is an indication of recovery from acute infection (Rehermann and Nascimbeni, 2005). HBsAb has a protective ability and thus provides immunity against subsequent infection but the protective ability of HBcAb and that of HBeAb has remained controversial despite reports in chimpanzees to that effect (Chang and Lewin, 2006).

2.8 MECHANISM OF HBV PERSISTENCE/CHRONIC INFECTION

2.8.1 Suppression of the immune system

The failure of the immune system to clear HBV after a primary infection may lead to chronic infection and subsequent development cirrhosis or hepatocellular carcinoma. A wide range of factors have been attributed to the development of persistent infection. Suppression of the immune system by HBV happens to be a major factor in the development of chronic infection

(Chang and Lewin, 2006; Chisari *et al.*, 2010). This occurs by the induction of a reduced CD4+ T-cell activity which eventually leads to an ineffective and insufficient CD8+ T cell response (Chisari *et al.*, 2010). The reduction in CD4+ T cell activity affects the ability of some APCs such as dendritic cells to produce certain cytokines including TNF- α , IFN- γ and IL-12 which are important in viral inhibition and degradation (Chang and Lewin, 2006). The reduced production of these cytokines also leads to reduction in the expansion and proliferation of CD8+ T cells which are required for viral clearance. The reduced CD8+ T cells also have a reduced capacity to further produce more of anti-viral cytokine, IFN- γ for viral clearance. Some of the HBV-specific CD8+ T cells may also develop immunological tolerance to HBV; that is they fail to recognise and bind HBV leading to failure to clear the virus (Chang and Lewin, 2006; Chisari *et al.*, 2010).

The expression of programmed cell death protein 1 (PD 1) of T cells have also been observed to suppress the effector function of T cells in the fight against HBV. Though T regulatory cells (T regs) have been shown to suppress CD8+ T cells and the production of antiviral cytokines in certain diseases like HIV and HCV, its role in HBV still remain unclear (Chang and Lewin, 2006).

2.8.2 Viral factors

HBx protein of the HBV is able to suppress immune response as well as antigen presentation during the course of HBV infection through the NF κ B pathway. They also have the ability to increase the expression of HLA 1 on liver cells with resultant recruitment of inflammatory cell causing damage to the liver and subsequently reducing its ability to clear the HBV virus (Chang and Lewin, 2006).

HBeAg has been linked the reduction of HBeAg- and HBcAg-specific CD4+ T-cells via Fas-mediated apoptosis and have also been shown to reduce the expression of toll-like receptor 2

(TLR 2) on monocytes. This effect leads to the reduce ability of dendritic cells to prime T cells and the production of important cytokines including IL-12, IFN- γ and TNF- α and thus leading to viral persistence (Chang and Lewin, 2006). Another viral factor affecting the outcome of HBV infection is the size of viral inoculum. It has been shown that the higher the inoculum, the higher the chance of viral clearance (Chisari *et al.*, 2010).

Other minor mechanisms that have been associated with the development of HBV persistence include HBV infection of immune privilege sites as well as the existence T cell escape mutants resulting from mutations in HBV and thus unable to be recognised by CD8+ T cells for viral clearance (Rehermann and Nascimbeni, 2005; Chisari *et al.*, 2010).

2.9 EXPRESSION OF SPECIFIC CYTOKINES IN HBV INFECTION

Cytokines are a group of soluble proteins that are secreted by a number of different cells and are involved in cell to cell interactions and communication (Cameron and Kelvin, 2003). They are also involved in multiple biological processes in the body which include but not limited to inflammatory process, immune regulation, embryogenesis, haematopoiesis, disease pathogenesis and the process of ageing. That is, they are pleiotropic in nature (Cameron and Kelvin, 2003; Dinarello, 2007). They are usually small proteins with molecular weight of 8 to 40,000 Daltons and the messenger RNA (mRNA) which synthesize these cytokines are known to have quite a short half-life (Peters, 1996; Dinarello, 2000).

Based on the cell they act upon, cytokines may be classified as autocrine, that is cytokine which act on the very cell that produce it, paracrine; cytokines that act upon cells close to the cell secreting it and endocrine; cytokines that act upon cell far from the cell secreting it (Zhang and An, 2007). Cytokines used to be known and called by different names according to their cellular

sources. For instance those secreted by lymphocytes used to be called lymphokines, monokines for those cytokines secreted by monocytes, interleukins for cytokines secreted by and act on leukocytes and chemokines for those group of cytokines having chemotactic activity. Cytokines may also be grouped into pro-inflammatory and anti-inflammatory (Dinarello, 2000; Zhang and An, 2007).

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2.10 CYTOKINE SIGNALLING AND EXPRESSION

Cytokines have specific receptors on the surface of cells unto which they bind (Peters, 1996). Once a cytokine binds to its receptor on a cell surface, they activate a group of tyrosine kinases which are associated with the cytokine receptor known as Janus associated kinases (JAK) (Heim, 1999). The activated JAK then phosphorylate and activate another group of cytoplasmic proteins known as the Signal Transducers and Activators of Transcription (STAT) family (Weber-nordt *et al.*, 1998). The phosphorylated STATs form dimers and are subsequently trans-located into the nucleus where they bind to specific response elements (5' untranslated regions) of the cytokine-responsive gene. This binding results in the rapid transcription of the associated gene (Peters, 1996; Heim, 1999). Despite the pleiotropic nature of cytokines, there are still some distinctions in their overall effect. There is specificity in cytokine-receptor binding in at least one receptor chain. Again, the different cytokine receptors activate different combinations of JAK. The JAKs also phosphorylate different combinations of STATs and lastly, different cytokines would have different response element on their cytokine-responsive (Peters, 1996). This JAK-STAT signalling pathway for cytokine expression is known to be regulated by groups of intracellular proteins called Suppressor of cytokine signalling (SOCS) proteins and cytokine-inducible SRC homology 2 (SH2)-domaincontaining proteins (CISHs) (Yoshimura *et al.*, 2007). For example, certain cytokines including

IL-6, IL-4, LIF, G-CSF are known to induce the production of SOCS proteins which eventually inhibit the JAK-STAT pathway by a negative feedback mechanism leading to the secretion of these cytokines (Heim, 1999; Yoshimura *et al.*, 2007).

2.11 ROLES OF SPECIFIC CYTOKINES IN HBV PATHOGENESIS

2.11.1 Role of interleukin 12 (IL-12) in the pathogenesis of HBV

Interleukin 12 (IL-12) falls under the IL-12 family of cytokines which are made up of heterodimers. This family includes cytokines such as IL-12, IL-23, IL-27 and IL-35. The biological active form of IL-12 is made up of p35 alpha chain and a p40 beta chain sub-units forming IL-12p70 (Liu *et al.*, 2003; Vignali and Kuchroo, 2012). It is a 70kD protein and was originally referred to as natural killer cell stimulatory factor (NKSF) (D'Andrea *et al.*, 1992). IL-12 exerts its biological effect by binding IL-12R β 1 and IL-12R β 2 before activating JAK2 and Tyk2. The activated Janus associated kinase 2 (JAK 2) and tyrosine kinase 2 (Tyk 2) then phosphorylate and activate STAT4 (Vignali and Kuchroo, 2012). It is a pro-inflammatory cytokine which is secreted mostly by APCs such as macrophages, monocytes, dendritic cells and B cells in response to infection. It plays a key role in the differentiation of T cells into T helper type 1 cells, stimulating T lymphocytes and NK cells to secrete IFN- γ as well as enhancing the cytotoxic activity of NK cells and cytotoxic T cells. This makes it a central cytokine in the fight against intracellular and parasitic infections (Gee *et al.*, 2009). In humans the genes encoding for the p35 subunit of IL-12 is located on chromosome 3 (3p12-p13.2) while the genes which encode the p40 subunit of IL-12 is located on chromosome 5 (5q31-33) (Cameron and Kelvin, 2003; Gee *et al.*, 2009).

IL-12 has been shown to be involved in non-cytopathic inhibition of hepatitis B viral replication in liver cells in transgenic mouse through the induction of IFN- γ . The induction of IFN- γ also enhances the activity of HBV specific-cytotoxic T lymphocytes which have also been shown to be involved in the non-cytopathic inhibition of HBV (Thimme *et al.*, 2003). Additionally, the administration of a murine recombinant IL-12 was shown to noncytopathically inhibit viral replication through the induction of IFN- γ and thus emphasizing the important role IL-12 plays in the fight against HBV (Cavanaugh *et al.*, 1997). In a related study, the induction of Th1 cytokines by IL-12 was linked to the clearance and sero-conversion of HBeAg in chronic hepatitis B patients and thus adding to the fact that a preferential shift towards Th1 types cytokine favoured HBV viral clearance and vice versa (Rossol *et al.*, 1997).

The liver induces immune tolerance as a mechanism of establishing CHB infection. IL-12 based vaccination has been shown to reverse this liver induced immune tolerance in mouse models of chronic hepatitis B and hence IL-12 may prove to be a great immunotherapeutic strategic which could be explored in CHB patients for potential treatment (Zeng *et al.*, 2013).

2.11.2 Role of interferon-gamma (IFN- γ) in the pathogenesis of HBV

Interferon- γ , originally referred to as macrophage activating factor, is the only type 2 interferon and is secreted by Th 1 cells, cytotoxic T lymphocytes, B cells, NK cells, NKT cells as well as some antigen presenting cells while type 1 interferons (IFN- α and IFN- β) are secreted by virally infected cells (Le Page *et al.*, 1999; Schroder *et al.*, 2004). The genes responsible for the secretion of IFN- γ is found on chromosome 12 in humans. It is a proinflammatory cytokine and a key T helper type 1 (Th 1) cytokine (Dinarello, 2000; Cameron and Kelvin, 2003). IFN- γ has been found to stimulate the activities of macrophages by enhancing their antimicrobial activity, anti-tumour activity and their antigen processing and presentation activity (Schroder *et al.*, 2004). The

biological effect of IFN- γ is mediated through binding to a 90 kD glycoprotein receptor on the surface of cells. The IFN- γ receptor (IFNGR) is made up of two subunits; IFNGR1 and IFNGR 2. The IFNGR1 and IFNGR 2 associate with JAK 1 and JAK 2 respectively. During ligand binding JAK 2 is autophosphorylated and activated, this subsequently phosphorylate JAK1. The activated JAKs then bind and phosphorylate STAT1 further forming homodimers. The STAT1-STAT1 homodimer is then translocated to the nucleus where it binds IFN- γ activated sites (GAS) which are present on the promoter region of some IFN-stimulate genes (ISGs) and thus initiating transcription of IFN- γ genes (Peters, 1996; Schroder *et al.*, 2004; Plataniias, 2005).

APCs on recognition of an antigen secretes certain cytokines and chemokines including IL-12, IL-18 and macrophage-inflammatory protein-1 α (MIP-1 α). The chemokine attracts NK cells and other immune cells to the site of infection. IL-12 and IL-18 subsequently combine to stimulate the secretion of IFN- γ from these immune cells. IL-4, IL-10 and TGF- β rather down regulate the activity of IFN- γ (Schroder *et al.*, 2004).

In cell mediated immunity, IFN- γ plays a very crucial role and together with other cytokines like IL-12 and TNF- α , it is able to inhibit HBV replication. Significantly, low expression of IFN- γ has been associated with impaired immune response to HBV infection while in other studies an increase in IFN- γ were associated with HbeAg negativity (Cavanaugh *et al.*, 1997; Rizvi *et al.*, 2012; Srivastava *et al.*, 2014). IFN- γ mediates non-cytopathic clearance of HBV by activating HBV-specific CD8⁺ and CD4⁺ T cell as well as NK and NKT cells. It has been found that IFN- γ is remarkably upregulated during acute episodes of HBV in chimpanzees lending credence to its involvement in viral clearance (Kakimi *et al.*, 2000; Thimme *et al.*, 2003). The inhibition of HBV by IFN- γ has also been shown to be dependent on the production of nitric oxide (NO) in mouse models (Guidotti *et al.*, 2000).

In addition to its role in the fight against HBV infection, IFN- γ has been shown to possess antifibrotic properties (Weng *et al.*, 2005). In CHB patients with hepatic fibrosis, intramuscular administration of 50 μ g for a period of nine months significantly improved upon the fibrotic score (Weng *et al.*, 2005). Gene polymorphism in IFN- γ has also been linked to the susceptibility to intrauterine infection with HBV as well as persistence of HBV (Yu *et al.*, 2006; Srivastava *et al.*, 2014).

A number of genes including genes encoding RNA-dependent protein kinase (PKR), Mx GTPases and RNase L are known to induce IFN- γ production in a number of viral infections including HBV though an Mx-independent mechanism of inhibition of HBV replication may exist. Genes mediating antiviral activity of IFN- γ in inhibiting HBV replication mainly require proteasome activity (Wieland *et al.*, 2003).

2.11.3 Role of Tumour necrosis factor alpha (TNF- α) in the pathogenesis of HBV

Tumour necrosis factor alpha (TNF- α) is a 17kD trimeric cytokine belonging to the TNF superfamily of cytokines. It is formed after the cleavage of a 26kD transmembrane tumour necrosis factor protein by a TNF converting enzyme (TACE). It is encoded by genes located on human chromosome 6 within the major histocompatibility region (MHC) (Peters, 1996; Schottelius *et al.*, 2004). TNF- α is mainly secreted by macrophages and monocytes but other immune cells including B cells, T cells, dendritic cells, natural killer cells, neutrophils, mast cells basophils, eosinophils also secrete TNF- α . Some non-immune cells like fibroblasts, keratinocytes, neurons, osteoblasts, smooth muscle, astrocytes, glial cells, granuloma cells, retinal pigment epithelial cells and spermatogenic cells as well as some tumour cells are also other sources of TNF- α (Schottelius *et al.*, 2004; Tse *et al.*, 2012). A number of stimuli including viruses, bacterial and parasitic products, tumour cells, complement, cytokines including

interleukin-1 β (IL-1 β), IL-2, interferon- γ (IFN- γ), macrophage CSF (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNF- α itself, ischemia, trauma, and irradiation are able to induce TNF- α genes to produce TNF- α (Schottelius *et al.*, 2004).

It is a proinflammatory pleiotropic cytokine with numerous roles including stimulating growth and differentiation of different cells, induction of apoptosis in tumour cells and cells infected with viruses. It stimulates the production of Th 1 type cytokines by inducing the production of IL-12 and IL-18 which in turn induces the production of IFN- γ (Schottelius *et al.*, 2004).

TNF- α is able to express its action by binding to two transmembrane glycoprotein receptors; TNF-RI (p60 or p55; CD120a) and TNF-RII (p80 or p75; CD120b). Although TNF-RI is found on a number of cell surfaces, TNF-RII is mostly found on immune cells (Wajant *et al.*, 2003; Schottelius *et al.*, 2004).

In the pathogenesis of HBV, TNF- α has been shown to be involved in noncytopathic viral clearance (Biermer *et al.*, 2003). One mechanism by which TNF- α achieves a noncytopathic inhibition of HBV is through the disruption of the formation of viral cytoplasmic capsids through the activation of the inhibitor of κ B kinase complex (IKK- α/β) and NF- κ B signalling pathway (Biermer *et al.*, 2003; Kuo *et al.*, 2009). TNF- α may also enhance the expression of HLA class I antigens which are required for immune recognition of HBV infected cells for subsequent clearance (Hussain *et al.*, 1994). Together with IFN- γ , TNF- α is also involved in the direct killing of HBV-infected cells (Hussain *et al.*, 1994). Indeed TNF- α has been shown to be upregulated in HBV infection although this rise in TNF- α levels have been linked to liver damage rather than hepatitis B viral replication (González-Amaro *et al.*, 1994; Hussain *et al.*, 1994).

Due to the important role TNF- α plays in the pathogenesis of numerous inflammatory diseases, anti-TNF- α administration has been used in treating a number of diseases including ankylosing spondylitis, rheumatoid arthritis and psoriasis. Treatment with anti-TNF- α agents such as

infliximab and etanercept may likely result in reactivation of hepatitis B in such patients with past history of hepatitis B and hence administration of an antiviral prophylaxis together with the anti-TNF- α has been recommended (Calabrese *et al.*, 2004; Cantini *et al.*, 2014).

2.11.4 Role of interleukin 6 (IL-6) in the pathogenesis of HBV

Interleukin 6(IL-6) as with many other cytokines is a pleiotropic cytokine which belongs to the interleukin 6 family of cytokines. It is a 26kDa cytokine made up of about 184 amino acids with the gene responsible for its production located on chromosome 7 (7p21) (Tanaka *et al.*, 2014; Xia *et al.*, 2015). It is involved in immune regulation, haematopoiesis, inflammatory reactions and oncogenesis. IL-6 has at various times been referred to with different names including B-cells stimulatory factor-2, hybridoma/plasmacytoma growth factor, IFN- β 2, 26kDa protein, and hepatocyte stimulating factor (HSF) (Kishimoto, 2010; Tanaka *et al.*, 2014).

