

**CADMIUM, ARSENIC AND LEAD LEVELS IN PERSONS WITH CHRONIC  
HEPATITIS B IN THE EJURA-SEKYEDUMASE DISTRICT, GHANA.**

**BY**

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**INTEGRI PROCEDAMUS**

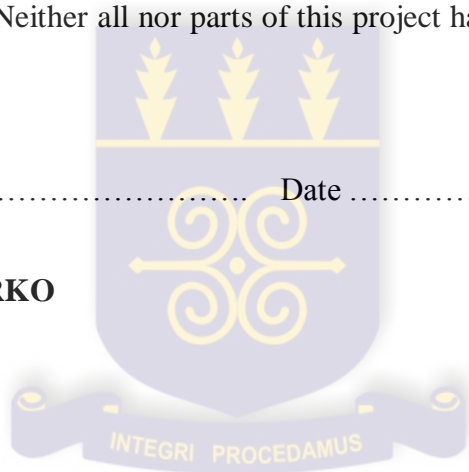
## DECLARATION

I, NYARKO ERIC do hereby declare that with the exception of references to other people's work, which have been duly acknowledged, this thesis is the outcome of my own research conducted at the Department of Chemical Pathology, School of Biomedical and Allied Health Sciences (SBAHS), University of Ghana, under the supervision of Dr. Henry Asare-Anane, Head, Department of Chemical Pathology, School of Biomedical and Allied Health Sciences (SBAHS), University of Ghana, and Dr. Mark Ofosuhen, Research Fellow at Noguchi Memorial Institute of Medical Research (NMIMR), University of Ghana. Neither all nor parts of this project have been presented for another degree elsewhere.

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## ABSTRACT

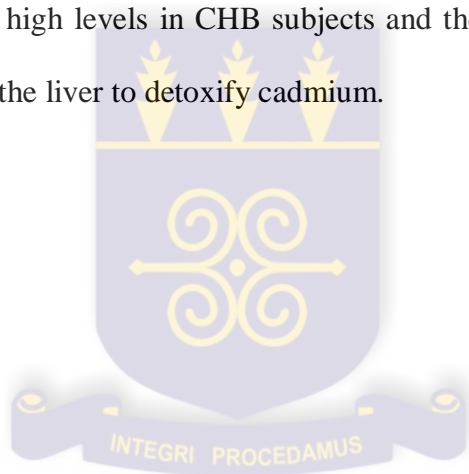
**Introduction:** Chronic hepatitis B (CHB) increases the risk of liver dysfunction and leads to complications such as cirrhosis and hepatocellular carcinoma (HCC). The rate of progression of CHB to these complications is attributed to hepato-toxicants, including heavy metals. Some heavy metals such as selenium and zinc have hepatoprotective benefits. However, others such as cadmium, arsenic and lead are hepatotoxic. The aim of this study was to measure the levels of cadmium, arsenic and lead in persons with CHB in the Ejura-Sekyedumase District (ESD) of the Ashanti region of Ghana.

**Subjects, Materials and Methods:** Eighty-two (82) subjects (comprising 41 CHB subjects and 41 controls) participated in the study. The CHB subjects were recruited from the Ejura-Sekyedumase District (ESD) hospital and 41 controls from the ESD community. Urine samples were collected for the measurement of cadmium, arsenic and lead. Blood samples were drawn for the measurement of total bilirubin, direct bilirubin, ALT, AST, Total protein, and Albumin. Sera from the blood were also analyzed for HBsAg, HBsAb, HBeAg, HBeAb, and HBcAb.

**Results:** Compared with the controls, the CHB subjects had significantly elevated levels of urinary cadmium ( $p = 0.004$ ), total bilirubin ( $p = 0.030$ ), direct bilirubin ( $p = 0.031$ ) and ALT ( $p = 0.005$ ). However, there was no significant difference between the levels of albumin ( $p=0.105$ ) and AST ( $p = 0.224$ ) in the CHB subjects and controls. There was no association between the urinary cadmium levels and ALT or AST ( $r^2 = 0.003$ ). However, there was a weak positive correlation between the urinary cadmium levels and serum total bilirubin ( $r^2 = 0.024$ ). There was a weak positive correlation between the urinary

arsenic concentration and ALT concentration ( $r^2 = 0.072$ ) and AST concentration ( $r^2 = 0.062$ ), however no correlation between the urinary arsenic concentrations and serum total bilirubin ( $r^2 = 0.018$ ). There was a weak positive correlation between the urinary lead concentrations and ALT concentration ( $r^2 = 0.065$ ) and total bilirubin concentration ( $r^2 = 0.063$ ), but no correlation between the urinary lead concentration and AST concentration ( $r^2 = 0.041$ ).

**Conclusion:** It is concluded that cadmium levels are high in persons with CHB than in persons without CHB. The study asserts that cadmium acts synergistically with HBV to cause its significantly high levels in CHB subjects and the HBV impairs liver function, making it difficult for the liver to detoxify cadmium.



## **DEDICATION**

I dedicate this work to my sweet and smart daughter Hochmah Maame Afua Ntiamoah Nyarko, and my colleague and friend Sarah N. Bortey (of blessed memory).



## ACKNOWLEDGEMENT

I would like to express my worship to the awesome Yaweh. My deepest gratitude to my supervisors, Dr. Henry Asare-Anane, Head of department of Chemical Pathology, School of Biomedical and Allied Health Sciences (SBAHS) and Dr. Mark Ofosuhene, Research Fellow, Department of Clinical Pathology, NMIMR for their corrections and constructive criticisms. Also to Mr. Emmanuel Ofori, Chemical Pathology, SBAHS, for his helping suggestions and inputs. I am also grateful to Dr. S.D Amanquah, Dr. Sylvester Y. Opong, Dr. Bartholomew Dzudzor, Sister Afua Bontu Adjei and Mr. Richmond Owusu Ateko for their encouragement, advice and support in diverse ways. My thanks also go to the entire staff of the department of Chemical Pathology for their help in many different ways.

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## LIST OF ABBREVIATIONS

A	Absorbance
AAS	Atomic Absorption Spectroscopy
AFP	Alpha-fetoprotein
ALA	Aminolevulinic acid
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
ATP	Adenosine Triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
BCG	Bromocresol green
C	Control
Cat. No.	Catalogue number
CHB	Chronic Hepatitis B
CD4 <sup>+</sup>	Cluster of Differentiation four-plus
CDC	Centre for Disease Control and Prevention
CVH	Chronic Viral Hepatitis
CYP450	Cytochrome P450
DNA	Deoxyribonucleic acid
DZA	Diazotized Sulphanilic Acid
ER	Endoplasmic Reticulum
ESD	Ejura-Sekyedumase District
ENHIS	European Environmental and Health Information System
FBC	Full Blood Count
GOT	Glutamic Oxaloacetic Transaminase
GPT	Glutamate Pyruvate Transaminase

GSH.....	Glutathione
GST .....	Glutathione-S-transferase
HBV.....	Hepatitis B Virus
HBV-CVH.....	Hepatitis B Virus associated Chronic Viral Hepatitis
HCC .....	Hepatocellular Carcinoma
HCV .....	Hepatitis C Virus
HIV .....	Human Immunodeficiency Virus
HDV .....	Hepatitis Delta Virus
HLA .....	Human Leucocyte Antigen
IARC .....	International Agency for Research on Cancer
IFCC .....	International Federation of Clinical Chemistry
IgG .....	Immunoglobulin G
Kbp .....	kilo base pairs
LBAT .....	Liver Bile Acid Transporter
LDH .....	Lactate dehydrogenase
LDL .....	Low Density Lipoprotein
mRNA .....	messenger Ribonucleic acid
MDH .....	Malate dehydrogenase
MHC .....	Major Histocompatibility Complex
NAD .....	Nicotinamide Adenine Dinucleotide
NADPH .....	Nicotinamide Adenine Dinucleotide Phosphate
NIH.....	National Institutes of Health
PgRNA .....	Pregenomic RNA
R .....	Reagent
RB .....	Reagent Blank

RNA .....	Ribonucleic acid
ROS .....	Reactive Oxygen Species
S .....	Sample
SB .....	Sample Blank
SH .....	Sulphahydral
T.....	Test
ULN .....	Upper Limit of Normal
VHPB .....	Viral Hepatitis Prevention Board
WHO.....	World Health Organization
WR .....	Working Reagent

## CHAPTER ONE

### 1.0 INTRODUCTION

#### 1.1 Background

Chronic Hepatitis B (CHB) results from inflammatory injury to the liver, caused by hepatitis B viruses, which has persisted for six or more months without resolution (WHO, 2015). Chronic Hepatitis B is generally the most prevalent form of all chronic viral hepatitis (CVH) (Hollinger *et al.*, 2001) and as such, a public health problem (WHO, 2008). The prevalence of CHB worldwide exceeds 7%, and about a quarter of the world's population lives in areas where there are high levels of infection (WHO, 2008). The severe pathological consequences of persistent and chronic Hepatitis B Virus (HBV) infections include the development of chronic hepatic insufficiency, cirrhosis, and hepatocellular carcinoma (HCC) (WHO, 2008). Studies have shown a link between several factors in the initiation, progression and complications of Chronic Hepatitis B. These include, race, gender, age, food, occupation and environmental factors (Chen and Morgan, 2006). Among the environmental factors which enhance the progression of CHB to fatal states are mycotoxins, food, excessive alcohol consumption, polluted soil and water contaminated with pesticides, fertilizers and herbicides (Beth *et al.*, 2010). Thus, these soil pollutants and water contaminants may contain heavy metals including Arsenic (As), Copper (Cu), Cadmium (Cd), Lead (Pd), Chromium (Cr), Mercury (Hg) and Zinc (Zn) (Schmidt, 2003). Excessive uptake of metals by plants through water, soil and agricultural inputs may produce toxicity in human nutrition, and can complicate acute and chronic diseases (Schmidt, 2003). Regardless of the source, heavy metals such as Cadmium, Arsenic and Lead may cause hepatomegally and disrupts hepatic function

(Schmidt, 2003). Heavy metals have toxic effects on various organs of the body including kidneys, heart, skin, bones and liver. Studies have indicated that lead (Pb) levels in blood and urine increases with liver diseases (Anuradha, 2007). Toxicity of cadmium has also been widely studied (Melgar *et al.*, 1997). Although various studies have been done on the levels of these heavy metals in water, food and even in persons living in high risk environments (Armah *et al.*, 1998; Gyasi *et al.*, 2014), no such work has been done in persons with CHB in Ghana.

## **1.2 Problem Statement**

Exposure to hepato-toxicants enhances the progression of viral hepatitis to liver failure (García-Niño and Pedraza-Chaverrí, 2014). Some of these hepato-toxicants include heavy metals such as Arsenic, Lead, Chromium and Cadmium (García-Niño and Pedraza-Chaverrí, 2014). Lead causes hepatocytes to become inflamed and enlarged, die more rapidly and are replaced by fatty deposits, leading to hepatosclerosis (Castilla *et al.*, 1995). Chronic exposure and accumulation of cadmium and arsenic leads to hepatitis and HCC (Taylor, 1997). In persons with CHB, cadmium, arsenic and lead chances weakened immune system, malnutrition, poor quality of health, HCC, cirrhosis, hepatic failure, reduced life expectancy and eventually death (Robinson, 1995). Management of CHB is very expensive. It has no cure and its progression leads to increased morbidity and mortality (WHO, 2008).

### **1.3 Justification**

Intoxication by heavy metals might enhance liver dysfunction, however, the levels of these heavy metals in persons with CHB has not been studied in Ghana. Such knowledge will be relevant to the understanding of the various co-morbidities to HCC and Cirrhosis and or the synergistic effect of persistent HBV infection and heavy metal concentrations on liver function. This will also help in public education of persons with CHB and the general public whose occupation involve the use of chemicals, food, water and soil with potential heavy metal contamination.

### **1.4 Hypothesis**

Heavy metals do not contribute to hepatic dysfunction in persons with chronic Hepatitis B.

### **1.5 Aim**

To measure the levels of Cadmium, Arsenic and Lead in persons with CHB in the Ejura-Sekyedumase District of Ashanti Region, Ghana and to find out if such levels have effects on the functions of the study subject liver.