IL-6 function is mediated through two receptor systems, a more specific IL-6 receptor (IL-6R) and a glycoprotein 130 (gp130) receptor which is shared by other members of the IL-6 family cytokines. Signalling through the gp130 is mediated by JAK-STAT pathway and the RasMAPK pathways. Suppressor of cytokine signalling (SOCS) proteins is known to have a negative feedback effect of IL-6 signalling. The pleiotropic and redundancy exhibited by IL-6 is because the gp130 receptor that it uses in signal transduction is found on numerous cell surfaces (Kishimoto, 2010).

Interleukin 6 is involved in the pathogenesis of HBV in many ways. It is thought to facilitate HBV infection by binding to the pre-S1 region of the HBV envelope protein (Galun *et al.*, 2000). In fact elevated concentrations of IL-6 has been found in patients with CHB infection and its associated liver cirrhosis and hepatocellular carcinoma (Kuo *et al.*, 2009; Sheng *et al.*, 2015a). Although the serum levels of IL-6 have been reported to be negatively correlated to the levels of

transaminases in the liver, it has been suggested as a good marker in acute liver damage in HBV as compared to other cytokines such as IL-10, IL-12 and IFN- γ (Wong *et al.*, 2009). IL-6 is also known to be able to inhibit HBV replication independent of IFN- α/β and IFN- γ . Although IL-6 does not disrupt HBV nucleocapsids and suppress the secretion of HBsAg and HBeAg, its ability to inhibit HBV replication is as a result of a slight reduction of HBV transcripts or core proteins as well as a marked reduction in the level of HBV genomecontaining nucleocapsids. IL-6 has also been shown to be able to reduce the accumulation of covalently closed circular DNA (cccDNA) of HBV, though it had no effect on existing cccDNA of HBV (Kuo *et al.*, 2009).

HBx proteins of HBV have been shown to be able to activate IL-6 genes for the production of IL-6 through NF- $\kappa\beta$ signalling pathways while the soluble interleukin 6 receptor (sIL-6R) has also been shown to possess antiviral activity which is mediated through the cyclooxygenase-2 pathway (Lee *et al.*, 1998; Wang *et al.*, 2013). Taken together, IL-6 and its receptors especially sIL-6R provide a novel therapeutic target for treating CHB. IL-6 can induce the production of acute phase proteins by acting on hepatocytes, differentiation of T and B cells and promote maturation of platelets by acting on the bone marrow (Tanaka *et al.*, 2014).

2.11.5 Role of interleukin 4 (IL-4) in the pathogenesis of HBV

Interleukin 4(IL-4) is a multifunctional cytokine which was originally referred to as B cell differentiation and stimulatory factor at discovery (Zamorano *et al.*, 2003). Its weight varies between 12 and 20 kDa and this is due to variation in natural glycosylation (Luzina *et al.*, 2012). Activated T lymphocytes are the main cellular source of IL-4 although basophils, mast cells and eosinophils do secrete some amount of IL-4. It takes a key part in the differentiation of naïve T lymphocytes into T helper type 2 (Th 2) cells and in proliferation, differentiation and apoptosis of a number of cells of both haematopoietic and non-haematopoietic sources, thus it plays an

important role in the control of the immune system (Zamorano *et al.*, 2003; Luzina *et al.*, 2012). It is also involved in B cell activation, IgE class switching, up-regulation of MHC class II expression and inhibition of pro-inflammatory cytokines. It also plays a significant role in the pathogenesis of asthma, allergy and fight against parasitic infections (Lin *et al.*, 2003). In humans, the IL-4 gene is found on chromosome 5q31 with more than 50 allelic variants known (Lu *et al.*, 2014). IL-4 shares some structural characteristics with IL-13 (Luzina *et al.*, 2012). IL-4's activity is mediated through the binding of IL-4 receptors (IL-4Rs) which are expressed on a number of different cells. Two types of IL-4Rs have been classified; type I which is made up of and alpha chain (IL-4R α) and the common gamma chain (γ c) while type II is still made up of IL-4R α and IL-13 low affinity receptor 1 (IL-13R α 1) (Zamorano *et al.*, 2003). The IL-4R α chain and the γ c chain associates with JAK1 and JAK3 respectively while the (IL-13R α 1 chain of the type II receptor associates with TYK2. Once IL-4 binds its appropriate receptors, there is activation of the associated JAK tyrosine kinases. This subsequently leads to the phosphorylation of STAT6, the phosphorylated STAT6 is then translocated into the nucleus where it binds the appropriate IL-4 response element within the interferon gamma (IFN- γ) activation sites (GAS) for onward gene expression (Pernis *et al.*, 1995; Zamorano *et al.*, 2003).

In the pathogenesis of HBV, IL-4 has been shown to suppress HBsAg mRNA expression and subsequent production of HBsAg (Lin *et al.*, 2003). The pregenomic RNA of HBV codes for the core and polymerase proteins and also serves as template for reverse transcription of the HBV DNA. IL-4 has been shown to suppress this pregenomic RNA of HBV thereby inhibiting HBV replication (Lin *et al.*, 2003). IL-4 can also suppress the precore mRNA which secretes the HBeAg (Lin *et al.*, 2003). Intrahepatic T lymphocytes, most of which are natural killer T lymphocytes (NKT) isolated from chronic HBV patients was shown to produce IL-4 and when IL-12 was present, IFN- γ was also produced (Trobonjaca *et al.*, 2001). When α galactosylceramide-presenting hepatocytes was used to stimulate NKT cells, they were found

to produce IL-4. These α -galactosylceramide activated NKT cells are also able to inhibit HBV replication in HBV transgenic mice (Kakimi *et al.*, 2000).

Genetic polymorphisms at different positions in the IL-4 gene have been associated with an increased susceptibility of HBV especially among Caucasians (Zheng *et al.*, 2013; Lu *et al.*, 2014). Modulating IL-4 levels, its receptors and its signalling pathways could therefore serve as alternate therapeutic as well as prognostic tool in managing hepatitis B infection and its associated liver diseases.

2.11.6 Role of interleukin 10 (IL-10) in the pathogenesis of HBV

Interleukin 10 (IL-10) is a 37 kDa multipurpose immunoregulatory cytokine formally referred to as cytokine synthesis inhibitory factor (CSIF) at its discovery. It is a Th 2 cytokine produced by Th 2 cells as well as macrophages, monocytes, B cells, mast cells and eosinophils.

The genes encoding for human IL-10 is located on the long arm of chromosome 1 (1q31-32) (Asadullah *et al.*, 2003). It is an anti-inflammatory cytokine able to inhibit inflammatory processes, able to inhibit the differentiation and proliferation of Th 1 cells thus inhibiting the secretion of Th 1 cytokines such as IL-2 and IFN- γ . It is also able to inhibit the proliferation of other cells of in the immune system including B cells, antigen-presenting cells, natural killer cells, granulocytes and mast cells (Asadullah *et al.*, 2003).

IL-10 shares structural homology with other cytokines such as IL-19, IL-20, IL-22, IL-24, IL26 and less related IL-28A, IL-28B and IL-29; together they form the IL-10 family of cytokines (Ouyang *et al.*, 2011). It also shares homology with certain viruses like Epstein-Barr virus (BCRF1), herpes virus type 2, cytomegalovirus and Orf virus (Asadullah *et al.*, 2003).

Interleukin-10's activity is mediated through binding to IL-10 receptors (IL-10R) which are mostly found on immune cells although they occasionally occur of other non-immune cells. The IL-10/IL-10R complex subsequently binds and activate JAK1 and TYK2 which further leads to

the phosphorylation of STAT 1, STAT 3 and STAT 5. This is translocated into the nucleus where they bind to cytokine response element on the interferon gamma (IFN- γ) activation sites (GAS) for onward gene activation (Asadullah *et al.*, 2003).

In the pathogenesis of hepatitis B, HBcAg has been shown to stimulate the secretion of IL-10 from T cells, B cells and monocytes in CHB patients. Although HBcAg has also been shown to stimulate the production of IFN- γ in CHB patients, much more IL-10 is produced compared to IFN- γ . Taking together, it is thought that the suppression of cellular immune response by IL-10 may contribute to protecting the liver from cellular damage while also contributing to the persistence of the HBV due to the suppression of cellular response (Hyodo *et al.*, 2004). Again, IL-10 concentrations have been shown to increase in patients with occult HBV infection. This has been attributed to the ability of IL-10 to suppress the immune response against HBV in such patients (Arababadi *et al.*, 2010). In one longitudinal study, there was a close temporal correlation between the serum IL-10 levels and fluctuations in hepatitis B viral load (Das *et al.*, 2012). Additionally, in vitro blockade of IL-10 led to the restoration of HBV-specific CD8⁺ T cell functionality (Das *et al.*, 2012).

Polymorphism of IL-10 at -1082 region have been associated with increased intrautrine viral clearance and lower viral load in children while polymorphisms in the genes encoding IL-10 genotypes have been found to have different HBV-HCC risk associations in different populations (Tunçbilek, 2014; Saxena and Kaur, 2015). Particularly, those who carry the 592A allele in the IL-10 promoter region are thought to be at higher risk of developing persistent HBV infection. There is a different school of thought who have also shown that no such association exists between HBV infection and IL-10 polymorphism (Cheong *et al.*, 2006; Sofian *et al.*, 2013)

The anti-inflammatory and the immunosuppressive properties of Interleukin-10 and its receptor (IL-10/IL-10R) complex can be explored to protect the liver of CHB patients from considerable damage and the development of cirrhosis and HCC.

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CHAPTER 3

MATERIALS AND METHOD

3.1 STUDY DESIGN AND STUDY SITE

This case control study was carried out at the Serology and Virology Unit of Komfo Anokye Teaching Hospital (KATH) between January 2015 and December 2015. KATH is a 1000 bed capacity tertiary referral hospital that serves the Kumasi metropolis, surrounding towns and northern sector of Ghana and it is located in Kumasi in the Ashanti region.

3.2 STUDY POPULATION AND RECRUITMENT

3.2.1 Sample size

Using Cochran's sample size formula for categorical data, sample size was calculated as follows:

$$n = \frac{Z^2 P(1-P)}{d^2}$$

n = required sample size

Z = confidence level at 95% (standard value of 1.96)

P = estimated prevalence of hepatitis B (8.68% in Kumasi; (Amidu *et al.*, 2012))

d = margin of error at 5% (standard value of 0.05)

$$n = \frac{(1.96)^2 0.0868(1-0.0868)}{(0.05)^2}$$

$$(0.05)^2$$

$$n = 122$$

Out of the total of 122 participants with past or present history of HBV infection that were required, we were able to recruit a total of 120 HBV patients as cases and 105 healthy blood donors as control after they had given written informed consent. Questionnaires were used to obtain socio-demographic information of study participants. Information pertaining to clinical care and other conditions were inferred from folders and data-base of the hospital.

3.2.2 Inclusion and exclusion criteria

Participants with HBV/history of HBV who gave informed consent were included in the study. Participants who were positive for HCV and HIV, on herbal medication, pregnant, consumed alcohol, with a history of immunosuppressive/antiviral therapy and co-existence of other chronic liver disease (as obtained from patient's folder), were excluded from the study.

3.3 ETHICAL CONSIDERATION

The study protocol was reviewed and granted for by Committee for Human Research, Publication and Ethics (CHRPE) of the Kwame Nkrumah University of Science & Technology and the Research and Development Unit of Komfo Anokye Teaching Hospital (CHRPE/AP/076/15).

3.4 SAMPLE COLLECTION

Five millilitres (5mls) of blood was taken from each participant and serum was separated (Hettich Universal 320 centrifuge- DJB Labcare Ltd, UK) by centrifuging at 1000 rpm for 10minutes. Aliquots of serum were stored in cryovials at -80°C until assayed.

3.5 SEROLOGICAL SCREENING

3.5.1 HBV Screening

Screening for HBV was done by detecting HBsAg in the serum of patients using ABON HBsAg One step kit (Abon Biopharm, Hangzhou,China). Detection of HBsAg was based on a lateral

flow immunoassay which had the ability to detect as low as 1ng/mL of HBsAg. Briefly, the test strip was immersed in the serum sample, it was taken out and placed on a flat surface and timed. The results was reported after 15 minutes as positive when a red line appeared at the test region of the test strip as well as at the control region and negative when only one line appeared at the control region. The relative sensitivity and specificity of the kit was >99% and 97% respectively as stated by the kit manufacturer.

3.5.2 HCV Screening

Screening for HCV was performed using Advance Quality Rapid Anti-HCV test kit (InTec Products, INC., China). The detection of HCV antibody in the sample was based on the binding of the HCV antibody to an HCV antigen-colloid gold conjugate which was embedded in the sample pad forming a conjugate/HCV antibody complex. This complex then migrated along the test strip till it was captured by an antibody-binding protein A which was immobilised at the test area. A colour was formed at the test region for positive samples and no colour was formed for negative samples. A coloured line develops at the control band of the test kit to validate the results of the test. The test procedure was carried out as follows; 10 μ L of serum was added to the sample pad on the test strip. Two drops of sample diluent was immediately added and result read after 15 minutes. The overall specificity of the kit has been known to be 97 to 99% while a 100% sensitivity has been reported.

3.5.3 HIV Screening

Anti HIV 1&2 antibody screening was performed using First Response HIV 1-2 rapid test kit (Premier Medical Corporation, Kachigan, India). This is an immunochromatographic qualitative

test for the detection of HIV-1 as well as group O and HIV-2 antibodies. The kit is labelled 1, 2 and C corresponding to HIV-1, HIV-2 and Control respectively. There is a nitrocellulose membrane inside the kit coated with capture antigens to HIV-1(gp41 and p24) at band labelled 1 and capture antigen to HIV-2(gp36) at band labelled 2 on the kit. HIV antibodies in the test sample bind to a recombinant HIV 1&2 conjugated with colloidal gold particles in the test pad. The addition of a diluent enables these conjugate-antibody complex to migrate along the nitrocellulose membrane and are captured by respective capture antigen at positions labelled 1 and 2 with subsequent development of a coloured line indicative a reactive results. The control band will appear irrespective of the reactive or non-reactive sample serving to validate the results.

The test was performed as follow; 10µL of serum was added to the sample well on the cassette. 35µL of Assay Diluent was then added to the sample well and timed for 15 minutes. The results was read by observing for the development of a colour from the results window. The sensitivity, specificity and total agreement of the kit has been reported as 100%.

3.5.4 Hepatitis B profile test

To categorize patients into acute, recovery and chronic cases, screening was done for HBsAg, HBsAb, HBeAg, HBeAb and HBcAb using DiaSpot Rapid Diagnostic Test kit (USA). This kit measures HBsAg and HBeAg by a dual-antibody sandwich method while the detection of HBsAb was based on a dual-antigen sandwich method. HBeAb and HBcAb were measured based on a neutralization competitive inhibition method. Though a qualitative test, it is able to detect HBsAg concentration of $\geq 2\text{ng/mL}$, HBeAg concentration of $\geq 2\text{NCU/mL}$, HBsAb concentration of $\geq 30\text{mIU/mL}$, HBeAb $\geq 1:8$ and HBcAb $\geq 1:5$.

The test was performed as follows: 25µL of serum was added to each of the 5 sample wells. A drop of buffer was then added to each of the sample wells. The results was read at 15 minutes. The results were interpreted as follows: for markers based on sandwich method (HBsAg, HBsAb and HBeAg), two lines at the _C' and _T' bands was recorded as positive while only one line at the _C' band was reported as negative. For the markers which were detected based on competition method (HBeAb and HBcAb), two coloured lines at the _C' and _T' was reported negative while only one coloured line appearing at the _Cl position was reported as positive.

3.5.5 HBcIgM Test

To differentiate between acute infection and HBeAg positive CHB, screening for HBcIgM was performed using Advance Quality TM One Step HBcIgM test kit (InTec Products, INC., China). It is a qualitative rapid immunochromatographic assay for the detection of IgM type antibody to the HBcAg. A capture antibody made up of IgM µ chain is immobilized on the nitrocellulose membrane at the test region. HBcIgM in the sample binds to antigen-colloidal gold conjugate in the test pad. The conjugate-antibody complex migrate along the nitrocellulose membrane and it is captured at the test band by the capture antibody forming a coloured line in positive cases. The test is negative if no coloured band appears at the test band. A coloured band appears at the control band to validate the results.

The test is performed as follows: Ten microliters (10µL) of serum is added to the test pad. Hundred microliters (100µL) of sample diluent is then added and results is read at 15 minutes.

3.6 DEFINITION AND CLASSIFICATION OF HBV INFECTION

Based on the various HBV serological markers, HBV infection was staged as acute infection, HBV recovery, HBeAg-negative chronic hepatitis B (HBeAg –ve CHB) and HBeAg-positive

chronic hepatitis B (HBeAg +ve CHB) as described by (Krajden *et al.*, 2005; Shepard *et al.*, 2006) in Table 1.

Table 1 Classification of HBV infection

| HBsAg | HBsAb | HBeAg | HBeAb | HBcAb | HBcIgM | COMMENT |
|----------|----------|----------|----------|----------|----------|---------------------|
| Positive | Negative | Positive | Negative | Positive | Positive | Acute infection |
| Negative | Positive | Negative | Negative | Positive | Negative | HBV recovery |
| Positive | Negative | Positive | Negative | Positive | Negative | HBeAg positive CHB |
| Positive | Negative | Negative | Positive | Positive | Negative | HBeAg negative CHB |
| Negative | Negative | Negative | Negative | Negative | Negative | Not exposed/control |

3.7 LIVER FUNCTION TEST (LFT)

The second part of the serum was used in accessing liver damage due to HBV infection by performing the liver function test (LFT) using Cobas® Integra™ 400plus Biochemistry Analyzer (Roche Diagnostics Ltd., Switzerland). The following markers were tested ALT, AST, ALP, GGT, total bilirubin, direct bilirubin, total protein, albumin and globulin.

Elevated hepatic enzymes were defined as follows; ALT > 40.0 U/L and > 31.0 U/L for male and female respectively, AST > 37.0 U/L and > 31.0 U/L for male and female respectively, ALP > 117.0 U/L for all adults and GGT > 51.0 U/L or > 33.0 U/L for male or female respectively (Osakunor *et al.*, 2015). Intrahepatic cholestasis was defined as participants with elevated ALP, GGT and total bilirubin with an AST/ALT ratio of >1.5 whilst extrahepatic cholestasis was defined as participants with elevated ALP, GGT and total bilirubin with an AST/ALT ratio of

<1.5. Participants with elevated AST and ALT with AST/ALT ratio <1 were grouped as non-alcoholic fatty liver disease (Hall and Cash, 2012)

3.8 CYTOKINE MEASUREMENT USING SANDWICH ELISA

Measurement of the various cytokines (IL-12p70, IFN- γ , TNF- α , IL-4, IL-6 and IL-10) was done using Human ELISA MAXTM Standard Set (BioLegend, San Diego, CA). The measurements were done according to the manufacturer's instruction with minor modifications as follows:

A day prior to running the ELISA, rat monoclonal antibodies (capture antibody) specific to the cytokine of interest were diluted in a carbonate coating buffer, pH 9.5. Hundred microliters (100 μ L) of this capture antibody solution was then added to each well of 96-well microtiter plate (Nunc Maxisorp, Roskilde, Denmark) for each cytokine. The plate was then sealed and incubated overnight at between 2°C and 8°C.

The following day, the plates were washed 4 times with Wash Buffer per well using PW40 Microplate Washer (Bio-Rad, Marnes-la-Coquette, France). The residual buffer was blot by firmly tapping plate upside down on absorbent paper. Two hundred microliters (200 μ L) of assay diluent was added to each well to block non-specific binding and reduce background. The plates were sealed and incubated at room temperature for 1 hour with shaking on a Heidolph Titramax 101 vibrating platform shaker (Heidolph Instruments, Germany) at 500 rpm. Test samples were thawed and appropriate dilutions for standards were also prepared. The standards for the various cytokines were diluted to the following concentrations: IL-12p70 (1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL and 15.6 pg/mL),

TNF- α (500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL and 15.6 pg/mL and 7.8 pg/mL), IFN- γ (500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL and

7.8 pg/mL), IL-4 (250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, 7.8 pg/mL and 3.9 pg/mL), IL-6 (500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL and 7.8 pg/mL) and IL-10 (250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, 7.8 pg/mL and 3.9 pg/mL).

The plates were washed 4 times as described above after the incubation. Subsequently, fifty microliters (50 μ L) of standards and test samples was added to the appropriate wells. The plates were sealed and incubated at room temperature for 2 hours with shaking as described above. The plates were washed 4 times with wash buffer after which 50 μ L of diluted detection antibody solution was added to each well, plates were sealed and incubated at room temperature for 1 hour with shaking. After washing the plate 4 times, 50 μ L of diluted AvidinHRP solution was added to each well. Plates were sealed and incubated at room temperature for 30 minutes with shaking. The plates were washed 5 times with Wash Buffer but for this final wash, the wells were soaked wells in Wash Buffer for 1 minute for each wash in order to minimize background. Fifty microliters (50 μ L) of TMB Substrate Solution was added to each well and incubated in the dark for 20 minutes. Different intensities of blue colour developed depending on the concentration of the particular cytokine. Fifty microliters (50 μ L) of stop solution was then added to each well to stop the reaction. Positive wells turned from blue to yellow. The absorbance was read at 450 nm within 15 minutes using ELx 800 Absorbance Microplate Reader (Bio-Tek Instruments. Inc., Winooski, VT, USA). A graph of the various standards were plotted against their corresponding absorbance. Concentrations of the various samples were then derived from the standard curves.

3.9 STATISTICAL ANALYSIS

Data was entered into excel worksheet (Microsoft Office, 2013 version) and was analysed using GraphPad Prism version 6.01 (GraphPad Software, Inc., CA, USA). Data was expressed as mean

± SD, median (inter quartile range) and frequency (proportion) where appropriate. Kruskal-Wallis test followed by Dunn's multiple comparisons test were used to compare liver biomarkers, Th1 and Th2 cytokines in acute HBV, HBV recovery, HBeAg-positive CHB and HBeAg-negative CHB and control. Area under the curve (AUC) and receiver operating characteristic (ROC) were used to determine diagnostic accuracy of the various cytokines. To determine the strength of association of the various cytokines in identifying the various stages of HBV infection, a multivariate logistic regression was performed to determine the odds ratio (OR) and its associated level of significance. To evaluate the use of cytokines as markers of hepatic injury or inflammation, a linear regression statistic was performed to assess the strength of relationship between markers of hepatic injury (ALT, AST, ALP and GGT) and Th1 and Th2 cytokines. *P* value of <0.05 was considered significant for all analyses.



CHAPTER 4

RESULTS

4.1 DEMOGRAPHICS

The demographics of the study population is summarized in Table 2. There was no difference between the ages of the cases and control subjects in this study. Males comprised 53.3% of study participants while females were 46.7% of the study population

Table 2. Study demographics

| Parameters | CASES(n=120) | CONTROL(n=105) | Total(n=225) | P-value |
|--------------|--------------|----------------|--------------|---------|
| Age(years) | 32.1±10.8 | 30.5 ±10.3 | 31.7±10.6 | 0.3867 |
| Male, %(n) | 47.5% (57) | 68.8% (72) | 53.3% (119) | |
| Female, %(n) | 52.5% (63) | 31.1% (33) | 46.7% (105) | |

Age expressed as mean ±SD

4.2 DISTRIBUTION OF THE VARIOUS STAGES OF HBV INFECTION

Figure 3 is a graphical presentation of the various stages of HBV infection by sex as observed in the study population. Acute HBV infection accounted for 3 (2.5%) of the total HBV infections, HBV recovery state accounted for 12 (10%) while HBeAg positive CHB and HBeAg negative CHB accounted for 5 (4.2%) and 100 (83.3%) respectively. In all HBeAg which is a marker for infectivity and HBV replication was present in 8 (6.67%) of HBV cases.

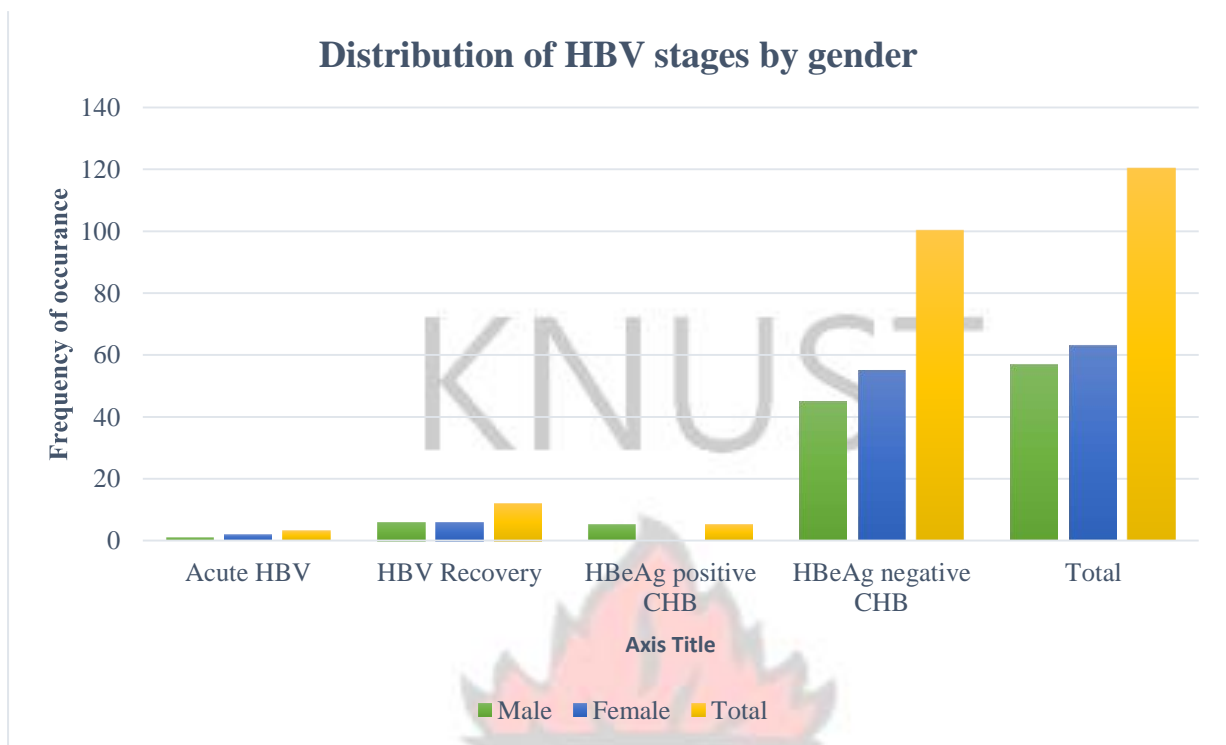


Figure 3. Distribution of the various stages of HBV infection

4.3 LIVER MARKERS IN THE VARIOUS STAGES OF HBV INFECTION

Table 3 is a summary of the liver chemistries in acute HBV, HBV recovery, HBeAg positive and HBeAg negative CHB infections as well as control group. Median alanine aminotransferase (ALT) concentration was significantly elevated in HBeAg positive chronic infection, elevated in acute HBV and HBeAg negative chronic infection but reduced in HBV recovery compared to the control group ($p=0.013$). The highest ALT concentration was recorded in HBeAg positive group and it was 2.5 times higher than that of the control group, 1.8 times compared to acute infections, 2.7 times higher than HBV recovery and 2 times higher than HBeAg negative CHB. Median albumin concentration was reduced in all the HBV groups, however, this reduction was only significant in the HBeAg negative chronic infection compared to the control group ($p=0.0001$). The median globulin concentration was elevated in all the HBV groups although significant elevations were observed in HBV recovery group and the HBeAg negative chronic

infection group compared to the control group ($p < 0.0001$), the highest concentration of 27.1 g/L was in the HBV recovery group. The median concentration of direct bilirubin was elevated in all the HBV groups although this elevated concentration was significant in the HBeAg negative chronic infection group compared to the control group ($p = 0.0316$).

There were no statistically significant differences in the median levels of AST, ALP, GGT, total protein, total bilirubin and indirect bilirubin among the various HBV groups and the control group.



Table 3 Liver function test in the various stages of HBV infection

| Parameters | 1. Controls | 2. Acute Infections (n=3) | 3. HBV Recovery | 4. HBeAg positive CHB | 5. HBeAg negative CHB | P-value | Significant pairs |
|----------------------------|-------------------|---------------------------|--------------------|-----------------------|-----------------------|--------------------|-------------------|
| | (n=105) | | (n=12) | (n=5) | (n=100) | | |
| AST (U/L) | 24.7(18.3-27.5) | 23.6(17.7-72.8) | 21.55(15.8-34.48) | 34.4(27.8-94) | 24.2(17.33-44.6) | 0.2859 | None |
| ALT(U/L) | 12.7(9.6-18.75) | 17.1(12.4-86) | 11.65(8.83-24.23) | 31.2(24.85-123.2) | 16.3(11.45-36.6) | 0.013 | 1 vs. 4 |
| ALP(U/L) | 58.4(40.6-74.5) | 83.9(62.4-87.6) | 76.85(48.53-141.2) | 99.6(45.45-151.7) | 67.6(47.33-100.7) | 0.0568 | None |
| GGT (U/L) | 26.1(20.35-36.5) | 31.8(16.7-43.6) | 27.9(17.73-85.38) | 32(22.9-96.25) | 27.4(15.53-52.7) | 0.8581 | None |
| Total Protein (g/L) | 66.5(64.5-69.2) | 67.3(63.1-71.08) | 63.9(52.5-77.7) | 69.6(67.1-75.25) | 68.7(65.15-73.6) | 0.4383 | None |
| Albumin (g/L) | 44.7(42.17-47.2) | 39.0(31-47.6) | 42.65(30.54-46.99) | 43.6(39.4-44.05) | 39.95(35.73-44.3) | 0.0001 | 1 vs. 5 |
| Globulin (g/L) | 20.9(19.15-22.95) | 24.9(21.5-30.2) | 27.1(22.48-39.18) | 26(23.3-35.45) | 26.45(23.05-30.1) | < 0.0001 | 1 vs. 3 & 5 |
| Total Bilirubin (µmol/L) | 8.4(4.65-12) | 9.70(6.8-18) | 11.05(3.55-16.78) | 12.5(8.1-21.6) | 9.3(5.63-15.6) | 0.5637 | None |
| Direct Bilirubin (µmol/L) | 2.4(1.8-3.2) | 2.50(1.8-5.7) | 3.45(2.2-6.07) | 3.2(2.45-5.49) | 3.5(1.95-5.85) | 0.0316 | 1 vs. 5 |
| Indirect Bilirubin(µmol/L) | 6.1(3.2-8.7) | 7.20(5-12.3) | 6.9(1.87-10.35) | 9.3(5.15-16.6) | 5.0(3-9.2) | 0.5392 | None |

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AST= Aspartate aminotransferase, ALT= Alanine aminotransferase, ALP= Alkaline phosphatase, GGT= Gamma-glutamyl transferase. Liver biomarkers are expressed as median (interquartile range). Kruskal-Wallis test followed by Dunn's *post-hoc* test were used to analyse the various stages of HBV infection and control. *P* value of <0.05 was considered significant.

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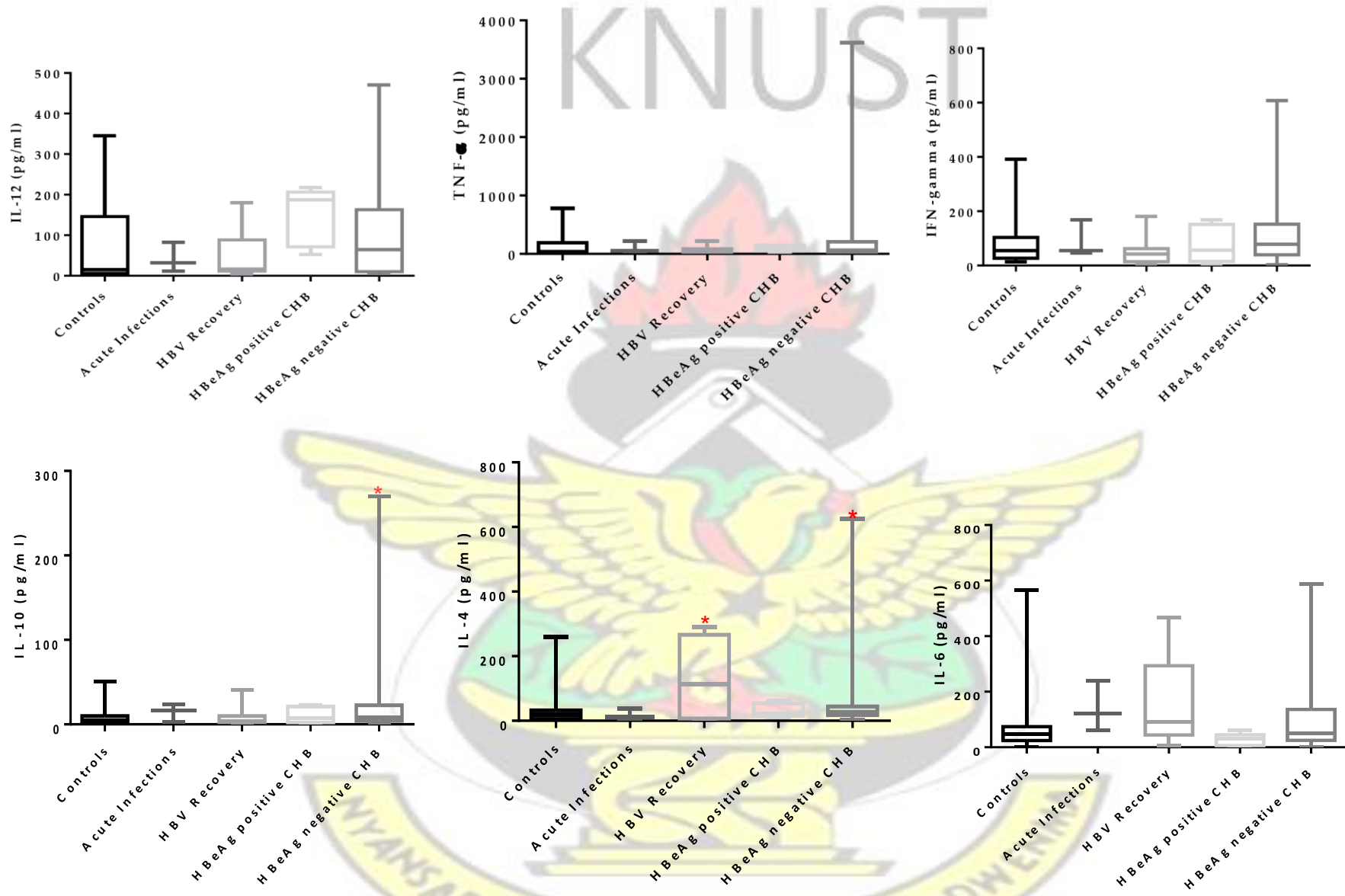
4.4 CYTOKINE PATTERN IN THE VARIOUS STAGES OF HBV INFECTION

The median concentration of IL-12p70 was elevated in the acute infection, HBV recovery, HBeAg positive CHB and HBeAg negative CHB groups compared to the control group ($p=0.1036$). Median TNF- α was elevated in acute infection, HBeAg positive CHB and HBeAg negative CHB groups but reduced in HBV recovery compared to the control group ($p=0.3984$). Median INF- γ concentration was not significantly different in acute infection compared to control group but was reduced in HBV recovery and elevated in both HBeAg positive CHB and HBeAg negative CHB groups compared to the control group ($p=0.0954$). Median IL-4 levels were reduced in acute infection but elevated in HBV recovery ($p<0.05$), HBeAg positive CHB and HBeAg negative CHB ($p<0.05$) groups compared to control. Median levels of IL-6 were elevated in acute infection, HBV recovery and in HBeAg negative CHB groups but reduced in HBeAg positive CHB compared to control group ($p=0.0702$). Median levels of IL-10 were reduced in HBV recovery but elevated in acute infection, HBeAg positive CHB group and significantly elevated in the HBeAg negative CHB group ($p=0.0253$) compared to control group.