## **1.6 Specific Objectives**

The specific objectives of the study were:

1. To compare urine levels of Cadmium, Arsenic and Lead of CHB subjects with controls.
2. To determine the association of Cadmium with ALT, AST and Bilirubin.
3. To determine the association of Arsenic with ALT, AST and Bilirubin.
4. To determine the association of Lead with ALT, AST and Bilirubin

## CHAPTER TWO

### 2.0 LITERATURE REVIEW

#### 2.1 Chronic Hepatitis B

Chronic Hepatitis (CHB) results from hepatitis B viral infections that fail to resolve after 6 months, and as such leads to consistent and progression of hepatic injury, leading to hepatocellular carcinoma (HCC), cirrhosis, fatty liver and sometimes liver failure (Bortoli *et al.*, 2010). Globally, increasing numbers of people are affected by CHB which may lead to HCC, cirrhosis and eventually liver failure (WHO, 2008; El-Serag and Rudolf, 2007). About 80% of those exposed to the virus develop a chronic infection (Nelson *et al.*, 2011).

##### 2.1.1 Aetiology of Chronic Hepatitis B

The primary cause of CHB is the Hepatitis B virus. However, the progression and severity of the condition is influenced by genetics, lifestyle and the environment of the host (NIH, 2009). Genetically, spontaneous clearance of an HBV infection is associated with vigorous polyclonal and multi-specific CD4<sup>+</sup>T-helper cell responses in contrast to the weak responses seen in persistent infections (Chisari and Ferrari, 1995). The human leucocyte antigen (HLA) molecules, encoded by Major Histocompatibility Complex (MHC) class II are responsible for presenting peptide epitopes to CD4<sup>+</sup> T-helper cells. It is therefore reasonable to postulate that polymorphism in the MHC class II region may explain the variation in outcome. Furthermore, CD4<sup>+</sup>T- cell responses in patients who have recovered from HBV are greater in those who carry the HLA-DRB1\*1302 allele

(Diepolder *et al.*, 1998). The ability to bind and present a wide range of epitopes is likely to generate a polyclonal and multi-specific T-helper cell response, as seen in individuals who spontaneously eliminate the infection. Moreover, a broad range of potential T cell epitopes would reduce the opportunity for the virus to evade recognition through sequence variation. The importance of this ability to present a wide range of epitopes is further underlined by the finding that individuals who are heterozygous at MHC class II loci are less likely to develop CHB infection than homozygous individuals (Chisari and Ferrari, 1995). Patients with chronic HBV infection are counseled regarding lifestyle changes to decrease the rate of progression of liver disease to fatal state. There are no specific dietary associations, however in countries where consumption of aflatoxin-contaminated food is prevalent, CHB is high (Udoh *et al.*, 2000). Moreover, heavy drinking of alcohol has been associated with increased risk of cirrhosis and HCC in persons with CHB. All patients with chronic HBV infection will benefit from a balanced diet, regular exercises, abstinence from smoking, no or minimal intake of alcohol; and protection of one's self from hepatotoxic polluted substances in air, water and food (Anna and McMahon, 2005). Research has shown a link between some factors of progression of CHB, including hepatotoxic agents. Exposure to heavy metals produce several adverse health effects. The common biochemical mechanisms that determine their toxicity and carcinogenicity are the generation of oxidative stress, which leads to hepatic damage (Garcia-Nino and Pendraza-Chaverri, 2014).

### 2.1.2 Epidemiology

The prevalence of chronic HBV infection in areas of high endemicity is at least 7% , with 10-20% prevalence in Africa or Far East (WHO, 2008). As of 2010, China had 120 million infected people, followed by India and Indonesia with 40million and 12 million, respectively. According to World Health Organization (WHO), an estimated 600,000 people die every year related to the infection (Komas *et al.*, 2013). In relation to CHB endemicity, the world can be divided into three areas. These are high (with prevalence >8%), intermediate (2-8%), and low (<2%) (Mahoney *et al.*, 1999). High endemic areas include south-east Asia and the Pacific Basin (excluding Japan, Australia, and New Zealand), sub-Saharan Africa, the Amazon Basin, parts of the Middle East and some countries in Eastern Europe. In these areas, between 70 to 90% of the population becomes infected before the age of 40; and about 20% of these persons have CHB (Hollinger and Liang, 2001). In countries such as China, Senegal, and Thailand, infection rates are very high in infants, and continue through early childhood. In Panama, New Guinea, and Solomon Islands, infection rates in infants are relatively low and increase rapidly during early childhood (Komas *et al.*, 2013). Low endemic areas include North America, Western and Northern Europe, Australia, and parts of South America, where the carrier rate is less than 2% (Hollinger and Liang, 2001). The primary method of transmission reflects the prevalence of chronic HBV infection in a given area. In the high-prevalence areas such as China and South East Asia, transmission during childbirth is most common, although in other areas of high endemicity (eg. Africa), transmission during childhood is a significant factor. In intermediate prevalence areas, the disease is predominantly spread among children (Hollinger and Liang, 2001). Moreover, prevalence

in areas such as the continental United States and Western Europe is mainly as a result of injection drug use and unprotected sex. The prevalence of chronic carriage in sub-Saharan Africa ranges between 3% and 22% in blood donors (Redd *et al.*, 2007). Hepatitis B can be transmitted horizontally or vertically (Custer *et al.*, 2004). If a pregnant woman is an HBV carrier and is also Hepatitis B e Antigen (HBeAg) – positive, her newborn baby has 90% likelihood to be infected and become a carrier (Hollinger and Liang, 2001). This form of transmission is called vertical transmission. Of these children, 25% will die later from liver cancer or other chronic liver diseases (Hollinger and Liang, 2001). Horizontal transmission occurs between individuals who exchange contaminated body fluid through work, play, fight or sex. Early life horizontal transmission routes in infants and early childhood includes bites, lesions, and sanitary habits. Horizontal transmission in adults is through sexual contact and intravenous drug use (Custer *et al.*, 2004).

### **2.1.3 Virology**

The Hepatitis B virus (HBV) (Figure 2.1) is a member of the hepadnavirus family. Its virus particle consists of an outer lipid envelope and an icosahedral nucleocapsid (or nucleocapsid core) composed of protein. These virions are about 42 nm in diameter (WHO, 2008). The nucleocapsid encloses the viral Deoxyribonucleic acid (DNA) and a DNA polymerase, that has reverse transcriptase activity (Locarnini, 2004).

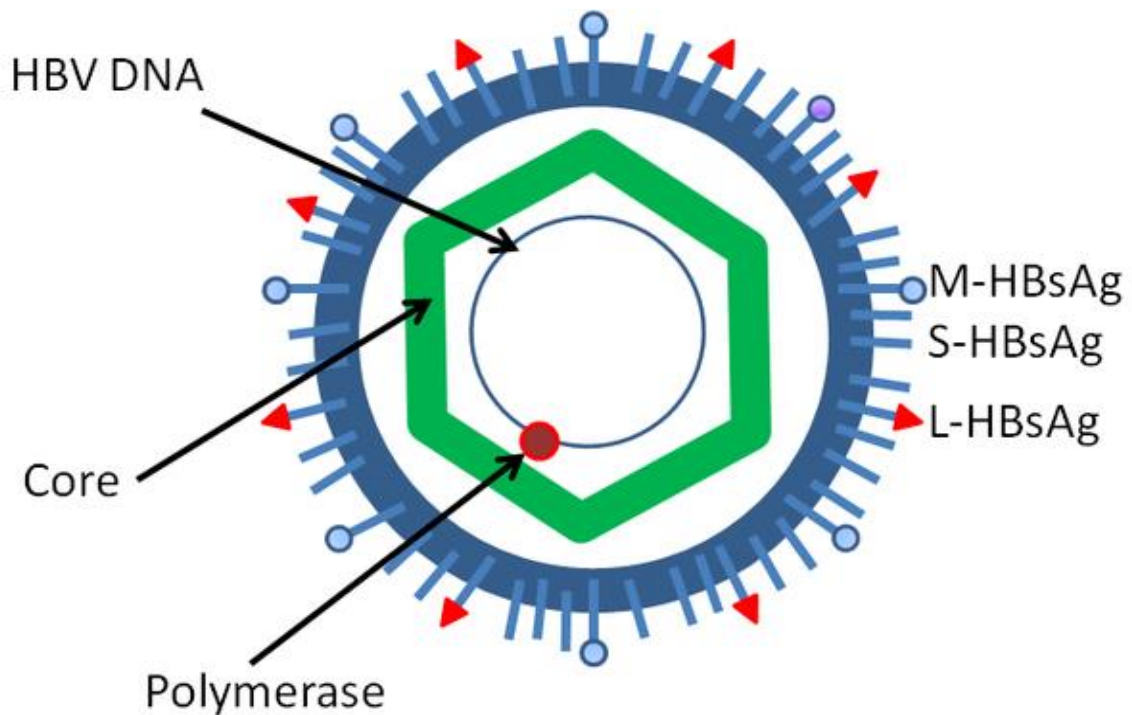


Figure 2.1 The Structure of Hepatitis B Virus (Locarnini, 2004)

### 2.1.3.1 Types of Hepatitis B Virus

There are ten genotypes of the hepatitis B virus according to overall nucleotide sequence variation of the genome. The genotypes are A, B, C, through to J (Huang *et al.*, 2013). The genotypes have a distinct geographical distribution and are used in tracing the evolution and transmission of the virus (Kramvis *et al.*, 2005). Some HBV genotypes are further classified as sub-genotypes. HBV sequence is characterized by > 8% nucleotide differences for genotype, and 4%-8% nucleotide differences for sub-genotype. Over 30 related sub-genotypes belonging to HBV genotypes have been determined to date, but the mechanisms of different pathogenic characteristics of these HBV genotypes are not known for certain (Huang *et al.*, 2013). Many studies have reported different genotypes (Moura *et al.*, 2013; Shaefar, 2007). These genotypes and their sub-genotypes show

different geographical distribution, and are related to disease progression, response to antiviral treatment, and prognosis. The “A-D” and “F” genotypes are divided into various sub-genotypes; however no sub-genotypes have been defined for “E”, “G” and “H” (Moura *et al.*, 2013). Genotype “A” is widespread in sub-Saharan Africa and Northern Europe; genotypes “B” and “C” are common in Asia; genotype “C” is primarily observed in Southeast Asia; genotype “D” is dominant in Africa, Europe, and India; genotype “G” is reported in France, Germany, and the United States; and genotype “H” is commonly encountered in Central and South America. Genotype “I” has recently been reported in Vietnam and Laos. The newest HBV genotype, “J”, has been identified in the Ryukyu Islands in Japan (Shaefar, 2007). Geographic distribution of HBV genotypes may be related to route of exposure. For example, genotypes “B” and “C” are more common in high-endemic regions of perinatal or vertical exposure, which plays an important role in viral transmission. Other genotypes are primarily observed in regions of horizontal exposure (Liu and Kao, 2013). Therefore, genotyping provides an epidemiological clue in the investigation of acquisition, because this lies in the geographical distribution of HBV (Cookly, 2010). Serotypically, there are four major types of HBV based on antigenic epitopes presented on its envelope proteins. This envelope (also called the HBsAg) is heterogeneously antigenic with a common antigen designated “a”, and two pairs of mutually exclusive antigens, “d” and “y”, and “w” and “r”. This has resulted in four subtypes “adr”, “adw”, “ayr” and “ayw” (Magnius and Norder, 1995). The distribution of these subtypes also varies geographically. Because of the common determinants, protection against one subtype appears to confer protection to the other subtypes, and no differences in clinical features have been related to subtypes (Zuckerman, 1996). In the

United States, Northern Europe, and Asia, the “d” determinant is common, but the “y” determinant is not. The “d” determinant to the near exclusion of “y” is found in Japan. The “y” determinant, and rarely “d”, is found in Africa and in Australia aborigines (Zuckerman, 1996). The “y” is also frequently found in India. In Europe, the US, India, Australia, and Oceania, the “w” determinant predominates, while in Japan, China, and Southeast Asia, the “r” determinant predominates. Subtypes “adw”, “ady”, and “adr” are each found in extensive geographic regions of the world. Subtype “ayr” is rare in the world, but it is commonly found in small populations in Oceania (Magnius and Norder, 1995).