Acute infection phase and HBeAg-positive CHB had the highest levels of inflammation as demonstrated by high levels of IL-6 and IL-12p70.

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Cytokine levels are expressed as median (interquartile range). Kruskal-Wallis test followed by Dunn's multiple comparisons test were used to compare the various stages of HBV infection and control. *P* value of <0.05 was considered significant.

Figure 4. Th1 and Th2 cytokine pattern in the various stages of HBV infection

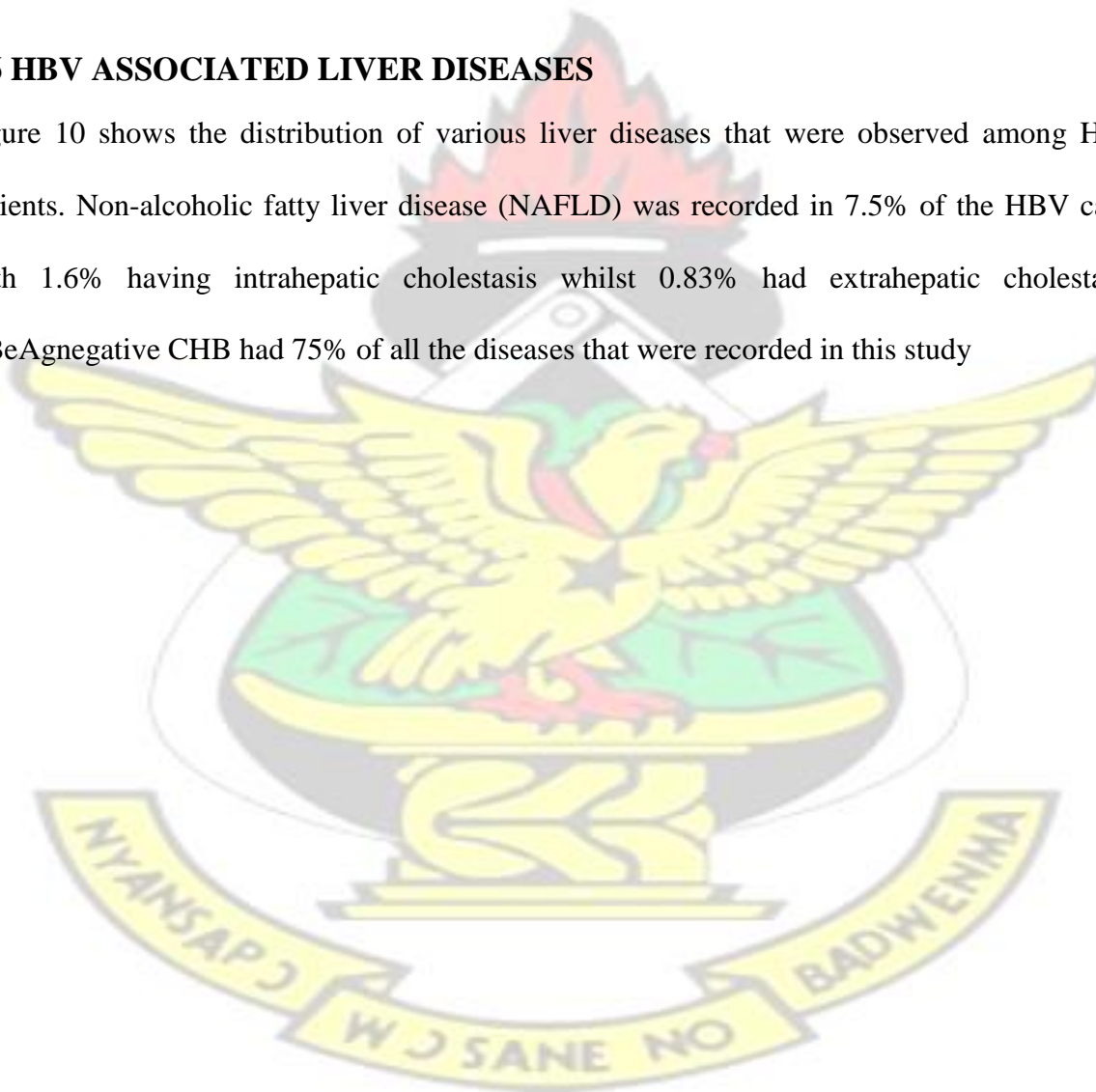


4.5 ELEVATED HEPATIC ENZYMES

Table 4 shows the proportion of HBV patients with elevated liver enzymes. Thirty percent (30%) of all the HBV patients had elevated AST levels whilst 23.3% having elevated ALT levels. ALP was elevated in 17.5% of all HBV cases whilst 30.8% had elevated GGT levels. Both AST and ALT were elevated in 17.5% whilst 12.5% had combine elevation of ALP and GGT. All the four enzymes were elevated in 5.8% of the total HBV cases.

4.6 HBV ASSOCIATED LIVER DISEASES

Figure 10 shows the distribution of various liver diseases that were observed among HBV patients. Non-alcoholic fatty liver disease (NAFLD) was recorded in 7.5% of the HBV cases with 1.6% having intrahepatic cholestasis whilst 0.83% had extrahepatic cholestasis. HBeAgnegative CHB had 75% of all the diseases that were recorded in this study



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Table 4 Participant with elevated liver enzymes (HBV associated Hepatic Injury)

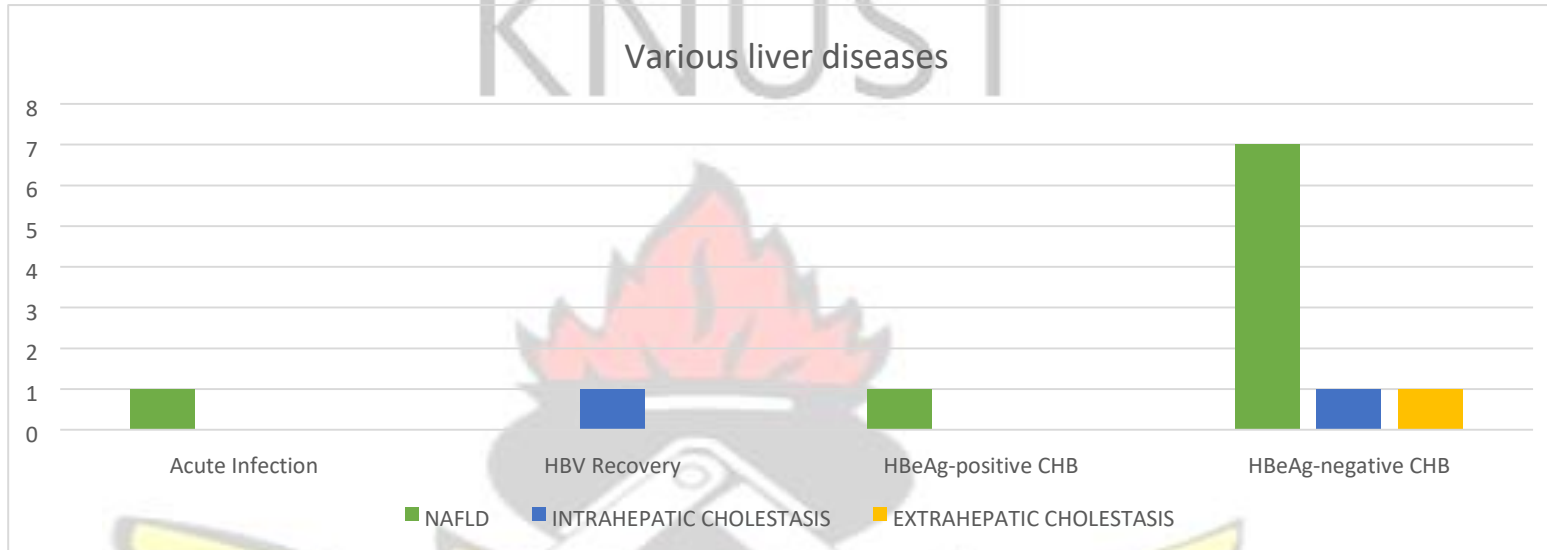
| Parameters | Acute(n=3) | HBV Recovery (n=12) | HBeAg positive CHB(n=5) | HBeAg negative CHB(n=100) | Total (n=120) |
|----------------------|------------|------------------------|----------------------------|------------------------------|---------------|
| AST | 1(33.3%) | 2(16.67%) | 2(40%) | 31(31%) | 36(30%) |
| ALT | 1(33.3%) | 2(16.67%) | 2(40%) | 23(23%) | 28(23.3%) |
| ALP | 0 | 3(25%) | 2(40%) | 16(16%) | 21(17.5%) |
| GGT | 1(33.3%) | 4(33.3%) | 1(20%) | 31(31%) | 37(30.8%) |
| AST & ALT | 1(33.3%) | 0 | 2(40%) | 18(18%) | 21(17.5%) |
| ALP & GGT | 0 | 2(16.67%) | 0 | 13(13%) | 15(12.5%) |
| AST, ALT,ALP &GGT | 0 | 0 | 0 | 7(7%) | 7(5.8%) |

AST= Aspartate aminotransferase, ALT= Alanine aminotransferase, ALP= Alkaline phosphatase, GGT= Gamma glutamyltransferase



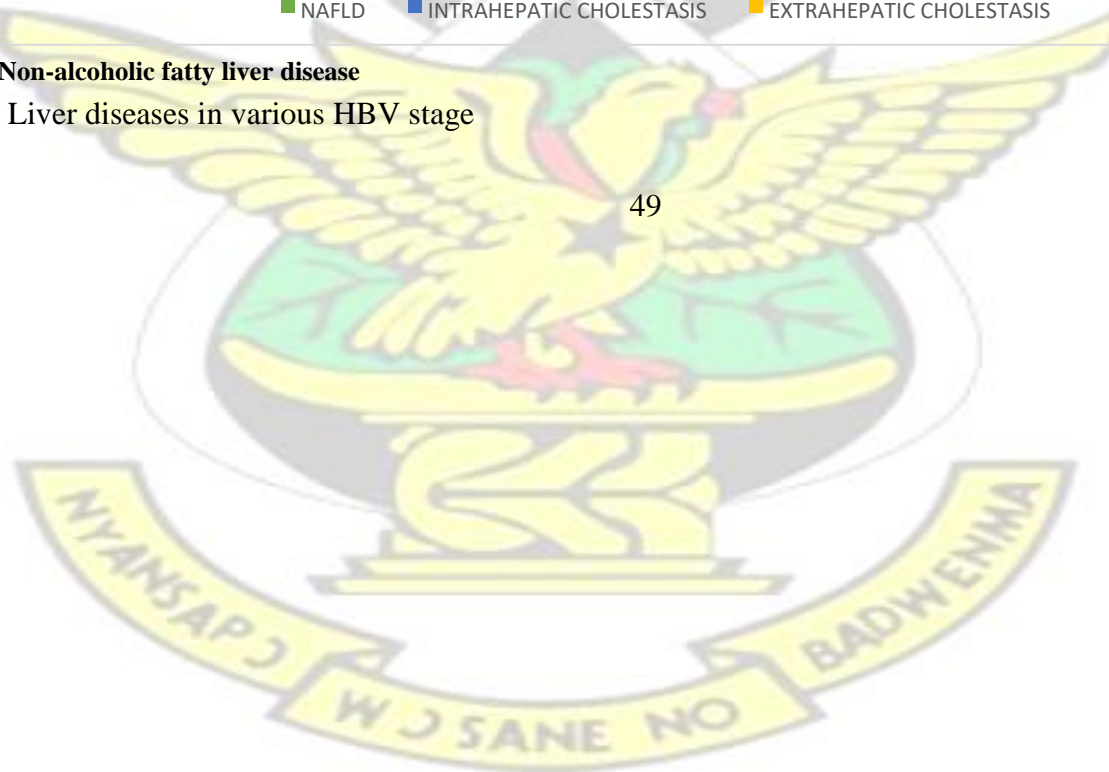
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Various liver diseases



NAFLD= Non-alcoholic fatty liver disease

Figure 5. Liver diseases in various HBV stage



4.7 DIAGNOSTIC ACCURACY OF CYTOKINES AND LIVER BIOMARKERS IN HBV INFECTION

To determine the diagnostic accuracy of the various Th1 and Th2 cytokines in identifying the various stages of HBV infections, a receiver operating characteristic curve (ROC) was performed and the area under the curve (AUC), sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were determined.

4.7.1 Acute HBV infection

In the acute infection group, IL-10 at a threshold of $>15\text{pg/ml}$ and at 95% confidence interval had the best combined AUC of 0.72, sensitivity of 66.7% the highest specificity of 86.7%, PPV of 25% and NPV of 97.5% in identifying this group of HBV infection compared to the other cytokines. IL-6 at a threshold of $>58.8\text{pg/mL}$ had a high AUC of 0.774($p=0.0222$) although its combined sensitivity, specificity, PPV and NPV of 100%, 55.56%, 13% and 100% respectively were not better than those of IL-10. Table 5 summarises the diagnostic accuracy of the various cytokines in identifying acute HBV infection.

4.7.2 HBV Recovery

In the HBV recovery group, IL-4 had the highest AUC of 0.716 and at a threshold value of $>37.5\text{pg/ml}$ and 95% confidence interval had a sensitivity of 66.7% and a specificity of 84.4% which was significantly different from the specificities of IL-12p70, TNF- α and IFN- γ . IL-4 had the best combined PPV and NPV of 53.3% and 90.5% respectively compared to the other cytokines. IL-12p70 had the highest sensitivity of 91.67% in this group although it had a specificity of 40.0% and an AUC of 0.512 ($p=0.8841$). Table 6 summarises the diagnostic accuracy for the various cytokines and liver biomarkers in the HBV recovery group.

4.7.3 HBeAg Positive CHB Infection

At a threshold of ≤ 30 pg/mL, IL-6 had an AUC of 0.75 ($p=0.0149$) with sensitivity, specificity, PPV and NPV of 80%, 71.1%, 23.5% and 96.9% respectively. IL-12p70 at a threshold value of >45 pg/mL had an AUC of 0.778 ($p=0.004$) and had a sensitivity, specificity, PPV and NPV of 100%, 62.2%, 22.7 and 100% respectively. Though IL-6 and IL-12p70 had similar AUC's, sensitivities, PPV and NPV, IL-6 had a better specificity compared to IL-12p70 making it a better maker for identifying this group of HBV infections. Table 7 summarizes the diagnostic accuracy of Th1 and Th2 cytokines in HBeAg positive CHB infection.

4.7.4 HBeAg Negative CHB Infection

The AUC of IL-10 was 0.66 ($p= 0.008$) and had a sensitivity, specificity, PPV and NPV of 67.0%, 60.0%, 78.8% and 45.0% at a threshold of >4.8 pg/ml. At a threshold of >13.8 pg/mL, IL-4 had an AUC of 0.642 ($p=0.0056$) a sensitivity, specificity, PPV and NPV of 84%, 46.67%, 77.8% and 56.8% respectively. Although IL-6 had a very good specificity (73.3%), its sensitivity was low (43%). Taken together, IL-10 was the best marker for identifying HBeAg negative CHB. Table 8 summarizes the diagnostic accuracy of cytokines and liver biomarkers in HBeAg negative chronic infection.

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Table 5. Diagnostic accuracies of Th1 and Th2 cytokines for acute HBV infections

| Parameters | Threshold value | AUC (95% CI) | Sensitivity% (95% CI) | Specificity% (95% CI) | PPV | NPV | LR+/LR- | p-value |
|---------------|-----------------|------------------------------|--------------------------|---------------------------|-------------|-------------|--------------------|---------------|
| IL-12p70 | >10 | 0.556(0.405 to 0.699) | 100(29.2 - 100.0) | 44.44(29.6 - 60.0) | 10.7 | 100.0 | 1.80 : 0.00 | 0.536 |
| TNF- α | >39 | 0.648(0.497 to 0.780) | 100(29.2 - 100.0) | 55.56(40.0 - 70.4) | 13.0 | 100.0 | 2.25 : 0.00 | 0.1338 |
| IFN- γ | >42 | 0.585(0.434 to 0.726) | 100(29.2-100.0) | 40(25.7 - 55.7) | 10.0 | 100.0 | 1.67 : 0.00 | 0.5792 |
| IL-4 | \leq 11.3 | 0.559 (0.409 to 0.702) | 66.67(9.4 - 99.2) | 60(44.3 - 74.3) | 11.1 | 94.9 | 1.67 : 0.56 | 0.7876 |
| IL-6 | >58.8 | 0.774(0.630 to 0.882) | 100(29.2-100.0) | 55.56(40.0 - 70.4) | 13.0 | 100.0 | 2.25 : 0.00 | 0.0222 |
| IL-10 | >15 | 0.722(0.574 to 0.842) | 66.67(9.4 - 99.2) | 86.67(73.2 - 94.9) | 25.0 | 97.5 | 5.00 : 0.38 | 0.2256 |

AUC = Area under the curve, CI = confidence interval, PPV = positive predictive value, NPV = Negative predictive value, LR+= positive likelihood ratio, LR- = negative likelihood ratio

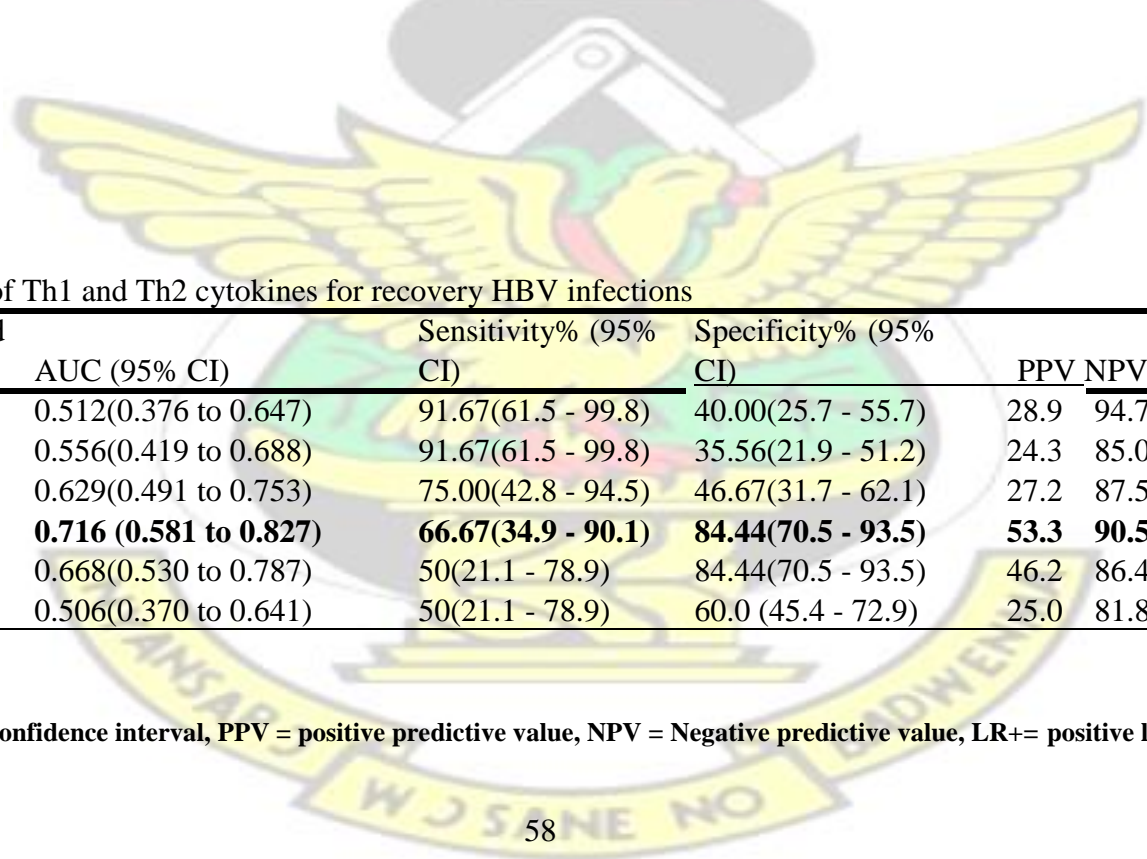


Table 6. Diagnostic accuracies of Th1 and Th2 cytokines for recovery HBV infections

| Parameters | Threshold value | AUC (95% CI) | Sensitivity% (95% CI) | Specificity% (95% CI) | PPV | NPV | LR+/LR- | p-value |
|---------------|-----------------|-------------------------------|---------------------------|---------------------------|-------------|-------------|--------------------|---------------|
| IL-12p70 | >8 | 0.512(0.376 to 0.647) | 91.67(61.5 - 99.8) | 40.00(25.7 - 55.7) | 28.9 | 94.7 | 1.53 : 0.21 | 0.8841 |
| TNF- α | \leq 102 | 0.556(0.419 to 0.688) | 91.67(61.5 - 99.8) | 35.56(21.9 - 51.2) | 24.3 | 85.0 | 1.42 : 0.23 | 0.500 |
| IFN- γ | \leq 59.4 | 0.629(0.491 to 0.753) | 75.00(42.8 - 94.5) | 46.67(31.7 - 62.1) | 27.2 | 87.5 | 1.41 : 0.54 | 0.1691 |
| IL-4 | >37.5 | 0.716 (0.581 to 0.827) | 66.67(34.9 - 90.1) | 84.44(70.5 - 93.5) | 53.3 | 90.5 | 4.29 : 0.39 | 0.0534 |
| IL-6 | >99 | 0.668(0.530 to 0.787) | 50(21.1 - 78.9) | 84.44(70.5 - 93.5) | 46.2 | 86.4 | 3.21 : 0.59 | 0.0724 |
| IL-10 | <3.6 | 0.506(0.370 to 0.641) | 50(21.1 - 78.9) | 60.0 (45.4 - 72.9) | 25.0 | 81.8 | 0.85 : 1.34 | 0.956 |

AUC = Area under the curve, CI = confidence interval, PPV = positive predictive value, NPV = Negative predictive value, LR+= positive likelihood ratio, LR- = negative likelihood ratio

Table 7. Diagnostic accuracies of Th1 and Th2 cytokines for HBeAg positive Chronic HBV infections

| Parameters | Threshold value | AUC (95% CI) | Sensitivity | Specificity | PPV | NPV | LR+/LR- | p-value |
|---------------|-----------------------------|------------------------------|---------------------------|---------------------------|-------------|-------------|--------------------|---------------|
| IL-12p70 | >45 | 0.778(0.638 to 0.883) | 100.00(47.8 - 100.0) | 62.22(46.5 - 76.2) | 22.7 | 100.0 | 2.65 : 0.00 | 0.0004 |
| TNF- α | >90 | 0.562(0.415 to 0.702) | 80.00(28.4 - 99.5) | 62.22(46.5 - 76.2) | 19.1 | 95.4 | 2.12 : 0.32 | 0.6192 |
| IFN- γ | \leq 126 | 0.511(0.366 to 0.655) | 60.00(14.7 - 94.7) | 17.78(8.0 - 32.1) | 7.5 | 80.0 | 0.73 : 2.25 | 0.9481 |
| IL-4 | >10 | 0.664(0.517 to 0.792) | 100.00(47.8 - 100.0) | 40.00(25.7 - 55.7) | 15.6 | 100.0 | 1.67 : 0.00 | 0.1681 |
| IL-6 | \leq30 | 0.753(0.611 to 0.864) | 80.00(28.4 - 99.5) | 71.11(55.7 - 83.6) | 23.5 | 96.9 | 2.77 : 0.28 | 0.0149 |
| <u>IL-10</u> | <u>\geq6.6</u> | <u>0.533(0.387 to 0.676)</u> | <u>60.00(14.7 - 94.7)</u> | <u>68.89(53.4 - 81.8)</u> | <u>17.6</u> | <u>93.9</u> | <u>1.93 : 0.58</u> | <u>0.8603</u> |

AUC = Area under the curve, CI = confidence interval, PPV = positive predictive value, NPV = Negative predictive value, LR+= positive likelihood ratio, LR- = negative likelihood ratio

Table 8. Diagnostic accuracies of Th1 and Th2 cytokines for HBeAg negative Chronic HBV infections

| Parameters | Threshold value | AUC (95% CI) | Sensitivity | Specificity | PPV | NPV | LR+/LR- | p-value |
|---------------|-----------------|------------------------------|---------------------------|---------------------------|-------------|-------------|--------------------|---------------|
| IL-12p70 | >15 | 0.595(0.510 to 0.675) | 67.00(56.9 - 76.1) | 53.33(37.9 - 68.3) | 76.1 | 42.1 | 1.44 : 0.62 | 0.0729 |
| TNF- α | >12 | 0.573(0.488 to 0.654) | 81.00(71.9 - 88.2) | 35.56(21.9 - 51.2) | 73.6 | 45.7 | 1.26 : 0.53 | 0.1723 |
| IFN- γ | >75 | 0.600(0.515 to 0.680) | 51.00(40.8 - 61.1) | 66.67(51.0 - 80.0) | 77.3 | 37.9 | 1.53 : 0.74 | 0.0506 |
| IL-4 | >13.8 | 0.642(0.558 to 0.720) | 84.00(75.3 - 90.6) | 46.67(31.7 - 62.1) | 77.8 | 56.8 | 1.57 : 0.34 | 0.0056 |
| IL-6 | >66 | 0.529(0.444 to 0.612) | 43.00(33.1 - 53.3) | 73.33(58.1 - 85.4) | 78.2 | 36.7 | 1.61 : 0.78 | 0.5665 |
| IL-10 | >4.8 | 0.659(0.576 to 0.736) | 67.00(56.9 - 76.1) | 60.00(44.3 - 74.3) | 78.8 | 45.0 | 1.67 : 0.55 | 0.0008 |

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AUC = Area under the curve, CI = confidence interval, PPV = positive predictive value, NPV = Negative predictive value, LR+= positive likelihood ratio, LR- = negative likelihood ratio



4.8 ASSOCIATION OF CYTOKINES AND LIVER BIOMARKERS WITH VARIOUS HBV INFECTION STAGES

4.8.1 ACUTE HBV INFECTION AND HBV RECOVERY

In acute HBV infection group, IL-10 levels at a threshold of $>15\text{pg/mL}$ was 26.89 times more likely to identify acute HBV infection ($p=0.0127$), the highest OR in this group. IL-12p70 levels at a threshold $>10\text{pg/mL}$ was on the other hand the least likely cytokine to identify acute HBV infection with OR of 1.6 ($p=0.901$).

In the HBV recovery group, IL-4 at a threshold of $>37.5\text{pg/mL}$ had the highest significant OR for identifying HBV recovery ($p=0.0022$). It was 8.686 times more likely to identify HBV infection whilst IL-12p70 at a threshold of $>8\text{pg/mL}$ was 7.333 times more likely to identify HBV recovery ($p=0.0452$). IL-6 at a threshold of $>99\text{pg/ml}$ was also 5.429 times more likely to identify HBV recovery ($p=0.0200$). Table 9 summarizes the odds ratio (OR) of the various cytokines and liver biomarkers as well as their level of significance in acute HBV infections and HBV recovery

4.8.2 HBeAg POSITIVE CHB AND HBeAg NEGATIVE CHB

In HBeAg positive CHB, IL-12p70 had the highest significant OR for identifying this group (0.0124). At a threshold of $>45\text{pg/ml}$, IL-12p70 was 17.91 times more likely to identify HBeAg positive CHB. Similarly, IL-6 at a threshold of $\leq 30\text{pg/mL}$ was 9.846 times more likely to identify HBeAg positive CHB ($p = 0.0400$).

In HBeAg negative CHB, IL-4 had the highest significant OR followed by IL-10, TNF- α and IL-12p70 in decreasing order for identifying this group. IL-4 at a threshold of $>13.8\text{pg/mL}$ was 4.594 more likely to identify HBeAg negative CHB ($p=0.0002$) whilst IL-10 at a threshold of $>4.8\text{pg/ml}$ was 3.045 times more likely to identify HBeAg negative CHB ($p=0.0034$). Table 10 summarizes the Odds Ratio (OR) of the various cytokines and liver

biomarkers at a specific threshold and their level of significance in HBeAg positive CHB and HBeAg CHB infections

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Table 9 Multivariate logistic regressions of Th1 and Th2 cytokines for Acute HBV Infections and HBV recovery

| Parameters | Threshold | OR (95% CI) | p-value | Threshold | OR (95% CI) | p-value |
|-------------------------|-------------|----------------------|----------------------------|-------------|---------------------|---------------|
| <u>Acute HBV</u> | | | <u>HBV Recovery</u> | | | |
| IL-12p70 (pg/ml) | >10 | 1.600(0.1351- 18.95) | 0.901 | >8 | 7.333(0.8691-61.88) | 0.0452 |
| TNF- α (pg/ml) | >39 | 8.707(0.4247- 178.5) | 0.1024 | \leq 102 | 6.069(0.7164-51.41) | 0.0851 |
| IFN- γ (pg/ml) | >42 | 4.709(0.2294- 96.69) | 0.2819 | \leq 59.4 | 2.625(0.627- 10.99) | 0.2078 |
| IL-4 (pg/ml) | \leq 11.3 | 3.000(0.2527- 35.61) | 0.5629 | >37.5 | 8.686(2.189- 34.46) | 0.0022 |
| IL-6 (pg/ml) | >58.8 | 7.977(0.3893- 163.5) | 0.2340 | >99 | 5.429(1.353- 21.79) | 0.0200 |
| IL-10 (pg/ml) | >15 | 26.89(0.574- 0.842) | 0.0127 | \leq 3.6 | 1.368(0.382- 4.908) | 0.7472 |

Table 10 Multivariate logistic regressions of Th1 and Th2 cytokines for HBeAg positive CHB infections and HBeAg negative CHB infections

| Parameters | Threshold | OR (95% CI) | p-value | Threshold | OR (95% CI) | p-value |
|----------------------------------|-----------|-------------|----------------------------------|-----------|-------------|---------|
| <u>HBeAg positive CHB</u> | | | <u>HBeAg negative CHB</u> | | | |

| | | | | |
|------|----------------------|---------------|-------|--------------------|
| >45 | 17.91(0.9317 -344.4) | 0.0124 | >15 | 2.320(1.131-4.762) |
| >90 | 6.588(0.6785 -63.98) | 0.1476 | >12 | 2.352(1.069-5.177) |
| ≤126 | 0.324(0.046 -2.271) | 0.2581 | >75 | 2.082(0.10-4.335) |
| >10 | 7.400(0.385- 142.1) | 0.1453 | >13.8 | 4.594(2.078-10.15) |
| ≤30 | 9.846(1.002 -96.72) | 0.0400 | >66 | 2.075(0.960-4.482) |
| >6.6 | 3.321(0.497 -22.16) | 0.3215 | >4.8 | 3.045(1.47 -6.306) |