#### **2.1.4 Hepatitis B Disease**

The secondary consequences of acute HBV infection include CHB, cirrhosis of the liver and hepatocellular carcinoma. The likelihood of progression to chronic infection is inversely related to age at the time of infection. The course of hepatitis B may be extremely variable. Hepatitis B virus infection has different clinical manifestations depending on the patient’s age at infection and immune status, and the stage at which the disease is recognized (Hyams, 1995). During the incubation phase of the disease (usually between 6 - 24 weeks), patients may feel unwell with possible nausea, vomiting, diarrhea, anorexia and headaches. Patients may become jaundiced, although loss of appetite may improve. Sometimes HBV infection produces neither jaundice nor obvious symptoms (Bell and Nguyen, 2009). These asymptomatic cases can be identified by detecting biochemical or virus-specific serologic alterations in the blood. Such persons may become silent carriers of the virus and constitute a reservoir for further transmission to

others (Dienstag, 2008). Most adult patients recover completely from their HBV infection, but others (about 5- 10% of persons infected) will not clear the virus, and will progress to become asymptomatic carriers or develop chronic hepatitis possibly resulting in cirrhosis or HCC. People who develop chronic hepatitis may develop significant and potentially fatal diseases like HCC and Cirrhosis (Dianstag, 2008). In general, the frequency of clinical disease increases with age, whereas the percentage of carriers decreases in that order (VHPB, 1997).

#### **2.1.4.1 Chronic Hepatitis B**

Chronic Hepatitis B is among the most common persistent viral infections in humans, affecting over 350 million people in the world today. A large fraction of these are in Eastern Asia and sub-Saharan Africa (WHO, 2008). About 2% of long-established chronic carriers apparently terminate their active infection and become HBsAg – negative (Robinson, 1995). Hepatitis Delta virus (HDV) is a defective virus that is only infectious in the presence of active HBV infection. Hepatitis Delta Virus infection occurs as either co-infection with HBV or super-infection of an HBV carrier. Co-infection usually resolves. Super-infection, however, causes frequently chronic HDV infection and chronic active hepatitis. Preventing acute and chronic HBV infection of susceptible persons by vaccination will also prevent HDV infection (CDC, 1991). Chronic Hepatitis B usually begins with mild symptoms. Infections of liver hepatocytes by these viruses inflame the cells, leading to formation of scar tissue. These scar tissues build up, replacing normal hepatic tissues (Aspinall *et al.*, 2011). More than 95% of people who become infected as adults will stage a full recovery and

develop protective immunity to the virus (Bell and Nguyen, 2009). It is however important to emphasize that, sustained increases in the concentrations of aminotransferases together with the presence of HBsAg for >6 months is regarded as indicative of chronic infection. During the initial phase of chronic HBV infection, serum HBV DNA levels are high and Hepatitis B e Antigen (HBeAg) is present. However, majority of carriers eventually lose the HBeAg and develop antibody to HBeAg (HBeAb) (McMahon, 2005). A large percent of HBeAg-positive patients have high serum HBV DNA but normal Alanine aminotransferase (ALT) levels. These patients are considered to be in the immune tolerant phase. Many of these patients develop HBeAg-positive CHB with elevated ALT levels as they age. Among carriers with elevated ALT levels, the rate of clearance of HBeAg averages between 8% and 12% per year, but is much lower in carriers who are in the immune tolerant phase (Lok and McMahon, 2007). These are mostly Asian children and young adults with normal ALT levels and also in immune-compromised subjects. Hepatitis B e Antigen (HBeAg) clearance may follow an aggression of hepatitis, manifested by an elevation of ALT levels. In old age, higher ALT, and HBV genotype B are associated with higher rates of spontaneous HBeAg clearance. After spontaneous HBeAg seroconversion, 67% to 80% of carriers have low or undetectable HBV DNA and normal ALT levels with minimal or no necro-inflammation on liver biopsy. This state is referred to as the inactive carrier state. Approximately 4% to 20% of inactive carriers have one or more reversions back to HBeAg- positive state (Bortolotti *et al.*, 2006). Among those who remain anti-HBe positive, 10% to 30% continue to have elevated ALT and high HBV DNA levels after HBeAg seroconversion, and roughly

10 to 20% of inactive carriers may have reactivation of HBV replication and exacerbations of hepatitis after years of quiescence. Therefore, serial testing is necessary to determine if an HBsAg-positive, HBeAg-negative carrier is truly in the “inactive carrier state” and lifelong follow-up is required to confirm that the inactive state is maintained (Liaw *et al.*, 2004). Clearance of HBeAg, whether spontaneous or after antiviral therapy, reduces the risk of hepatic decompensation and improves survival (Liaw *et al.*, 2004; Lin *et al.*, 1999). Moderate to high levels of persistent HBV replication or reactivation of HBV replication following a period of quiescence after HBeAg seroconversion leads to HBeAg-negative chronic hepatitis, which is characterized by HBV DNA levels >2,000 IU/ml and continued necroinflammation in the liver (Hadziyannis and Vasiolopoulos, 2001). Approximately 0.5% of HBsAg carriers will clear HBsAg yearly; most will develop anti-HBs antibodies. However, low levels of HBV DNA remain detectable in the serum in up to half of these persons. The prognosis is improved in carriers who cleared HBsAg, but HCC has been reported years after clearance of HBsAg, particularly in those who were older or had progressed to cirrhosis before HBsAg clearance (McMahon *et al.*, 2001).

### **2.1.5 Signs and Symptoms of Hepatitis B**

Acute infection with hepatitis B virus is associated with acute viral hepatitis. This illness begins with general ill-health, loss of appetite, nausea, vomiting, body aches, fever, and dark urine, and then progresses to the development of jaundice. It has been noted that itchy skin has been an indication as a possible symptom of all hepatitis virus types. The illness lasts for a few weeks and then gradually improves in most affected people. A few

people may have more severe liver disease and may die as a result (Terrault *et al.*, 2005). The acute infection may be entirely asymptomatic and may go unrecognized. Chronic infection with hepatitis B virus may either be asymptomatic or may be associated with a chronic inflammation of the liver (chronic hepatitis), leading to cirrhosis. This type of infection dramatically increases the incidence of HCC (liver cancer). In Europe, hepatitis B and C cause approximately 50% of hepatocellular carcinomas (El-Serag, 2011). Symptoms outside of the liver are present in 1–10% of HBV-infected people and it includes serum-sickness like syndrome, membranous glomerulonephritis, and papular acrodermatitis of childhood (Lok and McMahon, 2007). Chronic carriers are encouraged to avoid consuming alcohol as it increases their risk for cirrhosis and liver cancer (Liang, 2009).

### **2.1.6 Complications Associated with Chronic Hepatitis B**

Having a chronic HBV infection can lead to serious complications, such as cirrhosis (i.e. scarring of the liver) and HCC. People with chronic hepatitis B infection have an increased risk of liver cancer (Chang, 2007).

#### **2.1.6.1 Hepatocellular Carcinoma (HCC)**

Chronic infection with the hepatitis B virus (HBV) was noted to be associated with the development of hepatocellular carcinoma (HCC) as early as 1970. Studies have revealed that more than 80% of patients with HCC in high incidence areas (such as East Asia and sub-Saharan Africa) were seropositive for HBsAg. Moreover, population controls typically had rates of HBsAg of between 10% and 15% (DiBisceglie, 2009; El-Serag,

2012). Furthermore, more than 90% of HCC cases had antibody to hepatitis B core antigen (anti-HBc) detected in serum (Di Bisceglie, 2009). The detection of the HBcAb in sera of these patients is a serological evidence of active or prior HBV infection (Di Bisceglie, 2009). The frequency of HCC follows the same general geographic pattern as CHB (Di Bisceglie *et al.*, 2003). The age distribution of patients with clinically recognized tumours suggests that, these tumours appear after a mean duration of about 35 years of HBV infection (Di Bisceglie *et al.*, 2003). HBV causes about 80% of the world's primary liver cancer (Yang *et al.*, 2002). Host and viral risk factors for HCC include male gender, family history of HCC, old age, history of reversions from anti-HBe to HBeAg, presence of cirrhosis, HBV genotype C, core promoter mutation, and co-infection with HCV. Although cirrhosis is a strong risk factor for HCC, 30% to 50% of HCC associated with HBV occur in the absence of cirrhosis (Anna and McMahon, 2005).

#### **2.1.6.2 Cirrhosis**

In cirrhosis, hepatocytes die and are progressively replaced with fibrotic tissue leading to nodule formation. The internal structure of the liver is deranged leading to obstruction of blood flow and decrease in liver function. This damage is caused by recurrent immune responses stimulated by the presence of the HBV. Because liver inflammation can be symptomless, progression of inflammation to cirrhosis can occur without the knowledge of the patient (VHPB, 1996). Up to 20% of the chronic persistent hepatitis cases progress to cirrhosis. This is a serious liver disease associated with chronic and often widespread destruction of liver hepatocytes (WHO, 2008). Host and viral risk factors associated with increased rates of cirrhosis include older age, duration of HBV infection, HBV genotype

C, high levels of HBV DNA, habitual alcohol consumption, and concurrent infection with hepatitis C virus (HCV), hepatitis D virus (HDV) or human immunodeficiency virus (HIV) (Yim and Lok, 2006). Environmental factors that are associated with increasing risk of cirrhosis and HCC includes excessive alcohol consumption, carcinogens such as aflatoxin, smoking and toxic heavy metals (Fatovich, 2003).

### **2.1.7 Diagnosis and Monitoring of Hepatitis B Diseases**

Both acute and chronic hepatitis diseases are diagnosed by detection of viral antigens and antibodies; biochemical liver markers and imaging studies of the liver. Usually HBsAg – positive is an indication of infection. Persistence of the infection for more than six months generally means the condition is chronic (WHO, 2008). Emphatically, the differential diagnosis of acute or chronic HBV, active or passive chronic HBV infection state involves a battery of clinical, biochemical and immunological examinations and tests including the profiling of the HBV antigens and antibodies to determine the hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (HBsAb) (i.e. antibody to HBsAg), hepatitis B core antigen (HBcAg), HBcAb (i.e. antibody to HBcAg), hepatitis B e antigen (HBeAg) and antibody to HBeAg (HBeAb) (Hollinger and Liang, 2001; Robinson, 1995). Initial evaluation of CHB includes, history and physical examination; family history of hepatitis B viral infections and other liver diseases (including HCC and Cirrhosis); Laboratory tests to assess liver disease - Full Blood Count (FBC), Liver function tests (especially Alanine Aminotransferase (ALT)) and prothrombin time; tests for HBV replication - HBeAg/anti-HBe, HBV DNA; tests to rule out viral co-infections by other viruses such as: anti-HCV, anti-HDV and anti-HIV; tests to screen for HCC -

e.g. Alpha fetoprotein (AFP) and ultrasound scan. Also, for patients who meet criteria for chronic hepatitis, liver biopsy should be considered (Lok and McMahon, 2007). When patients are not eligible for treatment based on the above checks, but are HBeAg positive, with HBV DNA >20,000 IU/ml and ALT - normal, ALT levels should be measured every 3-6 months and the following inferences made: if ALT levels are between 1-2 times Upper Limit of Normal (ULN), person should recheck in 1-3 months. If it remains elevated, consider liver biopsy if age of patient  $\geq 40$  yrs. Consider treatment if biopsy shows moderate/severe inflammation or significant fibrosis. If ALT > 2 times ULN for 3-6 months and HBeAg is positive, with HBV DNA >20,000 IU/ml, consider liver biopsy and treatment (Lok and McMahon, 2007; Chu *et al.*, 2002). For patients who are in inactive HBsAg carrier state, ALT levels should be measured for every 3 months for 1 year. If it is persistently normal, then it should be measured twice the following year in 6 months intervals. If in either case, ALT is >1-2 times ULN, serum HBV DNA level should be checked. Liver biopsy should be considered if ALT borderline or mildly elevated on serial tests or if HBV DNA persistently >20,000 IU/ml. Treatment should be considered, if biopsy shows moderate or severe inflammation or significant fibrosis (Chu *et al.*, 2002; Lok and McMahon, 2007).

## **2.1.8 Management of Chronic Hepatitis B**

### **2.1.8.1 Treatment**

Chronically infected individuals with persistently elevated serum alanine aminotransferase (ALT), a marker of liver damage; and HBV DNA levels >20,000 IU/ml, are candidates for therapy (Lai *et al.*, 2007). Treatment lasts from six months to a

year, depending on medication and viral genotype (Alberti *et al.*, 20011). Although none of the available drugs can clear the infection, they can stop the virus from replicating, thus minimizing liver damage. As of 2008, there were seven medications licensed for treatment of hepatitis B infection. These include antiviral drugs lamivudine, adefovir, tenofovir, telbivudine and entecavir, and the two immune system modulators interferon alpha-2a and Pegylated interferon alpha-2a (Cao, 2009). The use of interferon, which requires injections daily or thrice weekly, has been supplanted by long-acting Pegylated interferon, which is injected only once weekly (Alberti *et al.*, 2011). However, some individuals are much more likely to respond than others, and this might be because of the genotype of the infecting virus or the person's heredity. The treatment reduces viral replication in the liver, thereby reducing the viral load (Promoolsinsup, 2002). Response to treatment differs between the viral genotypes (Cao, 2009).

#### **2.1.8.2 Vaccination and Preventive Methods**

Since 1991, vaccines for the prevention of hepatitis B have been routinely recommended for infants (Schillie *et al.*, 2013). Most vaccines are given in three doses over a course of 14 months (WHO, 2008). A protective response to the vaccine is defined as an anti-HBs antibody concentration of at-least 10 mIU/ml in the recipient's serum. The vaccine is more effective in children and 95% of those vaccinated have protective levels of antibody. The protection drops to around 90% at 40 years of age and to around 75% in those over 60 years. The protection afforded by vaccination is long lasting even after antibody levels fall below 10mIU/ml (Schillie *et al.*, 2013). Vaccination at birth is recommended for all infants of HBV infected mothers. A combination of hepatitis B

immune globulin and an accelerated course of HBV vaccine prevent around 90% of cases (Aspinall *et al.*, 2011). Apart from vaccination, it is recommended that persons who are HBsAg-positive should have their sexual partner(s) tested and vaccinated, use barrier protection during sexual intercourse if partner is not vaccinated or naturally immune, not to share tooth brushes or razors, cover open cuts and scratches, clean blood spills with detergent or bleach, not donate blood, organs or sperms (Lok and McMahon, 2007). A combination of harmful reduction strategies, such as the provision of new needles and syringes for treatment, decreases the risk of hepatitis B in intravenous drug users by about 75%.

## **2.2 Heavy Metals**

A heavy metal is any metal or metalloid with environmental concern and health consequence. The term originated with reference to the harmful effects of cadmium, mercury and lead (Duffus, 2002). It has since been applied to any other similarly toxic metal, or metalloid such as arsenic, regardless of density. Commonly encountered heavy metals are chromium, cobalt, nickel, copper, zinc, selenium, silver, antimony, mercury, thallium, arsenic, cadmium and lead (Barceloux and Barceloux, 1999). Some elements regarded as heavy metals are essential, in small quantities, for human health. These elements include vanadium, manganese, iron, cobalt, copper, zinc, selenium, strontium and molybdenum. A deficiency of these essential metals may increase susceptibility to other heavy metal poisoning (Chowdhury and Chandra, 1987). Others such as mercury, lead, cadmium and arsenic are toxic (Hanan *et al.*, 2005).

### **2.2.1 Cadmium**

Cadmium (Cd) is a soft, silver-white metal found naturally in small quantities in air, water and soil (Syers *et al.*, 2006). It is an extremely toxic metal commonly found in industrial workplaces. Due to its low permissible exposure limit, over exposures may occur even in situations where trace quantities are found. Sources of cadmium exposure include paints, cigarettes, food and air. People in occupation such as cadmium plating, e-waste collection and agriculture are highly exposed (Taylor, 1997).

#### **2.2.1.1 Toxicology of Cadmium**

Cadmium, upon entering the body, is widely distributed, with the major portion of the burden located in the liver and kidney. In these organs, cadmium has a long biological half-life, ranging from 17-30 years. After uptake from the lung or the gastrointestinal tract, cadmium is transported in blood plasma initially bound to albumin (ATSDR, 2012). This Cadmium-albumin complex is preferentially taken up by the liver. In the liver, cadmium induces the synthesis of metallothionein. A few days after exposure, metallothionein bound cadmium appears in the blood plasma. Because of its low molecular weight, cadmium-metallothionein complex is efficiently filtered through the glomeruli and thereafter taken up by the renal tubules, where it accumulates over a long time (ATSDR, 2012). Acute manifestation of cadmium poisoning includes chills, fever and muscle aches. Chronic accumulation leads to Chronic Kidney Diseases (CKD) and renal failure, osteoporosis, lung disorders, hepatitis and HCC (Taylor, 1997). There is some evidence that cadmium causes reproductive problems in humans including low birth weight and reduced sperm count (Maret and Moulis, 2013). People with higher

levels of cadmium in their urine (i.e evidence of chronic exposure to the heavy metal) appear to be nearly 3.5 times more likely to die of liver diseases than those with lower levels (Maret and Moulis, 2013). The biochemical mechanism of Cadmium poisoning is to increase oxidative stress by being a catalyst in the formation of reactive oxygen species, increasing lipid per-oxidation, and depleting glutathione and protein-bound sulphhydryl groups. Cadmium also can stimulate the production of inflammatory cytokines and down-regulates the protective function of nitric oxide formation (Navas-Acien *et al.*, 2004).

#### **2.2.1.1.1 Hepatotoxicity of Cadmium**

Cadmium is one of the known environment toxins that are detrimental to the liver (Jarup, 2003). Hepatotoxicity is indicated by changes such as swelling of hepatocytes, fatty changes, focal necrosis, hepatocytes degeneration and impaired liver function. Microscopic changes such as dilatation of ribosomes, damage of membrane-bounded lysosomes, nuclear pyknosis, have been reported (Thijissen *et al.*, 2007). The molecular mechanism that may be responsible for the hepatotoxicity of cadmium involves oxidative stress, disturbance of the antioxidant defense system and the generation of reactive oxygen species (Thijissen *et al.*, 2007; Stohs *et al.*, 2001). Several mechanisms have been suggested for the induction of cadmium-associated hepatotoxicity. One of the reported mechanisms of cadmium induced liver toxicity is mediated by the up-regulation of reactive oxygen species (such as hydroxyl groups, superoxides and hydrogen peroxides) which cause oxidative damage to membrane lipids (Packer and Cadenas, 2002). Primary injury of cells results from binding of cadmium to sulpherhydryl groups in mitochondria;

and secondary injury, initiated by the activation of kupffer cells have also been mentioned as possible mechanisms of toxic effect of cadmium on the liver (Rikans and Yamano, 2000).

### **2.2.2 Lead**

Lead (Pb) is a soft, malleable and corrosive-resistant metal. A member of the poisonous heavy metals, Lead exposure usually comes from the respiratory or digestive system, originating from lead-paint dust, air pollution, or food chain contamination (Cave *et al.*, 2010). Lead poisoning (also known as plumbism) is a type of metal poisoning and a medical condition caused by increased levels of lead in the body. Routes of exposure include contaminated air, water, soil, food, and consumer products such as cosmetics. Occupational exposure is a common cause of lead poisoning in adults (Needleman, 2004).

#### **2.2.2.1 Toxicology of Lead**

Acute poisoning by lead through any of the exposed routes causes typical neurological signs such as pain, muscle weakness, parasthesia, and rarely, encephalitis. Abdominal pain, nausea, vomiting, diarrhea, and constipation are other acute symptoms (Patrick, 2006). Gastrointestinal problems, such as constipation, diarrhea, poor appetite, and weight loss, are common in acute poisoning (Patrick, 2006). Absorption of large amounts of lead over a short time can cause shock due to loss of water from the gastrointestinal tract. Haemolysis due to acute poisoning can cause anaemia and haematuria. People who

survive acute poisoning often go on to display symptoms of chronic poisoning (Pearce, 2007). Chronic poisoning usually presents with symptoms affecting multiple systems. Central nervous, gastrointestinal system and neuromuscular system usually suffer more intensely. Signs of chronic exposure include loss of concentration, depression, nausea, abdominal pain, loss of coordination, and numbness and tingling in the extremities (Patrick, 2006). Fatigue, problems with sleep, headaches, stupor, slurred speech, and anaemia are also found in chronic lead poisoning. A blue line along the gum, with bluish black edging to the teeth, known as Burton line is another indication of chronic lead poisoning (Patrick, 2006). Anaemia may result when the cell membranes of red blood cells become more fragile as the result of damage to their membranes leading to haemolysis. Lead interferes with metabolism of bones and teeth and alters the permeability of blood vessels and collagen synthesis. It may also be harmful to the developing immune system of children (Flora *et al.*, 2008).

#### **2.2.2.1.1 Hepatotoxicity of Lead**

Autopsy studies of Pb-exposed humans indicate that the liver tissue is the largest repository (33%) of Pb from among the soft tissues followed by kidney cortex and medulla (Lyn, 2006). Lead causes liver cells to become enlarged into hyperplasia (initiating the formation of tumours in the liver), and causes inflammation, oxidation, and increased blood Low Density Lipoprotein (LDL) cholesterol levels (Lyn, 2006). High levels of lead are associated with a 3x-increase in liver damage (Cao *et al.*, 2009). The biochemical mechanism of lead toxicity includes the creation of reactive radicals which

damage cell structures including deoxyribonucleic acid (DNA) and cell membranes. Lead also interferes with DNA transcription, enzymes that help in the synthesis of vitamin D, and enzymes that maintain the integrity of the cell membrane (Flora *et al.*, 2008). A peer-reviewed study on lead exposure demonstrated another pathway for this metal's perpetuation of liver damage. A study found that lead could activate signals that increase tumor necrosis factor- $\alpha$ , a substance in the liver that causes inflammation, malignancy and cell death (ENHIS, 2006). Acute exposure to Pb in lead-acetate has been shown to decrease rat hepatic Cytochrome P450 (CYP450) content while increasing levels of urinary aminolevulinic acid (ALA). A reversal of the decreased CYP450 levels was observed when rats were co-treated with CYP450 inducers such as phenobarbital, suggesting that the CYP450 decrease may be due to Pb-induced inhibition of heme synthetic enzymes (Anuradha , 2007). A single dose of Pb nitrate (5 to 10 mmol/kg body wt) was found to decrease hepatic microsomal CYP450 enzyme levels as well as to decrease aminopyrene-*N*-demethylase activity. These decreases were followed by increase in levels of phase II enzyme components such as glutathione (GSH), glutathione-S-transferase (GST), and NAD(P)H:quinone oxidoreductase (DT diaphorase). This response mimicks the biochemical phenotype of hepatocyte nodules, suggesting a carcinogenic potential for lead nitrate and other Pb compounds (Anuradha , 2007).

### **2.2.3 Arsenic**

Arsenic (As) is a metalloid, which can exist in various allotropes, with the gray form having wide use in industry. It occurs in many minerals, usually in conjunction with

sulfur and also as a pure elemental crystal (Syers *et al.*, 2006). Arsenic poisoning is caused by elevated levels of arsenic in the body. The dominant basis of arsenic poisoning is from ground water that naturally contains high concentrations of arsenic. Other naturally occurring pathways of exposure include volcanic ash, weathering of arsenic-containing minerals and ores, and dissolved in groundwater. It is also found in food, water, soil, and air. Arsenic is absorbed by all plants, but is more concentrated in leafy vegetables, rice, apple and grape juice. Arsenic and its compounds, especially the trioxides, are used in the production of pesticides, treated wood products, herbicides, and insecticides. The toxicity of arsenic to insects, bacteria and fungi led to its use as a wood preservative (Rahman *et al.*, 2004).

#### **2.2.3.1 Toxicology of Arsenic**

Acute arsenic toxicity may be associated with hepatic necrosis and elevated levels of liver enzymes. Its intoxication may also result in toxic hepatitis with further elevated liver enzyme levels (IARC, 2004). Arsenic exposure may interfere with fetal development. Arsenic can cross the placenta into fetus and can be transported from mother to her child in breast milk. Unlike mercury, the naturally occurring forms of arsenic are the most toxic (ATSDR, 2007). Chronic arsenic exposure has been associated with birth defects and still births (Kwok *et al.*, 2006). Chronic arsenic ingestion may lead to cirrhotic portal hypertension. Case reports have also linked chronic high level arsenic exposure with hepatic angiosarcoma, a rare form of liver cancer. Biochemical mechanism of Arsenic poisoning includes the disruption of adenosine triphosphate (ATP) production through

several mechanisms. At the level of the citric acid cycle, arsenic inhibits lipoic acid, which is a cofactor for pyruvate dehydrogenase. In addition, by competing with phosphate, arsenate uncouples oxidative phosphorylation thus inhibiting energy-linked reduction of Nicotinamide Adenine Dinucleotide (NAD), mitochondrial respiration and ATP synthesis (Hughes, 2002). Hydrogen peroxide production is also increased, which, it is speculated, has potential to form reactive oxygen species and cause oxidative stress. These metabolic interferences lead to death from multi-system organ failure, including the kidneys and liver. The organ failure is presumed to be from necrotic cell death and non-apoptosis, since energy reserves have been too depleted for apoptosis to occur (Hughes, 2002).