IL-

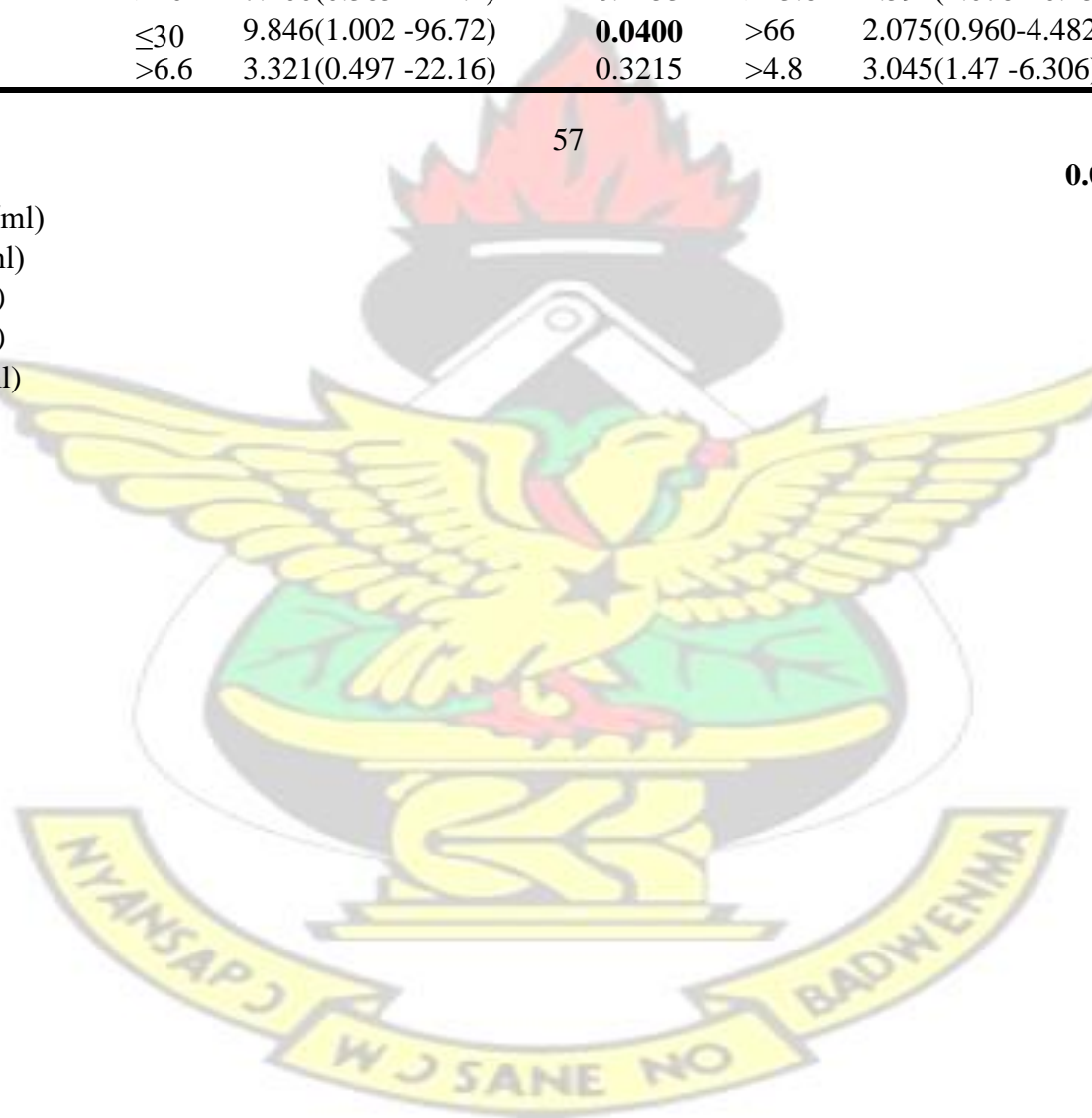
12p70(pg/ml)

57

0.0273

TNF- α (pg/ml)
 IFN- γ (pg/ml)
 IL-4(pg/ml)
 IL-6(pg/ml)
 IL-10(pg/ml)

0.0374
 0.0709
0.0002
 0.0669
0.0034



4.9 TH1 AND TH2 CYTOKINES AS MARKERS OF HEPATIC INJURY

4.9.1 Acute HBV infection and HBV Recovery

Table 11 is a summary of the relationships between various Th1 and Th2 cytokines and liver enzymes in acute HBV infection and HBV recovery. In the acute HBV group, there was a significant positive relation between TNF- α levels and ALT levels ($\beta=0.409$, $p=0.0016$). A unit increase in TNF- α will result in an increase in ALT levels by 0.409. Similarly, there was a significant positive relation between TNF- α levels and AST levels ($\beta=0.300$, $p=0.024$). In the HBV recovery group, there was no significant positive relation between any of the cytokines and liver enzymes.

4.9.2 HBeAg-positive CHB and HBeAg-negative CHB

Table 12 is a summary of the linear regression analysis between Th1 and Th2 cytokines and liver enzymes and in HBeAg-positive CHB and HBeAg-negative CHB. In the HBeAg-positive CHB, there was no significant positive relation between any of the Th1 and Th2 cytokines and liver enzymes.

In the HBeAg negative CHB group, there were significant positive relationships between IL10 levels and ALT levels ($\beta=0.443$, $p=0.034$) as well as between IL-10 levels and AST levels ($\beta=0.855$, $p=0.0005$). There was also a significant positive relation between TNF- α levels and ALP levels ($\beta=0.061$, $p=0.0006$).

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Table 11 Linear regression between Th1 and Th2 cytokines and liver enzymes in Acute HBV infections and HBV Recovery

| Parameters | AST | ALT | ALP | GGT |
|------------------|----------------------|-------------------------|----------------|-----------------------|
| Acute HBV | | | | |
| IL-12p70 | -0.112 (0.913) | -0.197 (0.888) | 0.232 (0.569) | -0.362 (0.104) |
| TNF- α | 0.300 (0.024) | 0.409 (0.0016) | 0.089 (0.542) | 0.001 (0.993) |
| IFN- γ | -0.208 (0.687) | -0.305 (0.661) | 0.063 (0.795) | -0.171 (0.330) |
| IL-4 | -0.296 (0.886) | -0.493 (0.860) | 0.442 (0.597) | -0.723 (0.132) |
| IL-6 | -0.031 (0.941) | -0.060 (0.915) | 0.098 (0.541) | -0.146 (0.076) |
| IL-10 | 0.768(0.831) | 0.890(0.857) HBV | 1.159(0.313) | -1.266 (0.152) |
| Recovery | | | | |
| IL-12p70 | 0.087 (0.5408) | -0.020 (0.828) | 0.183 (0.743) | 0.059 (0.824) |
| TNF- α | 0.016 (0.910) | -0.101 (0.241) | -0.148 (0.782) | 0.059 (0.819) |
| IFN- γ | -0.131 (0.427) | -0.110 (0.296) | -0.339 (0.603) | -0.298 (0.331) |
| IL-4 | -0.073 (0.272) | -0.0059(0.894) | -0.172 (0.514) | -0.107 (0.395) |
| IL-6 | 0.002 (0.967) | -0.034 (0.356) | 0.018 (0.935) | -0.050 (0.638) |
| IL-10 | 0.167 (0.816) | -0.407 (0.372) | 0.203 (0.942) | <u>-0.053 (0.968)</u> |

AST= Aspartate aminotransferase, ALT= Alanine aminotransferase, ALP= Alkaline phosphatase, GGT= Gamma-glutamyl transferase. Values presented as β (p-value)

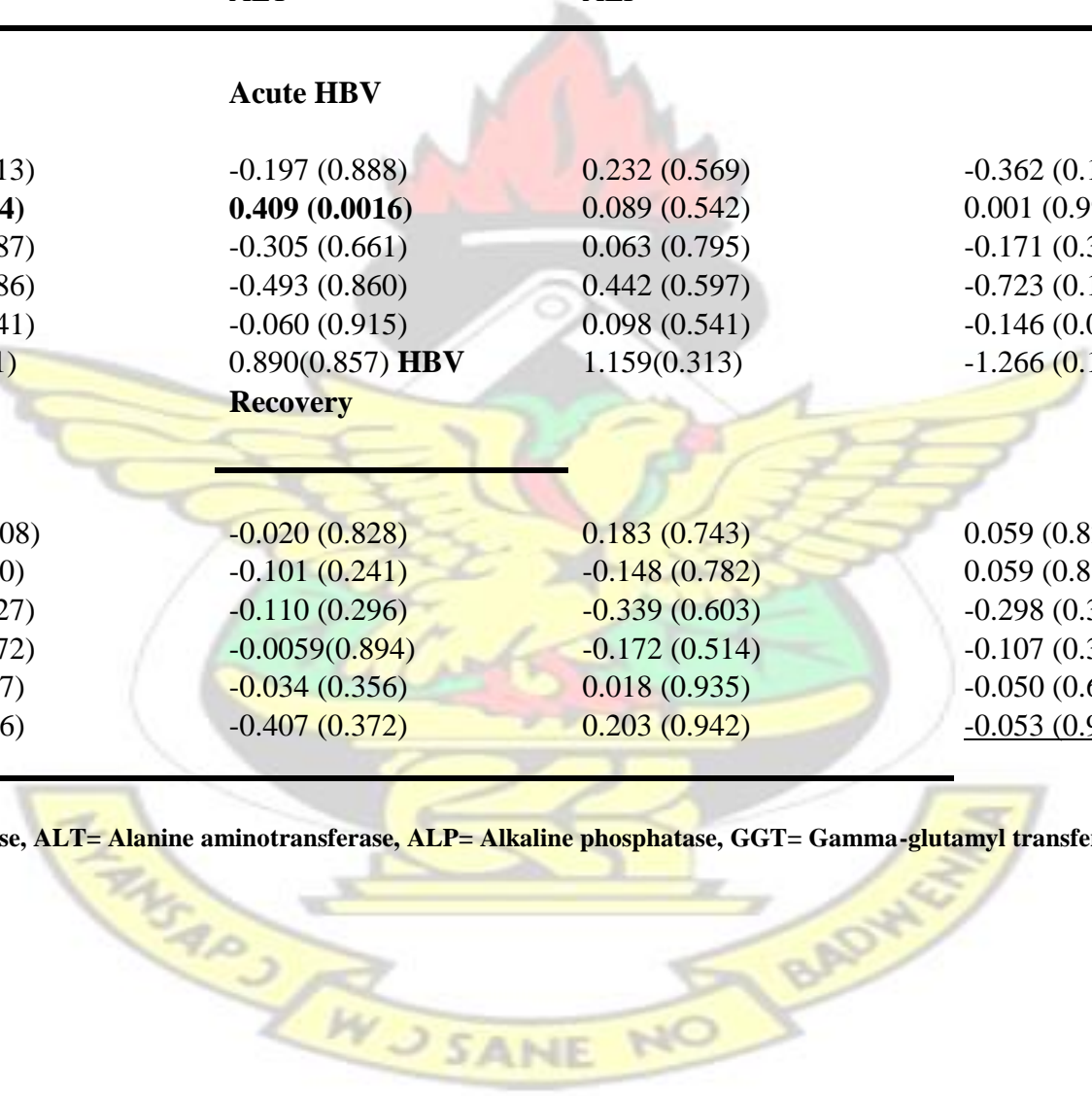


Table 12 Linear regression between Th1 and Th2 cytokines and liver enzymes in HBeAg positive CHB and HBeAg negative CHB

| Parameters | AST | ALT | ALP | GGT |
|---------------------------|-----------------------|----------------------|----------------------|----------------|
| <u>HBeAg positive CHB</u> | | | | |
| IL-12p70 | -0.367 (0.282) | -0.692 (0.211) | -0.205 (0.698) | -0.448 (0.243) |
| TNFa | 0.012 (0.979) | -0.061 (0.941) | -0.119 (0.862) | -0.204 (0.711) |
| IFNg | -0.281 (0.448) | -0.545 (0.371) | 0.647 (0.156) | -0.453 (0.253) |
| IL-4 | -0.810 (0.523) | -1.324 (0.535) | -1.753 (0.292) | -0.993 (0.491) |
| IL-6 | 1.527 (0.127) | 2.616 (0.115) | -0.418 (0.809) | 1.604 (0.182) |
| IL-10 | -2.349 (0.377) | -4.175 (0.345) | -2.186 (0.575) | -3.350 (0.246) |
| <u>HBeAg negative CHB</u> | | | | |
| IL-12p70 | -0.102 (0.350) | -0.087 (0.338) | -0.023 (0.723) | -0.187 (0.163) |
| TNFa | 0.030 (0.326) | 0.016 (0.531) | 0.061(0.0006) | 0.067(0.073) |
| IFNg | -0.099 (0.194) | -0.077 (0.227) | -0.031 (0.496) | -0.084 (0.371) |
| IL-4 | -0.063 (0.703) | -0.059 (0.668) | -0.022 (0.827) | 0.295 (0.148) |
| IL-6 | 0.018 (0.826) | 0.022 (0.750) | 0.023(0.644) | 0.034 (0.739) |
| IL-10 | 0.855 (0.0005) | 0.443 (0.034) | -0.025 (0.871) | -0.014 (0.964) |

AST= Aspartate aminotransferase, ALT= Alanine aminotransferase, ALP= Alkaline phosphatase, GGT= Gamma-glutamyl transferase. Values presented as β (pvalue)



CHAPTER 5

DISCUSSION

5.1 HBV STAGES

The various infection stages as identified in this study included acute HBV, HBV recovery, HBeAg positive CHB and HBeAg negative CHB. Majority of participant in our current study were HBeAg negative CHB patients (Figure 3). A study in Norway among immigrants found chronic hepatitis B as the most diagnosed HBV infection (Rimšėlienė *et al.*, 2011). At the onset of HBV infection, the host immune system mounts up a strong T-cell mediated cellular response in order to clear the virus. In situations where the immune response is not effective enough to clear the virus, the infection progresses from acute to chronic infection (Rehermann and Nascimbeni, 2005). HBeAg positivity was 6.67% among our HBV cases which was lower than the 13.3% which was reported in a cross-sectional study in Ghana (Rufai *et al.*, 2014). HBeAg and the HBV DNA are markers of HBV replication and infectivity (Krajden *et al.*, 2005). Our results therefore indicates that a lower proportion of HBV infected patients in the Ghanaian population are able to transmit the infection to others and the virus keeps replicating.

5.2 LIVER MARKERS IN VARIOUS STAGES OF HBV INFECTION

ALT which is a more specific liver enzyme was significantly higher in HBeAg positive CHB compared to control group (Table 3). Falasca *et al.* (2006) also observed a significantly high ALT levels among chronic hepatitis B patients in a cross-sectional study in Italy. In a large study in a Chinese cohort who were HBeAg positive, elevated ALT levels at presentation were more likely to sero-convert to HBeAb (Yuen *et al.*, 2003). Persistence of HBeAg for a longer period of time elicits cellular immune responses to fight the HBV. These immune responses

lead to destruction of hepatocytes resulting in the release of liver specific enzymes including ALT. As much as 23.3% of HBV cases had elevated ALT levels (Table 4).

Albumin was significantly lower in HBeAg negative CHB compared to controls (Table 3).

Albumin gives us an idea of the biosynthetic capacity of the liver (Busher, 1990). This presupposes that HBV infection lowers the liver's ability to synthesize albumin over a long

period of time. Globulins levels were also significantly high in HBV recovery and HBeAg negative CHB groups compared to the control group in this study (Table 3). Globulin levels

have correlated with development of hepatic fibrosis in chronic hepatitis B infection and has been shown to be a good predictive marker for fibrosis (Schmilovitz-Weiss *et al.*, 2006). In

HBV recovery group, HBsAb which are immunoglobulins are produced to neutralise the HBsAg. These immunoglobulins form a big part of the globulin that is measured as part of the

liver chemistries (Busher, 1990). The humoral immunity which predominate in HBeAg negative CHB elicits the production of HBsAb in an attempt to neutralise HBsAg which were

not cleared by the initial cellular arm of the immune system and provide subsequent protection (Rehermann and Nascimbeni, 2005). Direct bilirubin levels were also significantly higher in

HBeAg negative CHB compared to the control group (Table 3). Intrahepatic cholestasis and HBV infection has been identified as some of the causes of increased direct bilirubin in adults

(Roche and Kobos, 2004). Destruction of hepatocytes by HBV in severe form of liver disease such as cirrhosis leads to the build-up of direct bilirubin.

5.3 ELEVATED LIVER ENZYMES

The current study observed elevated levels of various liver associated enzymes including ALT, AST, ALP and GGT among a high number of HBV infected patients in this study (Table

4). This is an indication of an underlying inflammatory process and puts these patients at an increased risk of developing cirrhosis and hepatocellular carcinoma. The host immune response

to HBV is what destroys hepatocytes leading to the release of these liver enzymes and their levels usually gives an indication of the extent of liver injury (Lin and Kirchner, 2004; Ocama *et al.*, 2005).

5.4 HBV ASSOCIATED LIVER DISEASES

Nine cases of NAFLD, two cases of intrahepatic cholestasis and one case of extrahepatic cholestasis were identified among HBV infected patients in our study (Figure 10). In a nested-case control study of patients with cirrhosis in Ghana, 42.9% were found to be associated with HBV (Blankson *et al.*, 2005). Cirrhosis and HCC are among the severe forms of HBV associated liver pathologies with high morbidity and mortality rate. Up to 73% of all liver cancers and liver cirrhosis is as a result of HBV infection (Ott *et al.*, 2012). Majority of all the liver diseases were recorded in HBeAg-negative CHB. Majority of HBV associated cirrhosis have been reported to occur in the HBeAg-negative stage of HBV infection (Pan and Zhang, 2005). The longer HBV persist in the host, the more immune response is mounted against it resulting in destruction of hepatocytes and the development of various liver diseases.

5.5 TH1/TH2 CYTOKINE PATTERNS IN HBV STAGES

The present study found a significantly increased concentration of IL-4 among participants with HBV recovery and HBeAg negative CHB (Figure 7). The significant increase in IL-4 among HBV recovery group is consistent with a case-control study by Penna *et al.* (1997) among Italian cohort. He *et al.* (2013) in a case-control study among a Chinese population and Falasca *et al.* (2006) also in a case-control study among an Italian population all observed a significant elevation in IL-4 among CHB infections which is in agreement with this study.

Increased concentration of IL-4 have been shown to suppress HBsAg mRNA, the pregenomic RNA and the precore mRNA which secretes the HBeAg (Lin *et al.*, 2003). This may lead to

sero-clearance of HBsAg and HBeAg and subsequently sero-conversion to HBsAb and HBeAb respectively. Again, following HBV infection, there is an initial T-cell mediated response characterised by Th1 cytokines leading to the clearing of the HBV, IL-4 polarizes T-lymphocyte response towards Th2 type cytokines and activates humoral immune response. IL-4 is also able to stimulate the proliferation of B-cell and subsequently leading to the production of HBsAb in the recovery phase in the long term.