#### **2.2.3.1.1 Hepatotoxicity of Arsenic**

Arsenic-generated Reactive Oxygen Species (ROS) attack - Sulphydryl (SH) groups leading to their oxidation, damages proteins and enzymes requiring - SH groups. It impairs the metabolism of iron (Fe) by causing an increase of Fe in the liver with a concomitant decrease in haematocrit (Ramanathan *et al.*, 2002). The mechanism of arsenic hepatotoxicity to individual cell type has historically centered around the inhibitory effects on cellular respiration at the level of mitochondria. Disruption of oxidative phosphorylation and concomitant decrease in the cellular levels of ATP are thought to be important central events of arsenic-induced toxicity evoking increased production of hydrogen peroxide. These effects could cause the formation of reactive oxygen species resulting in oxidative stress (NRC, 1999). The increased lipid peroxidation and plasma membrane damage, as shown by a progressive reduction of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in

mice drinking arsenic-contaminated water is known to parallel Glutathione (GSH) depletion of the liver (Ramos *et al.*, 1995). . Glutathione depletion resulted in the accumulation of free radicals that initiated lipid peroxidation resulting in biochemical damage to hepatocytes by covalent binding to macromolecules (Ramos *et al.*, 1995). Peroxidative breakdown of polyunsaturated fatty acids and membrane phospholipids leads to biochemical changes culminating in the inactivation of membrane enzymes, which may lead to alteration of ion transport and cellular water content, eventually leading to cell death. Some studies have also implicated lipid peroxidation in the pathogenesis of hepatic fibrogenesis, which was seen as a relatively late outcome of chronic arsenic consumption. Considered together, these observations suggest that weakening of the antioxidant defense system of the liver and consequent peroxidative damage of the lipid membranes may play a central role in the causation of liver pathology in chronic arsenic toxicity (Mazumder, 2005).

## CHAPTER THREE

### 3.0 METHODOLOGY

#### 3.1 Area of Study

The study area was the Ejura-Sekyedumase District (ESD) in the Ashanti region of Ghana. The district is located in the middle belt of Ghana. It occupies an area of 1,289km<sup>2</sup> and has a population of 88,753 (GSS, 2011). The climatic conditions are those of savanna transition agro-ecological zone. The mean annual rainfall is 1400 mL which is received in two peak season but sometimes in a unimodal pattern. Inhabitants of this district are predominantly farmers, with hepatitis B viral infection prevalence rates between 15 – 22% (Afriyie-Gyawu, 2007). Their occupation involves the use of fertilizers, herbicides, pesticides and water which may be sources of heavy metals (Beth *et al.*, 2010). The main source of fertilizer - derived heavy metal in soils are phosphate fertilizers, manufactured from phosphate rocks that contain various metals as minor constituents in the ores. Analysis of fertilizers commercially marketed in Nigeria indicated that all phosphatic fertilizers contained Cadmium and Lead concentrations ranging from 2.84 to 11.32 mg/kg and 7.43 to 9.02 mg/kg respectively (Ukpabi *et al.*, 2012).

#### 3.2 Sampling Population

A total of eighty-two (82) participants were recruited into the study. 41 Chronic Hepatitis B (CHB) study subjects were recruited from the Ejura District Hospital (EDH) from a list of blood donors who had tested positive to HBsAg more than 6 months earlier and were

still positive at the time of this research. The 41 controls were from the hospital and the communities within the district.

### 3.3 Study Design

The design was a hospital based case - control study comprising CHB subjects as cases and apparently healthy subjects without acute or chronic hepatitis B as controls.

### 3.4 Minimum Sample Size Calculation:

Sample size, N, was calculated by

$$N = \frac{r + 1}{r} * \frac{(P^*)(1 - P^*)(Z_{\beta} + Z_{\alpha/2})^2}{(P_1 - P_2)^2}$$

Where, r = is the ratio of number of control to cases

P\* = average proportion exposed = (proportion of exposed cases + proportion of control exposed)/2.

Z<sub>β</sub>= standard normal variate for power. 90% power was selected for this study = 1.28

Z<sub>α/2</sub> = Standard normal variate for level of significance, 1.96 for 95% confidence level

P1 is the proportion in cases.

P2 is the proportion in controls.

P1-P2 = Different in proportion.

$r = 1$ , since equal number of cases and controls were taken.(Jaykaran and Tamoghna, 2013)

$$P^* = (50\% + 15\%)/2 = 0.325.$$

$$N = 37.59850 \approx 40$$

For this study, a total of 82 including 41 cases and 41 controls were recruited.

### **3.5 Subjects Selection**

#### **3.5.1 Inclusion Criteria:**

Chronic Hepatitis B subjects, who had previously reported to the Ejura District Hospital (EDH) between 2010 and 2013. These were persons who were found to be infected with HBV during a blood donation exercise, whose infection had failed to resolve over six (6) months and are of age 18yrs and above. Their chronic status was confirmed by re-screening for HBsAg during the subject selection this year (2015) and comparing with their results between 2010 and 2013. Participants who tested positive to HBsAg in both cases were included in the CHB subjects.

Persons without CHB were selected as controls. These were persons who tested negative to HBsAg during the screening of blood donors at the Ejura District Hospital and others who volunteered to participate from the community. Their status were confirmed by re-screening for HBsAg during the subject selection.

### **3.5.2 Exclusion criteria:**

- Persons unable or unwilling to provide informed consent
- Persons who were below 18yrs.

## **3.6 Ethical Issues**

### **3.6.1 Ethical Consideration**

Ethical approval was sought from the Ethical and Protocol Review Committee of the University of Ghana School of Medicine and Dentistry, College of Health Sciences (Protocol Identification Number: MS-Et/M.5 – P 4.2/2014-2015). All data were handled anonymously and confidentially, using numbers instead of subjects' name.

### **3.6.2 Consent form**

Informed written consents were obtained from all participants in accordance with the human experimentation guidelines of the University of Ghana (Ethical and Protocol Review Committee, 2012). Confidentiality was observed throughout the research process and the data made accessible only to the research team.

## **3.7 Sample Collection and Preparation**

Eight (8) milliliters (ml) of venous blood sample was drawn from each participant into two (2) 4ml serum separator tubes. The samples were allowed to stand at room temperature for 45 minutes, and centrifuged at 2000g for 5mins to obtain sera. The sera

were aliquoted into two Eppendorf tubes and stored at -20° C till assayed. About 50 mls urine samples were collected from each participant into sterile urine containers and subsequently analyzed.

### **3.8 Biochemical Analysis**

#### **3.8.1 Measurement of Total and Direct Bilirubin**

Total and Direct serum bilirubin concentrations were determined as described by Jandrossik and Grof (1938), using the Bilirubin (Total +Direct) kit (Human Diagnostica, Germany). The kit was made up of reagent 1 (consisting of sulphanilic acid, hydrochloric acid, caffeine and sodium benzoate), reagent 2 (sodium nitrite), reagent 3 (consisting of sulphanilic acid and hydrochloric acid), and reagent 4 (sodium nitrite)

##### **3.8.1.1 Measurement of Total Bilirubin**

One thousand microlitres (1000 µl) of the Total bilirubin reagent (Reagent 1) was pipetted into each of two test tubes labeled Sample Blank (SB) and Sample (S). Forty microlitres (40 µl) of Reagent 2 was added to the sample tube (S) and the contents mixed thoroughly and incubated at 25<sup>0</sup>C for 5 minutes. Hundred microlitres (100 µl) of the sera sample were added to each of the tubes SB and S, mixed and incubated at 25<sup>0</sup>C for 20 minutes. The absorbance of the sample was measured against the sample blank at 546nm using the Semi-automated Humalyzer Primus (Human Diagnostica, Germany).

**Principle of method:** Sulphanilic acid reacts with sodium nitrite to form diazotized sulphanilic acid (DSA)

Sulphanilic Acid + Sodium Nitrite → Diazotized Sulphanilic Acid (DSA)

Bilirubin then reacts with diazotized sulphanilic acid (DSA), in the presence of an accelerator (Caffeine), to form a pink-red azobilirubin dye

Bilirubin + DSA + Caffeine → Total Azobilirubin

The absorbance of the total azobilirubin dye formed is proportional to the concentration of total bilirubin in the serum at 546nm (Jandrassik and Grof, 1938).

### **3.8.1.2 Measurement of Direct Bilirubin**

One thousand microliters (1000 µl) of the direct bilirubin reagent (Reagent 3) was pipetted into each of two test tubes labeled – SB and S. 40 µl of Reagent 4 was added to the sample tube (S) and the content were mixed thoroughly. The tubes were incubated at 25<sup>0</sup>C for 5minutes. 100 µl of the serum sample was added immediately to each of the tubes SB and S, mixed and incubated at 25<sup>0</sup>C for exactly 5 minutes. The absorbance of the sample was measured against the sample blank at 546nm using the Semi-automated Humalyzer Primus (Human Diagnostica, Germany).

**Principle of method:** Sulphanilic acid reacts with sodium nitrite to form diazotized sulphanilic acid (DSA)

Sulphanilic Acid + Sodium Nitrite → Diazotized Sulphanilic Acid (DSA)

Bilirubin then reacts with diazotized sulphanilic acid (DSA), to form a pink-red direct azobilirubin dye. The absorbance of the direct azobilirubin dye formed is proportional to the concentration of bilirubin in the serum at 546nm (Jandrassik and Grof (1938).

### 3.8.2 Measurement of Total Protein

Serum total protein was measured (using the Biuret method) as described by Flack and Woollen (1984) with the Protein Total Liquicolor kit (Human Diagnostica, Germany). 20 µl of serum sample and standard were pipetted into each of the test tubes labeled Standard (S) and Sample (T) respectively. 1000µl of the colour reagent was added to each of the tubes labeled standard (S), sample (T) and a third empty test tube previously labeled Reagent Blank (RB). The contents were mixed and incubated at 25<sup>0</sup>C for 10 minutes. The absorbance of the sample (T) and the standard (S) were measured against the sample blank (B) at 540nm using the Semi-automatic Spectrophotometer PD-303S (APEL Company Limited, Japan).

**Principle of method:** The total protein colour reagent is composed of Sodium hydroxide, Potassium sodium tartrate, Copper sulphate and Potassium iodide. The peptide bonds of protein react with the copper II ions in alkaline solution to form a blue-violet colour complex. Each of the copper ions complexes with five or six peptide bonds. The higher the protein concentration, the more copper ions are complexed and the greater the intensity of the colour-complex.

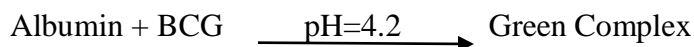
Copper (II) ions + protein → Blue – violet Biuret complex

The tartrate is added as a stabilizer whilst iodide is used to prevent auto-reduction of the alkaline copper complex. The intensity of the blue-violet colour formed is proportional to the protein concentration in the serum at 540nm (Flack and Woollen, 1984).

### 3.8.3 Measurement of Serum Albumin

Serum Albumin was measured using the Bromocresol green (BCG) method as described by Rockley (1964) and Dumas *et al*(1971) with the Albumin Liquicolor kit (Human Diagnostica, Germany). 10 µl of standard and serum samples were pipetted into each of the test tubes labeled Standard (S) and Sample (T) respectively. 1000 µl of Reagent 1(i.e the colour reagent) was added to each of the tubes labeled standard (S), sample (T) and a third test tube previously labeled Reagent Blank (RB). The tubes were mixed and incubated at 25<sup>0</sup>C for 5 minutes. The absorbance of the sample (T) and the standard (S) were measured against the reagent blank (B) at 546nm using the Semi-automatic Spectrophotometer PD-303S (APEL Company Limited, Japan), and albumin concentrations were calculated.

**Principle of method:** At a pH of 4.2, Bromocresol green (BCG) and citrate buffer forms a green coloured complex with Albumin:

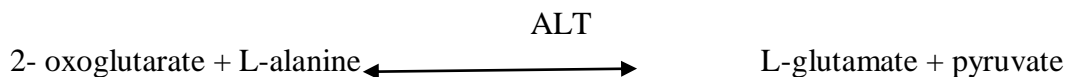


The intensity of the green colour formed is proportional to the albumin concentration in the serum at 546nm (Rockley, 1964)

### 3.8.4 Measurement of Serum Alanine Aminotransferase (ALT) activity

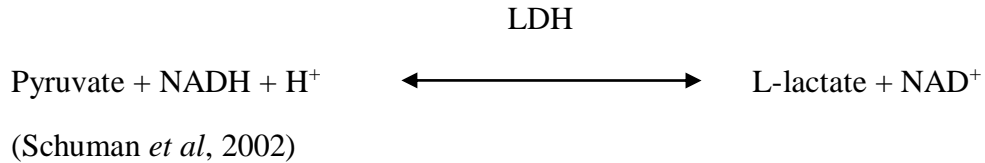
Serum alanine aminotransferase (ALT) activity was determined based on Kinetic method as recommended by the Expert Panel of the International Federation of Clinical Chemistry (IFCC), using Human GPT (ALAT) IFCC modified kit (Cat no. 12032) (Schuman *et al.*, 2002). To constitute working reagent (WR), one bottle of starting reagent (R2, consisting of 2-oxoglutarate and NADH) was poured into one bottle of enzyme reagent (R1, consisting of TRIS buffer-pH 7.5, L-alanine, and LDH ) and mixed thoroughly. 100 µl of sample was pipetted into cuvette set up in a water bath at 37°C and 1000 µl of the WR was added. The content was mixed, and absorbance, A<sub>1</sub> was read after 1 minute at 340nm with the Semi-automated Humalyzer Primus (Human Diagnostica, Germany). The absorbance A<sub>2</sub> and A<sub>3</sub> were read at the second (2) and third (3) minutes respectively. The ALT concentration in the sera samples were calculated by comparing the difference in absorbance at the times measured and multiplying that difference by the factor 2143 (Human Diagnostica, Germany).

**Principle of method:** ALT catalyzes the reversible transfer of the amino group from alanine to 2-oxoglutarate, forming pyruvate and L- glutamate.



Lactate dehydrogenase (LDH) then catalyses the conversion of Pyruvate to L-lactate as it converts Nicotinamide adenine dinucleotide (NADH) to NAD<sup>+</sup> in a reversible reaction.

The rate of NADH oxidation is proportional to the ALT activity.



### 3.8.5 Measurement of serum aspartate aminotransferase (AST) activity

Serum aspartate aminotransferase (AST) Activity was determined based on the Kinetic method as recommended by the Expert Panel of the International Federation of Clinical Chemistry (IFCC), using Human GOT (ASAT) IFCC modified kit (Cat no. 12021) (Schuman *et al.*, 2002). To constitute working reagent (WR), one bottle of starting reagent (R2, consisting of 2-oxoglutarate and NADH) was poured into one bottle of enzyme reagent (R1, consisting of TRIS buffer-pH 7.8, L-aspartate, LDH, and MDH) and mixed thoroughly.

100µl of sera sample was pipetted into a cuvette set up in a water bath at 37<sup>0</sup>C and 1000 µl of the WR was added. The mixture was mixed, and absorbance, A<sub>1</sub> was read after 1minute at 340nm with the semi-automated Humalyzer Primus (Human Diagnostica, Germany). The absorbances A<sub>2</sub> and A<sub>3</sub> were read at the second (2) and third (3) minutes respectively. The AST concentration in the sera samples were calculated by comparing the difference in absorbance at the times measured and multiplying that difference by the factor 2143 (Human Diagnostica, Germany).

**Principle of method:** AST catalyzes the reversible transfer of the amino group from aspartate to 2-oxoglutarate, forming oxaloacetate and L- glutamate.

AST

$$2\text{-oxoglutarate} + \text{L-aspartate} \rightleftharpoons \text{L-glutamate} + \text{oxaloacetate}$$

Malate dehydrogenase (MDH) then catalyses the conversion of oxaloacetate to L-malate as it converts Nicotinamide adenine dinucleotide (NADH) to  $\text{NAD}^+$  in a reversible reaction. The rate of NADH oxidation is proportional to the AST activity.

MDH

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{L-malate} + \text{NAD}^+ \text{ (Schuman } et al., 2002).$$

### 3.9 Detection of hepatitis B antigens and antibodies

Screening of all participants and profiling of the HBV-associated antigens and antibodies, were done using the Hexagon HBsAg (Human Diagnostica, Germany); and the Clinogen HBV-5 Card (Clinogen Diagnostics, Japan) respectively.

#### 3.9.1 Screening for HBsAg

HBsAg was detected with the Immunochromatographic (dual-antibody sandwich) method (Barr *et al.*, 1979), using the Hexagon HBsAg test strips (Human Diagnostica, Germany). The tests were performed at  $25^{\circ}\text{C}$ . The test strip was immersed into the tube containing the sera for 15 minutes, with the strip arrows pointing downwards and read. Samples positive for the HBsAg was recognized by two pink lines (indicating test (T) line 1 and control (C) line 2).

**Principle of method:** HBsAg present in serum reacts with colloidal gold particles which have been coated with monoclonal antibodies against HBsAg. The resulting immunocomplex migrate along the membrane and are bound in the test zone by a second monoclonal anti-HBsAg antibody (mouse antibody), which is fixed there in the form of horizontal line (T). Excessive immunocomplex and/or un-reacted colloidal gold particles migrate further and are bound in a second line by anti-mouse IgG antibodies (rabbit), forming the control (C) line 2 (Barr *et al*, 1979).

### **3.9.2 Hepatitis B Profiling**

The Clinogen HBV-5 card used in this work detects HBsAg, HBsAb, HBeAg, HBeAb and HBcAb, using colloidal gold and membrane chromatography technology.

#### **3.9.2.1 Detection of HBsAg**

HBsAg was detected with the Immunochromatographic (dual-antibody sandwich) method (Kumiko *et al.*, 1996), using Clinogen HBV-5 Card (Clinogen Diagnostics, Japan). The tests were performed at 25<sup>0</sup>C. The test card was removed from the pouch and laid horizontally on the bench, with the arrows facing right to left. 70 µl of the sera was pipetted and added to the yellow well (labelled HBsAg). The test was read at 15 minutes of adding the sera to the well. Sample positive for the HBsAg was recognized by two purple lines (indicating test (T) line 1 and control (C) line 2).

**Principle of Method:** Colloidal conjugated monoclonal antibodies reactive to HBsAg (sAb-Au), are dry immobilized onto a nitrocellulose membrane strip. When the serum sample is added, it migrates by capillary diffusion through the strip, rehydrating the gold conjugate. If present, the HBsAg will bind with the gold-conjugated antibodies forming particles. These particles will continue to migrate along the strip until the test zone (T), where they are captured by anti-HBs antibodies immobilized there and a visible pink to purple line will appear in the T zone. The gold conjugate will continue to migrate alone until it is captured in the control (C) zone from immobilized goat, anti-mouse IgG antibody and aggregating in a pink to purple line, which indicates the validity of the test (Kumiko *et al.*, 1996).

### **3.9.2.2 Detection of HBsAb**

HBsAb was detected with the Immunochromatographic (dual-antigen sandwich) method (Kumiko *et al.*, 1996), using Clinogen HBV-5 Card (Clinogen Diagnostics, Japan). The test was performed at 25<sup>0</sup>C. The test card was removed from the pouch and laid horizontally on the bench, with the arrows facing right to left. 70 µl of the sera was added to the green well (labelled HBsAb). The stop watch was simultaneously started at fifteen minutes (15 mins). The test was read at 15 minutes of adding the sera sample to the well. Sample positive for the HBsAb was recognized by two purple lines (indicating test (T) line 1 and control (C) line 2).

**Principle of method:** When the sera sample is added to the sample well, it moves through the conjugate well and mobilizes gold HbsAg conjugate that is coated on the conjugate pad. The mixture moves along the membrane by capillary action and reacts with HBsAg that is coated on the test region. If anti-HBsAg antibody is present, the result is the formation of coloured band in the test (T) region. If there is no HBsAg antibody in the sample, the area will remain colourless. The sample continues to move to the control (C) zone and forms a pink to purple colour. The formation of this colour at the C zone indicates that the test is valid (Kumiko *et al*, 1996).

### 3.9.2.3 Detection of HBeAg

HBeAg was detected with the Immunochromatographic (dual-antibody sandwich) method (Kumiko *et al.*, 1996), using Clinogen HBV-5 Card (Clinogen Diagnostics, Japan). The test was performed at 25<sup>0</sup>C. The test card was removed from the pouch and laid horizontally on the bench, with the arrows facing right to left. 70 µl of the sera was added to the lemon green well (labelled HBeAg). The test was read at 15minutes of adding the sample to the well. Sample positive for the HBeAg was recognized by two purple lines (indicating test (T) line 1 and control (C) line 2).

**Principle of method:** When sera specimen is added to the well, it moves through the conjugate pad in the well and mobilizes gold anti-HBeAg conjugate that is coated on the conjugate pad. The mixture moves along the membrane by capillary action and reacts with anti-HBeAg antibody that is coated on the test region. If HBeAg is present at the level of 1ng/ml or greater, the result is the formation of a coloured band in the test (T)

region. If there is no HBeAg in the sample, the area will remain colourless. The sample continuous to move to the control (C) area and forms a pink to purple colour, indicating the test is working and the results is valid (Kumiko *et al*, 1996).

#### **3.9.2.4 Detection of HBeAb**

HBeAb was detected by the neutralization competitive inhibition method (Par *et al.*, 1992;Kumiko *et al.*, 1996), using Clinogen HBV-5 Card (Clinogen Diagnostics, Japan). The test was performed at 25<sup>0</sup>C. The test card was removed from the pouch and laid horizontally on the bench, with the arrows facing right to left. 70 µl of the sera was added to the violet well labelled HBeAb. The test was read at 15 minutes of adding the sera to the well. Sample positive for the HBeAb was recognized by one pink to purple line at the control (C) zone and a colourless or no line at the test (T) zone.

**Principle of method:** The nitrocellulose membrane is immobilized with anti-HBeAg antibody on the test region. During the assay, the presence of anti-HBeAg antibody (HBeAb) in the serum sample will compete with gold- antiHBeAg antibody conjugated for the limited amount of HBeAg that is pre-coated on the sample pad. The mixture moves along the membrane by capillary action and reacts with anti-HBeAg antibody that is coated on the test region. If anti-HBeAg antibody is present, the test region will remain colourless (i.e. the precoated HBeAg is neutralized by anti-HBe existed in the sera sample). If there is no anti-HBeAg antibody in the sample, the test region will form a colour band. The sample continuous to move to the control area and forms a pink to

purple colour, indicating that the test is working and the result is valid (Kumiko *et al.*, 1996; Par *et al.*, 1992).

### 3.9.2.5 Detection of HBcAb

HBcAb was detected by the neutralization competitive inhibition method (Par *et al.*, 1992; Kumiko *et al.*, 1996), using Clinogen HBV-5 Card (Clinogen Diagnostics, Japan). The test was performed at 25<sup>0</sup>C. The test card was removed from the pouch and laid horizontally on the bench, with the arrows facing right to left. 70µl of the sera was added to the brown well labelled HBcAb. The test was read at 15minutes of adding the sera sample to the well. Sample positive for the HBcAb was recognized by one pink to purple line at the control (C) zone and a colourless or no line at the test (T) zone.

**Principle of method:** HBcAb is a competitive immunoassay test, for the detection of anti-Hepatitis B Virus core antigen in human blood specimen. When the specimen is added to the sample well, it moves through the conjugate pad and mobilizes gold-HBcAg conjugate that is coated on the conjugate pad. The mixture moves along the membrane by capillary action and reacts with anti-HBcAg that is coated on the test region. If anti-HBcAg antibody (HBcAb) is absent, the result is the formation of a coloured band in the test region. If there is HBcAb antibody in the sample, it will compete with anti-HBcAg coated on the membrane to bind Au-HBcAg conjugate. When the anti-HBcAg antibody level is higher than the cut-off, it will prevent Au-HBcAg to bind to anti-HBcAg coated on test region. The test band will remain colourless. The sample continues to move to the control zone and forms a pink to purple colour,

indicating the test is working and the result is valid ((Kumiko *et al.*, 1996; Par *et al.*, 1992).

### **3.10 Measurement of Urine levels of Cadmium, Lead and Arsenic**

Urine levels of Arsenic, Cadmium and Lead were determined using the VARIAN AA240 Atomic Spectrometer (Varian Incorporated, Netherland) with the Fast Sequential method (Varian, 1997; Kozłowska *et al.*, 2003).

#### **3.10.1 Digestion of Urine Sample**

Five grams (5g) of each of the urine sample was weighed into a glass beaker and five millilitres (5mls) of Aqua regia (made of 4.5mls of concentrated Hydrochloric acid (HCl) and 0.5mls of Nitric acid (HNO<sub>3</sub>)), was added. Each beaker was covered with a thin film and digested for 3 hours on a hot plate. The digested sample was transferred into 30mls measuring cylinder and topped up to 30ml with deionized water. The entire content in the measuring cylinder was then transferred into well-labeled containers for AAS reading (Kozłowska *et al.*, 2003).