Again, this study observed a reduction in IL-10 levels among participant who had recovered from HBV infection but elevated in acute infections, HBeAg positive CHB and significantly in HBeAg negative CHB compared to controls (Figure 9). The significant elevation in IL-10 among participants with HBeAg negative CHB is consistent with findings by Khan *et al.* (2011) in a case control study in an Indian population and Falasca *et al.* (2006) in a case control study among an Italian population. IL-10 has immune-regulatory properties able to inhibit the secretion of Th1 cytokines and suppressing cellular immune response to HBV thus shifting the balance towards a Th2 type profile which favours persistent or chronic infection (Asadullah *et al.*, 2003; Das *et al.*, 2012).

There was a shift towards pro-inflammatory cytokines in acute phase and HBeAg-positive phase of HBV infection as seen by the high levels of IL-12p70 and IL-6 in these phases. In a case-control study among Chinese population, He *et al.* (2013) observed a significant elevation in IL-12p70 in HBV patients compared to controls. This difference in our findings could be due to the differences in the study population used. Whilst our study used adult participants with HBV, their study used children infected with HBV with normal ALT levels. The HBeAg-positive phase coincides with the immune tolerant and early immune active phases of chronic HBV infection. IL-12 mediates a strong T-cell mediated response against HBV through the

proliferation of Th1 cells and production of Th1 cytokines such as IFN- γ for viral clearance (Rossol *et al.*, 1997).

There was an overall significant shift towards a Th2 cytokine pattern in HBV recovery and HBeAg-negative CHB as demonstrated by significant elevation in the levels of IL-4 and IL10. Jiang *et al.* (2002) and (Penna *et al.*, 1997) have demonstrated that chronic hepatitis B and HBV recovery phases may be associated with Th2 type cytokines.

5.6 DIAGNOSTIC ACCURACIES OF TH1/TH2 CYTOKINES

IL-4 showed the best accuracy (AUC of 0.72) for identifying HBV recovery compared to the other cytokines. It had the best specificity and sensitivity when a threshold point of $>37.5\text{pg/ml}$ was applied. It was 8.7 times more likely associated with HBV recovery (Table 9). Th2 cytokine like IL-4 are able to stimulate B cell culminating in an increased production of hepatitis B antibodies.

In this study IL-6 was identified as the best accurate marker (AUC of 0.75) for identifying HBeAg positive CHB. Its sensitivity and specificity were the best at a threshold point of $\leq 30\text{pg/ml}$. (Table 7). It was also found to be 9.8 times more likely to be associated with HBeAg positive CHB (Table 10). Increase levels of IL-6 cytokine has however been shown to be a probable predictor of hepatocellular carcinoma in chronic hepatitis B infection (Porta *et al.*, 2008; Kuo *et al.*, 2009).

IL-10 was identified as the best marker for identifying acute HBV (AUC of 0.72). It also had the best sensitivity and specificity when a threshold of $>15\text{pg/ml}$ was applied (Table 5). IL-10 was 27 times more likely to be associated with acute HBV infection (Table 9). IL-10 was also the best marker for identifying HBeAg negative CHB (AUC of 0.66). At a threshold of >4.8

pg/ml IL-10 was the most sensitive and specific in identifying HBeAg negative CHB (Table 8). IL-10 was 3 times more likely to be associated with HBeAg negative CHB (Table 10).

Gora-Gebka *et al.* (2003) demonstrated that there was no correlation in the serum levels of IL-6 and IL-12 in pre-treatment and post-treatment with interferon-alpha (IFN- α) among children with chronic hepatitis B. IL-10 on the other hand has been shown to predict acute on chronic liver failure in HBV infection (Wang *et al.*, 2014). Again, IL-6 has been shown to be a good prognostic marker for HCC in HBV (Sheng *et al.*, 2015b). However this is the first time, we are describing the diagnostic accuracies of these Th1 and Th2 cytokines in identifying acute HBV infection, HBV recovery, HBeAg-positive CHB and HBeAg-negative CHB in Ghana.

5.7 TH1 AND TH2 CYTOKINES AS MARKERS OF HEPATIC INJURY

Our results show that there is a significant positive relation with TNF- α and ALT as well as AST among persons with acute HBV infection (Table 11) as well as between TNF- α and ALP among patients who were HBeAg negative CHB (Table 12). Studies among patients with HCV suggest TNF- α as potential surrogate marker for liver damage and indeed is able to induce apoptosis in infected liver cells (Moura *et al.*, 2009). Significant levels of TNF- α have also been found in CHB patients compared to healthy population (González-Amaro *et al.*, 1994). Together with the fact that our results showed a positive relation among ALT and AST with TNF- α levels in acute HBV and HBeAg negative CHB, TNF- α could be used as a surrogate marker for hepatic injury in acute HBV and HBeAg negative CHB infections. There was also a significant positive relation between IL-10 and ALT as well as between IL10 and AST among persons who were HBeAg negative CHB (Table 12). A study among an Indian population revealed as increasing association of IL-10 with increasing severity of HBV associated liver

disease (Saxena *et al.*, 2014). IL-10 significantly correlated with the level of necroinflammation and fibrosis among HBV patients who were HBeAg negative in Thailand (Poovorawan *et al.*, 2013). With IL-10 significantly relating to other markers of liver injury, it could therefore be used as a surrogate marker for liver injury among HBeAg negative CHB in Ghana.

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CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

This study explored the disease pattern of Th1 and Th2 cytokines and their diagnostic accuracy in identifying the various stages of HBV infections as well as HBV associated liver pathology among Ghanaian adult population. Majority of persons with HBV infection were HBeAg negative CHB and the overall HBeAg positivity was low. There was a significant elevation in ALT, globulin and direct bilirubin levels in persons with HBV infection compared to control groups whilst there was a significant reduction in albumin among HBV infection compared to healthy controls. A high proportion of HBV patients had elevated liver enzymes while NAFLD was the commonest liver disease found. It stands that although a good number of HBV patients have elevated liver enzymes, there was no severe form of liver disease found again not many of HBV patients are infectious. HBV patients and the general public should therefore not be alarmed.

IL-4 was significantly elevated in both HBV recovery and HBeAg negative CHB whilst IL-10 was significantly elevated in HBeAg negative CHB compared to controls. Levels of IL12p70, TNF- α , IFN- γ and IL-6 were not statistically significant different in HBV cases compared to controls. For the first time, IL-4 was identified as the best diagnostic marker for identifying HBV recovery, IL-6 for identifying HBeAg positive CHB whilst IL-10 identified

acute HBV and HBeAg negative CHB infections. These cytokines could therefore be added in the work up in the diagnosis of the various stages of HBV infection.

The positive relationship between TNF- α and IL-10 with liver enzymes (ALT, AST and ALP) suggest that they could also be used as surrogate markers for liver injury in HBV infection.

The correlation of these cytokines to the various stage of HBV infections highlights the critical role of these molecules in the pathogenesis of HBV from infection. This could be a useful information to improve the clinical management of HBV infected patients.

6.2 LIMITATIONS

The current study did not carry out HBV DNA viral load estimation in classifying the various cases of HBV, participants with occult HBV who may stand the chance of transmitting HBV through blood transfusion may have been missed. Also the number of participants in various HBV groups were not proportional and could have affected the analysis. Lastly, platelet count, international normalize ratio (INR), liver ultrasound and liver histology were also not used in assessing liver disease hence some forms of liver disease may have also been missed and as such should be considered in future studies.

6.3 RECOMMENDATIONS

Screening for HBcAb should be considered in potential blood donors in order to reduce proportion of persons with occult HBV who could transmit HBV through blood transfusion.

Future studies should consider increasing the number of study participants proportionally in these various groups of HBV. In order to gain fair view of the prognostic value of Th1 and Th2 cytokines in HBV infection, we recommend a longitudinal study design for future studies.

REFERENCES

- Amidu N., Alhassan A., Obirikorang C., Feglo P., Majeed S., Timmy-Donkoh E. and Afful D. (2012) Sero-prevalence of hepatitis B surface (HBsAg) antigen in three densely populated communities in Kumasi, Ghana. *Journal of Medical and Biomedical Sciences* 1(2).
- Arababadi M.K., Pourfathollah A.A., Jafarzadeh A. and Hassanshahi G. (2010) Serum Levels of IL-10 and IL-17A in Occult HBV-Infected South-East Iranian Patients. *Hepat Mon* 10(1), 31-35.
- Asadullah K., Sterry W. and Volk H.D. (2003) Interleukin-10 Therapy—Review of a New Approach. *Pharmacological Reviews* 55(2), 241-269.
- Bertoletti A. and Gehring A.J. (2006) The immune response during hepatitis B virus infection. *J Gen Virol* 87(Pt 6), 1439-1449.
- Bessone F. (2014) Re-appraisal of old and new diagnostic tools in the current management of chronic hepatitis B. *Liver International* 34(7), 991-1000.
- Biermer M., Puro R. and Schneider R.J. (2003) Tumor Necrosis Factor Alpha Inhibition of Hepatitis B Virus Replication Involves Disruption of Capsid Integrity through Activation of NF- κ B. *Journal of Virology* 77(7), 4033-4042.
- Blankson A., Wiredu E.K., Gyasi R.K., Adjei A. and Tettey Y. (2005) Sero-Prevalence of Hepatitis B and C Viruses in Cirrhosis of the Liver in Accra, Ghana. *Ghana Med J* 39(4), 132-137.
- Busher J.T. (1990) Serum albumin and globulin. In *Clinical Methods: The History, Physical, and Laboratory Examinations* [H.K. Walker, W.D. Hall and J.W. Hurst, editors]. Boston: Butterworths.

- Calabrese L.H., Zein N. and Vassilopoulos D. (2004) Safety of antitumour necrosis factor (anti-TNF) therapy in patients with chronic viral infections: hepatitis C, hepatitis B, and HIV infection. *Annals of the Rheumatic Diseases* 63(suppl 2), ii18-ii24.
- Cameron M.J. and Kelvin D.J. (2003) Cytokines and chemokines--their receptors and their genes: an overview. *Adv Exp Med Biol* 5208-32.
- Cantini F., Boccia S., Goletti D., Iannone F., Leoncini E., Panic N., Prignano F. and Gaeta G.B. (2014) HBV Reactivation in Patients Treated with Antitumor Necrosis FactorAlpha (TNF- α) Agents for Rheumatic and Dermatologic Conditions: A Systematic Review and Meta-Analysis. *International Journal of Rheumatology* 20149.
- Cavanaugh V.J., Guidotti L.G. and Chisari F.V. (1997) Interleukin-12 inhibits hepatitis B virus replication in transgenic mice. *Journal of Virology* 71(4), 3236-3243.
- Chang J.J. and Lewin S.R. (2006) Immunopathogenesis of hepatitis B virus infection. *Immunol Cell Biol* 85(1), 16-23.
- Cheong J.Y., Cho S.W., Hwang I.L., Yoon S.K., Lee J.H., Park C.S., Lee J.E., Hahm K.B. and Kim J.H. (2006) Association between chronic hepatitis B virus infection and interleukin-10, tumor necrosis factor- α gene promoter polymorphisms. *J Gastroenterol Hepatol* 21(7), 1163-1169.
- Chevaliez S. and Pawlotsky J.-M. (2008) Diagnosis and management of chronic viral hepatitis: antigens, antibodies and viral genomes. *Best Practice & Research Clinical Gastroenterology* 22(6), 1031-1048.
- Chisari F.V., Isogawa M. and Wieland S.F. (2010) Pathogenesis of hepatitis B virus infection. *Pathol Biol (Paris)* 58(4), 258-266.
- D'Andrea A., Rengaraju M., Valiante N.M., Chehimi J., Kubin M., Aste M., Chan S.H., Kobayashi M., Young D. and Nickbarg E. (1992) Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. *J Exp Med* 176(5), 1387-1398.

- D'Souza R. and Foster G.R. (2004) Diagnosis and treatment of chronic hepatitis B. *Journal of the Royal Society of Medicine* 97(7), 318-321.
- Das A., Ellis G., Pallant C., Lopes A.R., Khanna P., Peppas D., Chen A., Blair P., Dusheiko G., Gill U., Kennedy P.T., Brunetto M., Lampertico P., Mauri C. and Maini M.K. (2012) IL-10-producing regulatory B cells in the pathogenesis of chronic hepatitis B virus infection. *J Immunol* 189(8), 3925-3935.
- Dinarello C.A. (2000) Proinflammatory cytokines. *Chest* 118(2), 503-508.
- Dinarello C.A. (2007) Historical Review of Cytokines. *European journal of immunology* 37(Suppl 1), S34-S45.
- Dunn C., Brunetto M., Reynolds G., Christophides T., Kennedy P.T., Lampertico P., Das A., Lopes A.R., Borrow P., Williams K., Humphreys E., Afford S., Adams D.H., Bertolotti A. and Maini M.K. (2007) Cytokines induced during chronic hepatitis B virus infection promote a pathway for NK cell-mediated liver damage. *J Exp Med* 204(3), 667-680.
- Falasca K., Ucciferri C., Dalessandro M., Zingariello P., Mancino P., Petrarca C., Pizzigallo E., Conti P. and Vecchiet J. (2006) Cytokine patterns correlate with liver damage in patients with chronic hepatitis B and C. *Ann Clin Lab Sci* 36(2), 144-150.
- Galun E., Nahor O., Eid A., Jurim O., Rose-John S., Blum H.E., Nussbaum O., Ilan E., Daudi N., Shouval D., Reisner Y. and Dagan S. (2000) Human interleukin-6 facilitates hepatitis B virus infection in vitro and in vivo. *Virology* 270(2), 299-309.
- Ganem D. and Prince A.M. (2004) Hepatitis B virus infection--natural history and clinical consequences. *N Engl J Med* 350(11), 1118-1129.
- Garcia-Tsao G., Friedman S., Iredale J. and Pinzani M. (2010) Now There Are Many (Stages) Where Before There Was One: In Search of a Pathophysiological Classification of Cirrhosis. *Hepatology* 51(4), 1445-1449.

- Gee K., Guzzo C., Che Mat N.F., Ma W. and Kumar A. (2009) The IL-12 family of cytokines in infection, inflammation and autoimmune disorders. *Inflamm Allergy Drug Targets* 8(1), 40-52.
- González-Amaro R., García-Monzón C., García-Buey L., Moreno-Otero R., Alonso J.L., Yagüe E., Pivel J.P., López-Cabrera M., Fernández-Ruiz E. and Sánchez-Madrid F. (1994) Induction of tumor necrosis factor alpha production by human hepatocytes in chronic viral hepatitis. *J Exp Med* 179(3), 841-848.
- Gora-Gebka M., Liberek A., Szydłowska-Lysiak W., Bako W. and Korzon M. (2003) Serum interleukin 6 and interleukin 12 levels in children with chronic hepatitis HBV treated with interferon alpha. *Ann Hepatol* 2(2), 92-97.
- Guidotti L.G., McClary H., Loudis J.M. and Chisari F.V. (2000) Nitric Oxide Inhibits Hepatitis B Virus Replication in the Livers of Transgenic Mice. *J Exp Med* 191(7), 1247-1252.
- Hahné S., van Houdt R., Koedijk F., van Ballegooijen M., Cremer J., Bruisten S., Coutinho R. and Boot H. (2013) Selective Hepatitis B Virus Vaccination Has Reduced Hepatitis B Virus Transmission in The Netherlands. *PLoS ONE* 8(7), e67866.
- Hall P. and Cash J. (2012) What is the Real Function of the Liver _Function‘ Tests? *The Ulster Medical Journal* 81(1), 30-36.
- He D., Li M., Guo S., Zhu P., Huang H., Yan G., Wu Q., Tao S., Tan Z. and Wang Y. (2013) Expression Pattern of Serum Cytokines in Hepatitis B Virus Infected Patients with Persistently Normal Alanine Aminotransferase Levels. *Journal of Clinical Immunology* 33(7), 1240-1249.
- Heim M.H. (1999) The Jak-Stat Pathway: Cytokine Signalling from the Receptor to the Nucleus. *Journal of Receptors and Signal Transduction* 19(1-4), 75-120.
- Hou J., Liu Z. and Gu F. (2005) Epidemiology and Prevention of Hepatitis B Virus Infection. *Int J Med Sci* 2(1), 50-57.