#### **3.10.2 Measurement of Urine levels of Cadmium**

The fuel outlet of the Varian AA240 FS was connected to the Acetylene cylinder and the support to air. The slit width was set to 0.5nm, the lamp current 4mA and the wavelength

228.8nm. The probe of the AAS was put into the cadmium standards and aspirated. The AAS analyzed the standards and the concentrations of cadmium and standard curve were displayed on the monitor connected to the detector. The probe was cleaned through several changes of deionized water, put into the sample digested and reconstituted as in section 3.9.1 and aspirated. The AAS analyzed the aspirated sample in heptaplicates and the mean concentrations of cadmium were displayed on the monitor connected to the detector (Varian, 1997; PelkinElmer, 1996).

### **3.10.3 Measurement of Urine levels of Arsenic**

The fuel outlet of the Varian AA240 FS was connected to the Acetylene cylinder and the support to Argon. The slit width was set to 0.5nm, the lamp current 10mA and the wavelength 193.7nm. The probe of the AAS was put into the Arsenic standards and aspirated. The AAS analyzed the standards and the concentrations of arsenic and standard curve were displayed on the monitor connected to the detector. The probe was then put into the sample digested (after cleaning through several changes of deionized water) and reconstituted as in section 3.9.1 and aspirated. The AAS analyzed the aspirated sample in heptaplicates and the mean concentrations of arsenic were displayed (Varian, 1997; Pelkin-Elmer, 1996).

#### 3.10.4 Measurement of Urine levels of Lead

The fuel outlet of the Varian AA240 FS was connected to the Acetylene cylinder and the support to air. The slit width was set to 1.0nm, the lamp current 5mA and the wavelength 217.0nm. The probe of the AAS was put into the lead standards and aspirated. The AAS analyzed the standards and the concentrations of lead and standard curve were displayed on the monitor connected to the detector. The probe was then lowered into the sample digested (after cleaning through many exchanges of deionized water) and reconstituted in section 3.9.1 and aspirated. The AAS analyzed the aspirated sample in heptaplicates and the mean concentrations of lead were displayed (Varian, 1997; PelkinElmer, 1996).

**Principle of methods:** The Principle is based on the Beer- Lamberts (Lambert, 1760; Beer, 1854) law and makes use of absorption spectrometry to assess the concentration of the heavy metal-analyte in a sample. The light source from the metals hollow cathode lamp emits a spectrum specific to the element of which it is made. The ground state atom of the metal absorbs the light energy at a specific wavelength as it enters the excited state. That is the electrons of the atoms in the atomizer are promoted to higher orbitals or excited state for a short period of time by absorbing a defined quantity of energy at a specific wavelength. This amount of energy is specific to a particular electron transition in a particular element. In general, each wavelength corresponds to only one element. As the number of atoms in the light path increases, the amount of light absorbed also increases. By measuring the amount of light absorbed, a quantitative determination of the amount of analyte can be made by the detector either as absorbance or by converting the absorbance to analyte concentration (PelkinElmer, 1996).

### **3.11 Data Analysis**

Data was entered into a spread sheet using Microsoft offices excel 2010. The Statistical Package for the Social Sciences (SPSS) version 20.0 was used for most of the statistical and analytical work. The unpaired student t-test was used to evaluate significant differences between the mean concentrations of the various liver markers in CHB subjects and controls; and also the mean concentrations of the heavy metals in the CHB subjects and the controls. P-values  $< 0.05$  were considered significant. The results were summarized using tables and graphs. Pearson product moment correlation coefficient ( $r$ ) was used to find the association between the concentrations of urinary heavy metals and the liver markers.

## CHAPTER FOUR

### 4.0 RESULTS

#### 4.1 Sociodemographic Characteristics of the Study Population

Table 4.1 shows the sociodemographic characteristics of the study population. A total of 82 subjects took part in the study, with age range of 18-50 years. The subjects were made up of 41 persons with CHB (cases) and 41 persons with neither acute nor chronic hepatitis B (controls). The CHB subjects were made up of 35 males (85%) and 6 females (15%), while the control subjects comprised of 27 males (66%) and 14 females (34%). There was no significant difference between mean age of cases ( $28.7 \pm 6.7$ ) and mean age of controls ( $30.6 \pm 8.3$ ). Most of the subjects in the study groups were farmers- 78.1% (32) of the CHB subjects and 76.6% (31) of the controls. All the CHB subjects tested positive for the HBsAg, however, 3(7.3%) out of the 41 were in the persistent (active) carrier state (HBeAg – positive) while 38 (92.7%) were of the inactive carrier state.

**Table 4.1: Sociodemographic characteristics of the study population (N = 82)**

<b>Sociodemographic</b>	<b>CHB Subjects (n = 41)</b>	<b>Controls (n = 41)</b>
<b>Characteristic</b>	<b>Number (%)</b>	<b>Number (%)</b>
<b>Gender</b>		
Males	35 (85.4)	27 (65.9)
Females	6 (14.4)	14 (34.1)
<b>Age</b>		
18 – 35	37 (90.2)	30 (73.2)
36 – 50	4 (9.8)	11 (26.8)
<b>Occupation</b>		
Farmers	32 (78.1)	31 (75.6)
Traders	6 (14.6)	9 (22.0)
Others	3 (7.3)	1 (2.4)
<b>CHB Status</b>		
Active Carrier state	3 (7.3)	<b>N/A</b>
Passive Carrier state	38 (92.7)	<b>N/A</b>

*Table 4.1 shows the sociodemographic characteristics of the study population. Data is presented as number of subjects (Percentage equivalent). N = the total number of study population (82), n=total number of people in the study group and N/A = Non applicable.*

## **4.2 Serum Biochemical Analytes**

Table 4.2 shows values for the various serum biochemical analytes for both cases and controls. There was no statistical significant difference in total protein ( $p = 0.153$ ), albumin ( $p = 0.105$ ) and AST ( $p = 0.224$ ) between the CHB subjects (cases) and controls. However, persons with chronic hepatitis B (CHB) had significantly higher total bilirubin ( $p = 0.030$ ), direct bilirubin ( $p = 0.031$ ) and ALT ( $0.005$ ), compared to controls.

**Table 4.2 Serum Biochemical Analytes**

<b>Serum Biochemical Analytes</b>	<b>CHB subjects (n=41)</b>	<b>Controls (n=41)</b>	<b>(RR)</b>	<b>p-value</b>
Total Bilirubin ( $\mu\text{mol/l}$ )	10.22 $\pm$ 7.55	7.81 $\pm$ 4.40	(2.00-20.00)	0.030*
Direct Bilirubin ( $\mu\text{mol/l}$ )	3.53 $\pm$ 2.20	2.69 $\pm$ 1.42	(0.10-6.80)	0.031*
Total Protein (g/l)	69.99 $\pm$ 6.87	1.85 $\pm$ 7.85	(60.00-81.00)	0.153
Albumin (g/l)	39.12 $\pm$ 7.36	39.48 $\pm$ 6.11	(33.00-55.00)	0.105
ALT (U/L)	28.11 $\pm$ 13.36	18.00 $\pm$ 7.53	( $\leq$ 40)	0.005*
AST (U/L)	27.52 $\pm$ 9.35	21.92 $\pm$ 7.82	( $\leq$ 40)	0.224

*Table 4.2 shows a comparison of serum biochemical analytes between the CHB subjects and controls. Data is presented as mean  $\pm$  SD. RR = Reference Range. p-values < 0.05 were considered statistically significant.*

### **4.3 Urinary Heavy Metals of the Study Population**

Results of urinary heavy metals between the CHB subjects and the controls are shown in the table 4.3. There was no statistical significant difference between the cases and controls for Lead ( $p = 0.693$ ) and Arsenic ( $p = 0.384$ ). However, persons with Chronic Hepatitis B (CHB) had significantly higher cadmium levels ( $p = 0.004$ ) compared with the controls.

**Table 4.3 Urinary Heavy Metals**

<b>Urinary Heavy Metal</b>	<b>CHB subjects (n = 41)</b>	<b>Controls (n = 41)</b>	<b>RR</b>	<b>p value</b>
Cadmium (ug/l)	0.113 ± 0.051	0.097 ± 0.031	≤ 0.195	0.004*
Lead (ug/l)	0.383 ± 0.244	0.252 ± 0.240	≤ 0.677	0.693
Arsenic (ug/l)	43.689 ± 19.711	37.976 ± 21.281	≤ 100	0.384

*Table 4.3 shows a comparison of urinary heavy metals between the cases and controls. Data is presented as mean ± SD. RR = Reference Ranges. p-values < 0.05 were considered as statistically significant.*

#### **4.4 Association of urinary Cadmium with ALT, AST and Bilirubin**

Figure 4.4abc shows the association of urinary cadmium with ALT, AST and Bilirubin respectively of the CHB subjects. There was no correlation between the urinary cadmium levels and ALT (Figure 4.4a) or AST (Figure 4.4b) ( $r^2 = 0.003$ ), but weak correlation between the urinary cadmium levels and serum total bilirubin ( $r^2 = 0.024$ ).

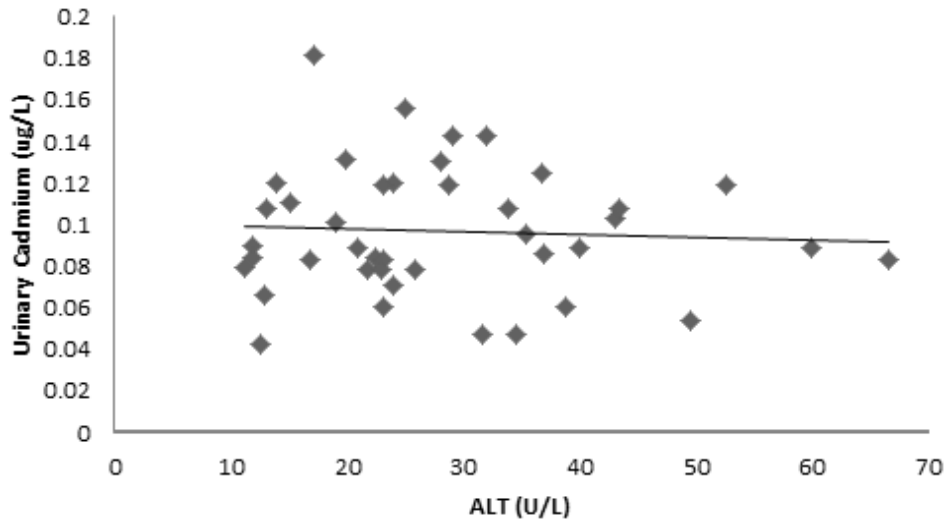


Figure 4.4a shows the correlation of urinary cadmium and ALT. A little correlation ( $r^2=0.003$ ) was found between the urinary cadmium levels and serum ALT. Correlation coefficient,  $r = 0.00-0.25$  shows little correlation,  $r = 0.25-0.50$  shows fair correlation,  $r = 0.50-0.75$  shows moderate to good correlation, whilst  $r = 0.75-1.00$  shows a strong positive correlation.