- Hussain M.J., Lau J.Y., Williams R. and Vergani D. (1994) Hepatic expression of tumour necrosis factor-alpha in chronic hepatitis B virus infection. *Journal of Clinical Pathology* 47(12), 1112-1115.
- Hyodo N., Nakamura I. and Imawari M. (2004) Hepatitis B core antigen stimulates interleukin-10 secretion by both T cells and monocytes from peripheral blood of patients with chronic hepatitis B virus infection. *Clinical and Experimental Immunology* 135(3), 462-466.
- Jiang R., Feng X., Guo Y., Lu Q., Hou J., Luo K. and Fu N. (2002) T helper cells in patients with chronic hepatitis B virus infection. *Chin Med J (Engl)* 115(3), 422-424.
- Kakimi K., Guidotti L.G., Koezuka Y. and Chisari F.V. (2000) Natural killer T cell activation inhibits hepatitis B virus replication in vivo. *J Exp Med* 192(7), 921-930.
- Khan S., Bhargava A., Pathak N., Maudar K.K., Varshney S. and Mishra P.K. (2011) Circulating Biomarkers and their Possible Role in Pathogenesis of Chronic Hepatitis B and C Viral Infections. *Indian Journal of Clinical Biochemistry* 26(2), 161-168.
- Kishimoto T. (2010) IL-6: from its discovery to clinical applications. *International Immunology* 22(5), 347-352.
- Koziel M.J. (1999) Cytokines in viral hepatitis. *Semin Liver Dis* 19(2), 157-169.
- Krajden M., McNabb G. and Petric M. (2005) The laboratory diagnosis of hepatitis B virus. *The Canadian Journal of Infectious Diseases & Medical Microbiology* 16(2), 65-72.
- Kuo T.-M., Hu C.-p., Chen Y.-L., Hong M.-H., Jeng K.-S., Liang C., Chen M.-L. and Chang C. (2009) HBV replication is significantly reduced by IL-6. *J Biomed Sci* 16(1), 41.
- Le Page C., Genin P., Baines M. and Hiscott J. (1999) Interferon activation and innate immunity. *Reviews in immunogenetics* 2(3), 374-386.
- Lee Y., Park U.S., Choi I., Yoon S.K., Park Y.M. and Lee Y.I. (1998) Human interleukin 6 gene is activated by hepatitis B virus-X protein in human hepatoma cells. *Clinical*

- Cancer Research* 4(7), 1711-1717.
- Lin K.W. and Kirchner J.T. (2004) Hepatitis B. *American family physician* 69(1), 75-82.
- Lin S.-J., Shu P.-Y., Chang C., Ng A.-K. and Hu C.-p. (2003) IL-4 suppresses the expression and the replication of hepatitis B virus in the hepatocellular carcinoma cell line Hep3B. *The Journal of Immunology* 171(9), 4708-4716.
- Liu J., Cao S., Herman L.M. and Ma X. (2003) Differential Regulation of Interleukin (IL)-12 p35 and p40 Gene Expression and Interferon (IFN)- γ -primed IL-12 Production by IFN Regulatory Factor 1. *J Exp Med* 198(8), 1265-1276.
- Lok A.S., Heathcote E.J. and Hoofnagle J.H. (2001) Management of hepatitis B: 2000—summary of a workshop. *Gastroenterology* 120(7), 1828-1853.
- Lok A.S. and McMahon B.J. (2001) Chronic hepatitis B. *Hepatology* 34(6), 1225-1241.
- Lu Y., Wu Z., Peng Q., Ma L., Zhang X., Zhao J., Qin X. and Li S. (2014) Role of IL-4 Gene Polymorphisms in HBV-Related Hepatocellular Carcinoma in a Chinese Population. *PLoS ONE* 9(10), e110061.
- Luzina I.G., Keegan A.D., Heller N.M., Rook G.A.W., Shea-Donohue T. and Atamas S.P. (2012) Regulation of inflammation by interleukin-4: a review of —alternatives. *Journal of Leukocyte Biology* 92(4), 753-764.
- Mahoney F.J. (1999) Update on Diagnosis, Management, and Prevention of Hepatitis B Virus Infection. *Clinical Microbiology Reviews* 12(2), 351-366.
- Merrill R.M. and Hunter B.D. (2011) Seroprevalence of markers for hepatitis B viral infection. *International Journal of Infectious Diseases* 15(2), e78-e121.
- Mkandawire P., Richmond C., Dixon J., Luginaah I.N. and Tobias J. (2013) Hepatitis B in Ghana's upper west region: a hidden epidemic in need of national policy attention. *Health Place* 2389-96.

- Moura A.S., Carmo R.A., Teixeira A.L. and Rocha M.O.d.C. (2009) Soluble inflammatory markers as predictors of hepatocellular damage and therapeutic response in chronic hepatitis C. *Brazilian Journal of Infectious Diseases* 13375-382.
- Nkrumah B., Owusu M., Frempong H.O. and Averu P. (2011) Hepatitis B and C viral infections among blood donors from rural Ghana. *Ghana Med J* 45(3), 97-100.
- Ocama P., Opio C.K. and Lee W.M. (2005) Hepatitis B virus infection: Current status. *The American Journal of Medicine* 118(12), 1413.e1415-1413.e1422.
- Osakunor D.N.M., Obirikorang C., Fianu V., Asare I. and Dakorah M. (2015) Hepatic Enzyme Alterations in HIV Patients on Antiretroviral Therapy: A Case-Control Study in a Hospital Setting in Ghana. *PLoS ONE* 10(8), e0134449.
- Ott J.J., Stevens G.A., Groeger J. and Wiersma S.T. (2012) Global epidemiology of hepatitis B virus infection: New estimates of age-specific HBsAg seroprevalence and endemicity. *Vaccine* 30(12), 2212-2219.
- Ouyang W., Rutz S., Crellin N.K., Valdez P.A. and Hymowitz S.G. (2011) Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu Rev Immunol* 2971-109.
- Pan C.Q. and Zhang J.X. (2005) Natural History and Clinical Consequences of Hepatitis B Virus Infection. *Int J Med Sci* 2(1), 36-40.
- Penna A., Del Prete G., Cavalli A., Bertoletti A., D'Elia M.M., Sorrentino R., D'Amato M., Boni C., Pilli M., Fiaccadori F. and Ferrari C. (1997) Predominant T-helper 1 cytokine profile of hepatitis B virus nucleocapsid-specific T cells in acute self-limited hepatitis B. *Hepatology* 25(4), 1022-1027.
- Pernis A., Witthuhn B., Keegan A.D., Nelms K., Garfein E., Ihle J.N., Paul W.E., Pierce J.H. and Rothman P. (1995) Interleukin 4 signals through two related pathways. *Proceedings of the National Academy of Sciences* 92(17), 7971-7975.

- Peters M. (1996) Actions of cytokines on the immune response and viral interactions: An overview. *Hepatology* 23(4), 909-916.
- Platanias L.C. (2005) Mechanisms of type-I-and type-II-interferon-mediated signalling. *Nature Reviews Immunology* 5(5), 375-386.
- Poovorawan K., Tangkijvanich P., Chirathaworn C., Wisedopas N., Treeprasertsuk S., Komolmit P. and Poovorawan Y. (2013) Circulating Cytokines and Histological Liver Damage in Chronic Hepatitis B Infection. *Hepatitis Research and Treatment* 20137.
- Porta C., De Amici M., Quaglini S., Paglino C., Tagliani F., Boncimino A., Moratti R. and Corazza G.R. (2008) Circulating interleukin-6 as a tumor marker for hepatocellular carcinoma. *Annals of Oncology* 19(2), 353-358.
- Rehermann B. and Nascimbeni M. (2005) Immunology of hepatitis B virus and hepatitis C virus infection. *Nat Rev Immunol* 5(3), 215-229.
- Rimšeliene G., Nilsen Ø., Kløvstad H., Blystad H. and Aavitsland P. (2011) Epidemiology of acute and chronic hepatitis B virus infection in Norway, 1992-2009. *BMC Infect Dis* 11(1), 1-7.
- Rizvi M., Azam M., Ajmal M., Malik A., Shukla I. and Afroz N. (2012) Role of Interferongamma and interleukin-12 in the immunopathogenesis of hepatitis B virus infection. *Euroasian Journal of Hepato-Gastroenterology* 2(1), 5-9.
- Roche S.P. and Kobos R. (2004) Jaundice in the adult patient. *American family physician* 69(2), 299-304.
- Rossol S., Marinos G., Carucci P., Singer M.V., Williams R. and Naoumov N.V. (1997) Interleukin-12 induction of Th1 cytokines is important for viral clearance in chronic hepatitis B. *Journal of Clinical Investigation* 99(12), 3025-3033.
- Rufai T., Mutocheluh M., Kwarteng K. and Dogbe E. (2014) The prevalence of hepatitis B virus E antigen among Ghanaian blood donors. *Pan Afr Med J* 1753.

- Sablon E. and Shapiro F. (2005) Advances in Molecular Diagnosis of HBV Infection and Drug Resistance. *Int J Med Sci* 2(1), 8-16.
- Saxena R., Chawla Y.K., Verma I. and Kaur J. (2014) Association of interleukin-10 with hepatitis B virus (HBV) mediated disease progression in Indian population. *The Indian journal of medical research* 139(5), 737.
- Saxena R. and Kaur J. (2015) Th1/Th2 cytokines and their genotypes as predictors of hepatitis B virus related hepatocellular carcinoma. *World J Hepatol* 7(11), 1572-1580.
- Schmilovitz-Weiss H., Tovar A., Halpern M., Sulkes J., Braun M., Rotman Y., Tur-Kaspa R. and Ben-Ari Z. (2006) Predictive value of serum globulin levels for the extent of hepatic fibrosis in patients with chronic hepatitis B infection. *J Viral Hepat* 13(10), 671-677.
- Schottelius A.J., Moldawer L.L., Dinarello C.A., Asadullah K., Sterry W. and Edwards C.K., 3rd (2004) Biology of tumor necrosis factor-alpha- implications for psoriasis. *Exp Dermatol* 13(4), 193-222.
- Schroder K., Hertzog P.J., Ravasi T. and Hume D.A. (2004) Interferon- γ : an overview of signals, mechanisms and functions. *Journal of Leukocyte Biology* 75(2), 163-189.
- Schuppan D. and Afdhal N.H. (2008) Liver Cirrhosis. *Lancet* 371(9615), 838-851.
- Sheng T., Wang B., Wang S.-y., Deng B., Qu L., Qi X.-s., Wang X.-l., Deng G.-l. and Sun X. (2015a) The Relationship Between Serum Interleukin-6 and the Recurrence of Hepatitis B Virus Related Hepatocellular Carcinoma after Curative Resection. *Medicine* 94(24), e941.
- Sheng T., Wang B., Wang S.Y., Deng B., Qu L., Qi X.S., Wang X.L., Deng G.L. and Sun X. (2015b) The Relationship Between Serum Interleukin-6 and the Recurrence of Hepatitis B Virus Related Hepatocellular Carcinoma after Curative Resection. *Medicine (Baltimore)* 94(24), e941.

- Shepard C.W., Simard E.P., Finelli L., Fiore A.E. and Bell B.P. (2006) Hepatitis B virus infection: epidemiology and vaccination. *Epidemiol Rev* 28:112-125.
- Sitia G., Aiolfi R., Di Lucia P., Mainetti M., Fiocchi A., Mingozzi F., Esposito A., Ruggeri Z.M., Chisari F.V., Iannacone M. and Guidotti L.G. (2012) Antiplatelet therapy prevents hepatocellular carcinoma and improves survival in a mouse model of chronic hepatitis B. *Proc Natl Acad Sci U S A* 109(32), E2165-2172.
- Sofian M., Kalantar E., Aghakhani A., Hosseini S., Banifazl M., Eslamifar A., Jourabchi A., Farazi A.A. and Ramezani A. (2013) No Correlation Between Interleukin-10 Gene Promoter Polymorphisms and Hepatitis B Virus Infection Outcome. *Hepatitis Monthly* 13(5), e8803.
- Srivastava M., Ranjan A., Choudhary J.K., Tripathi M.K., Verma S., Dixit V.K., Nath G. and Jain A.K. (2014) Role of Proinflammatory Cytokines (Interferon Gamma) and Anti-inflammatory Cytokine (Interleukin-10) Gene Polymorphisms in Chronic Hepatitis B Infection: An Indian Scenario. *Journal of Interferon & Cytokine Research* 34(7), 547-551.
- Stadler L.P., Mezoff A.G. and Staat M.A. (2008) Hepatitis B virus screening for internationally adopted children. *Pediatrics* 122(6), 1223-1228.
- Tanaka T., Narazaki M. and Kishimoto T. (2014) IL-6 in Inflammation, Immunity, and Disease. *Cold Spring Harbor Perspectives in Biology* 6(10).
- Thimme R., Wieland S., Steiger C., Ghayeb J., Reimann K.A., Purcell R.H. and Chisari F.V. (2003) CD8(+) T Cells Mediate Viral Clearance and Disease Pathogenesis during Acute Hepatitis B Virus Infection. *Journal of Virology* 77(1), 68-76.
- Trobonjaca Z., Leithäuser F., Möller P., Schirmbeck R. and Reimann J. (2001) Activating immunity in the liver. I. Liver dendritic cells (but not hepatocytes) are potent activators of IFN- γ release by liver NKT cells. *The Journal of Immunology* 167(3), 1413-1422.

- Tse B.W.C., Scott K.F. and Russell P.J. (2012) Paradoxical Roles of Tumour Necrosis Factor-Alpha in Prostate Cancer Biology. *Prostate Cancer* 20128.
- Tunçbilek S. (2014) Relationship between cytokine gene polymorphisms and chronic hepatitis B virus infection. *World Journal of Gastroenterology : WJG* 20(20), 62266235.
- Vignali D.A.A. and Kuchroo V.K. (2012) IL-12 family cytokines: immunological playmakers. *Nat Immunol* 13(8), 722-728.
- Wajant H., Pfizenmaier K. and Scheurich P. (2003) Tumor necrosis factor signaling. *Cell Death Differ* 10(1), 45-65.
- Wang K., Wu Z.B., Ye Y.N., Liu J., Zhang G.L., Su Y.J., He H.L., Zheng Y.B. and Gao Z.L. (2014) Plasma Interleukin-10: A Likely Predictive Marker for Hepatitis B Virus Related Acute-on-Chronic Liver Failure. *Hepat Mon* 14(7), e19370.
- Wang Q., Chen X., Feng J., Cao Y., Song Y., Wang H., Zhu C., Liu S. and Zhu Y. (2013) Soluble Interleukin-6 Receptor-Mediated Innate Immune Response to DNA and RNA Viruses. *Journal of Virology* 87(20), 11244-11254.
- Weber-nordt R.M., Mertelsmann R. and Finke J. (1998) The JAK-STAT Pathway: Signal Transduction Involved in Proliferation, Differentiation and Transformation. *Leukemia & Lymphoma* 28(5-6), 459-467.
- Weng H.-L., Wang B.-E., Jia J.-D., Wu W.-F., Xian J.-Z., Mertens P.R., Cai W.-M. and Dooley S. (2005) Effect of Interferon-Gamma on Hepatic Fibrosis in Chronic Hepatitis B Virus Infection: A Randomized Controlled Study. *Clinical Gastroenterology and Hepatology* 3(8), 819-828.
- Wieland S.F., Vega R.G., Müller R., Evans C.F., Hilbush B., Guidotti L.G., Sutcliffe J.G., Schultz P.G. and Chisari F.V. (2003) Searching for Interferon-Induced Genes That Inhibit Hepatitis B Virus Replication in Transgenic Mouse Hepatocytes. *Journal of Virology* 77(2), 1227-1236.

- Wong V.W.-S., Yu J., Cheng A.S.-L., Wong G.L.-H., Chan H.-Y., Chu E.S.-H., Ng E.K.-O., Chan F.K.-L., Sung J.J.-Y. and Chan H.L.-Y. (2009) High serum interleukin-6 level predicts future hepatocellular carcinoma development in patients with chronic hepatitis B. *International Journal of Cancer* 124(12), 2766-2770.
- Xia C., Liu Y., Chen Z. and Zheng M. (2015) Involvement of Interleukin 6 in Hepatitis B Viral Infection. *Cellular Physiology and Biochemistry* 37(2), 677-686.
- Yan W.J., Lian W.X. and Pei L. (1999) Detection of serum TNF α , IFN γ , IL 6 and IL 8 in patients with hepatitis B*[J]. *WORLD JOURNAL OF GASTROENTEROLOGY* 1.
- Yoshimura A., Naka T. and Kubo M. (2007) SOCS proteins, cytokine signalling and immune regulation. *Nat Rev Immunol* 7(6), 454-465.
- Yu H., Zhu Q.-R., Gu S.-Q. and Fei L.-E. (2006) Relationship between IFN-gamma gene polymorphism and susceptibility to intrauterine HBV infection. *World Journal of Gastroenterology : WJG* 12(18), 2928-2931.
- Yuen M.F., Yuan H.J., Hui C.K., Wong D.K.H., Wong W.M., Chan A.O.O., Wong B.C.Y. and Lai C.L. (2003) A large population study of spontaneous HBeAg seroconversion and acute exacerbation of chronic hepatitis B infection: implications for antiviral therapy. *Gut* 52(3), 416-419.
- Zamorano J., Rivas M. and Perez G. (2003) Interleukin-4: A multifunctional cytokine. *Immunologia* 22215-224.
- Zeng Z., Kong X., Li F., Wei H., Sun R. and Tian Z. (2013) IL-12-based vaccination therapy reverses liver-induced systemic tolerance in a mouse model of hepatitis B virus carrier. *J Immunol* 191(8), 4184-4193.
- Zhang J.-M. and An J. (2007) Cytokines, Inflammation and Pain. *International anesthesiology clinics* 45(2), 27-37.

Zheng Z., Li X., Li Z. and Ma X.-C. (2013) IL-4- 590C/T polymorphism and susceptibility to liver disease: a meta-analysis and meta-regression. *DNA and cell biology* 32(8), 443-450.

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