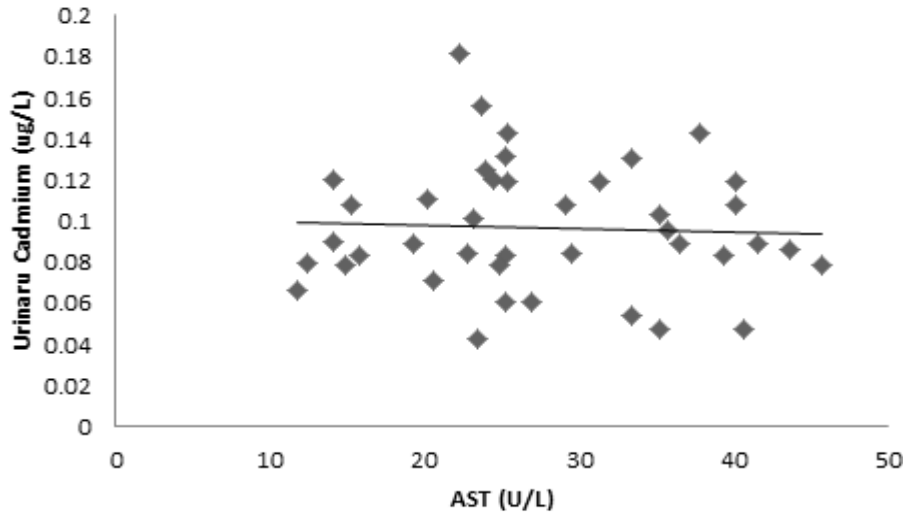


Figure 4.4b,  $r^2 = 0.003$

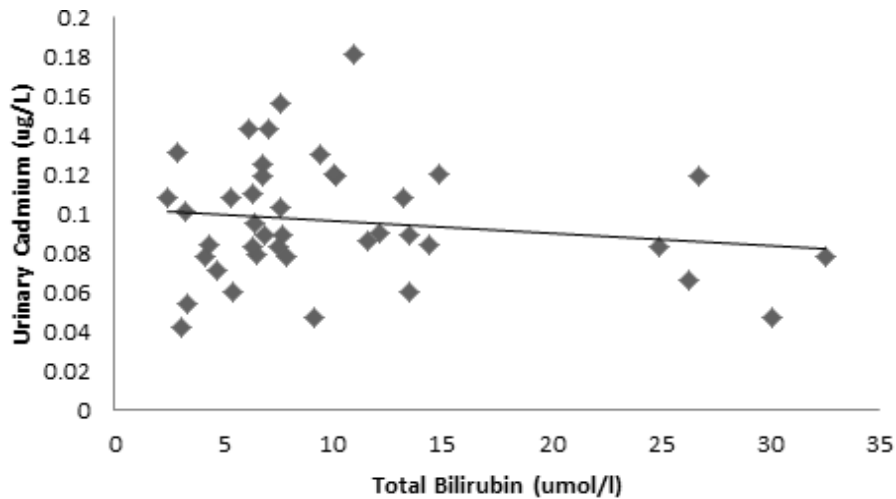


Figure 4.4c,  $r^2 = 0.0244$ .

Figures 4.4b and 4.4c shows the correlation of urinary cadmium with AST and Bilirubin respectively. A weak correlation was found between the urinary cadmium levels and serum ALT ( $r^2 = 0.003$ ) and also between the urinary cadmium levels and serum total bilirubin ( $r^2 = 0.0244$ ). Correlation coefficient,  $r = 0.00-0.25$  shows weak/little correlation,  $r = 0.25-0.50$  shows fair correlation,  $r = 0.50-0.75$  shows moderate to good correlation, whilst  $r = 0.75- 1.00$  shows a strong positive.

#### **4.5 Association of Urinary Arsenic with ALT, AST and Bilirubin**

Figures 4.5abc shows the association of urinary arsenic with ALT, AST and Bilirubin respectively of the CHB subjects. There was a weak correlation between the urinary arsenic concentration and ALT concentration ( $r^2 = 0.072$ ) and AST concentration ( $r^2 = 0.062$ ), but no correlation between the urinary arsenic concentrations and serum total bilirubin ( $r^2 = 0.018$ ).

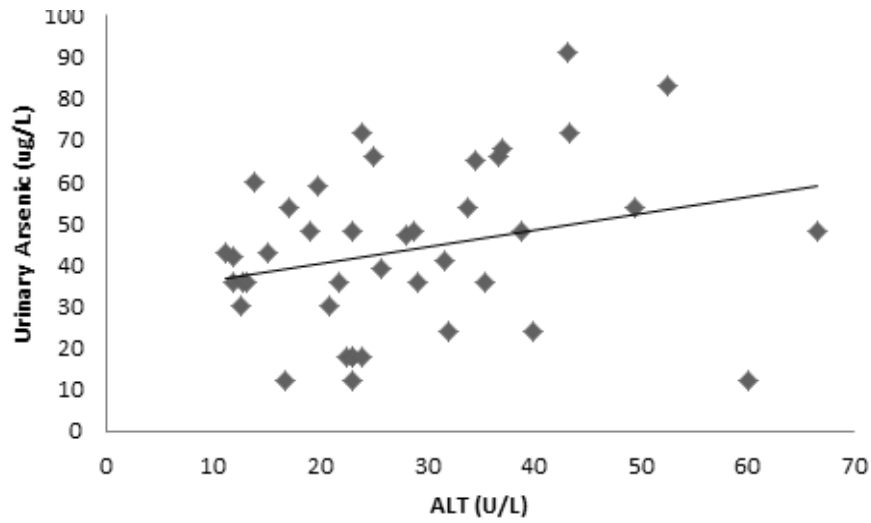


Figure 4.5a

Figure 4.5a shows the correlation of urinary arsenic with ALT. A weak correlation was observed between the urinary arsenic levels and serum ALT ( $r^2 = 0.072$ ). Correlation coefficient,  $r = 0.00-0.25$  shows weak/little correlation,  $r = 0.25-0.50$  shows fair correlation,  $r = 0.50-0.75$  shows moderate to good correlation, whilst  $r = 0.75-1.00$  shows a strong positive correlation..

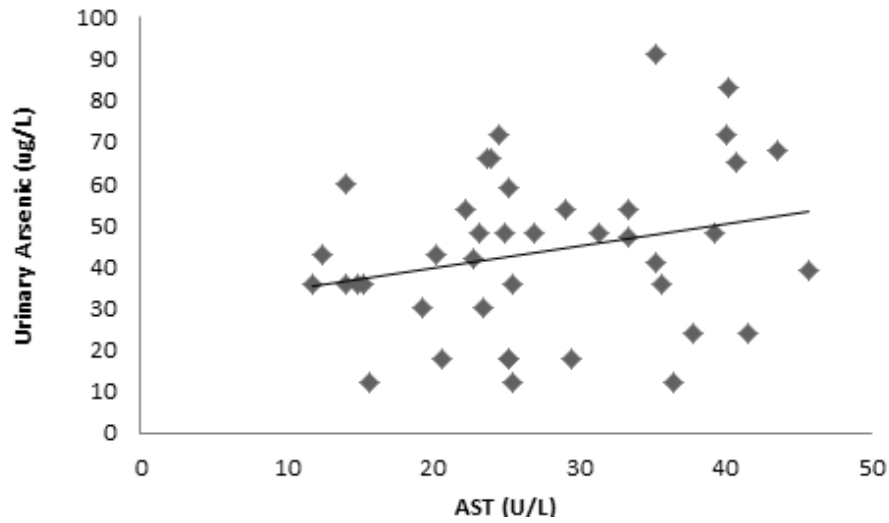


Figure 4.5b,  $r^2=0.062$ .

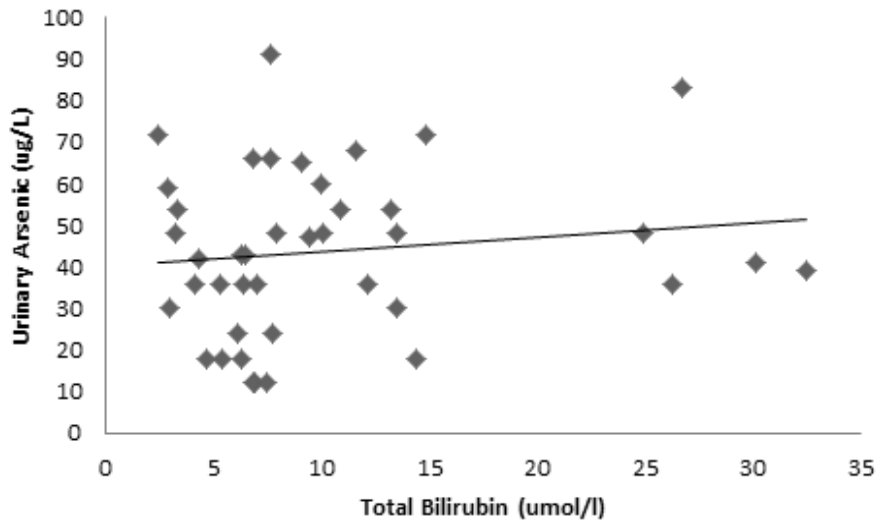


Figure 4.5c,  $r^2=0.018$ .

Figures 4.5b and 4.5c shows the correlation of urinary arsenic concentrations with AST and Bilirubin respectively. A weak correlation was observed between the urinary arsenic levels and serum AST ( $r^2 = 0.062$ ) and also between the urinary arsenic levels and serum total bilirubin ( $r^2 = 0.018$ ). Correlation coefficient,  $r = 0.00-0.25$  shows weak/little correlation,  $r = 0.25-0.50$  shows fair correlation,  $r = 0.50-0.75$  shows moderate to good correlation, whilst  $r = 0.75- 1.00$  shows a strong positive.

#### **4.6 Association of Urinary Lead with ALT, AST and Bilirubin**

Figures 4.6abc shows the association of urinary lead with AST, ALT and Bilirubin respectively of the CHB subjects. There was no correlation between the urinary lead concentration and AST concentration ( $r^2 = 0.041$ ). However, there was a weak positive correlation between the urinary lead concentrations and ALT concentration ( $r^2 = 0.065$ ) and total bilirubin concentration ( $r^2 = 0.063$ ).

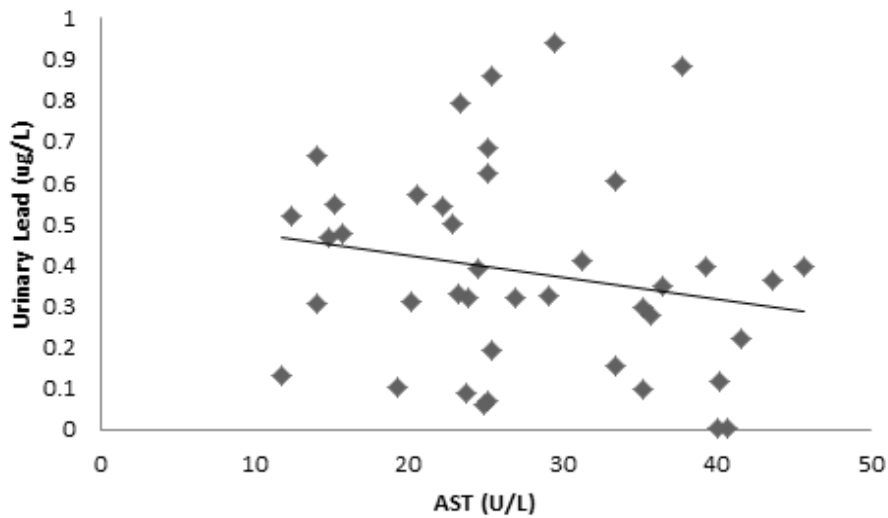


Fig. 4.6a.

Figure 4.6a shows the correlation of urinary lead and AST. A weak correlation ( $r^2 = 0.041$ ) was observed between the urinary lead levels and serum AST. Correlation coefficient,  $r = 0.00-0.25$  shows little correlation,  $r = 0.25-0.50$  shows fair correlation,  $r = 0.50-0.75$  shows moderate to good correlation, whilst  $r = 0.75-1.00$  shows a strong positive correlation.

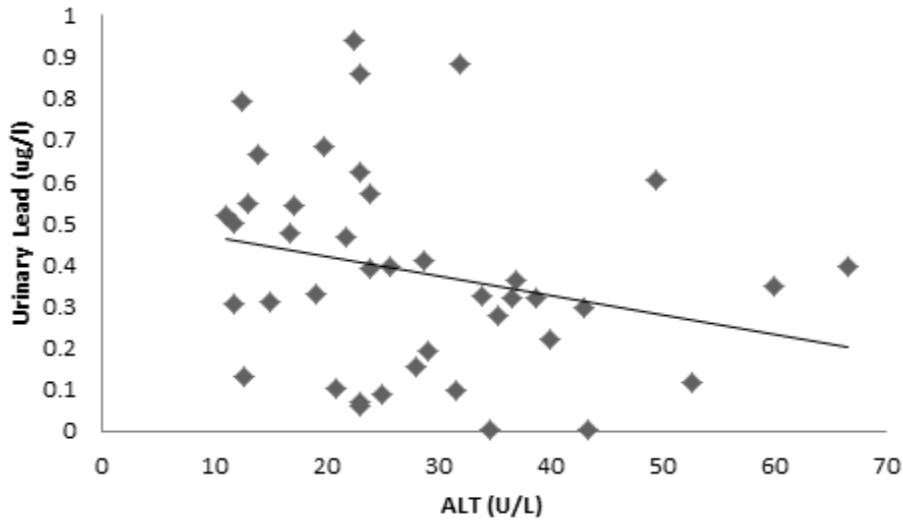


Figure 4.6b,  $r^2 = 0.065$ .

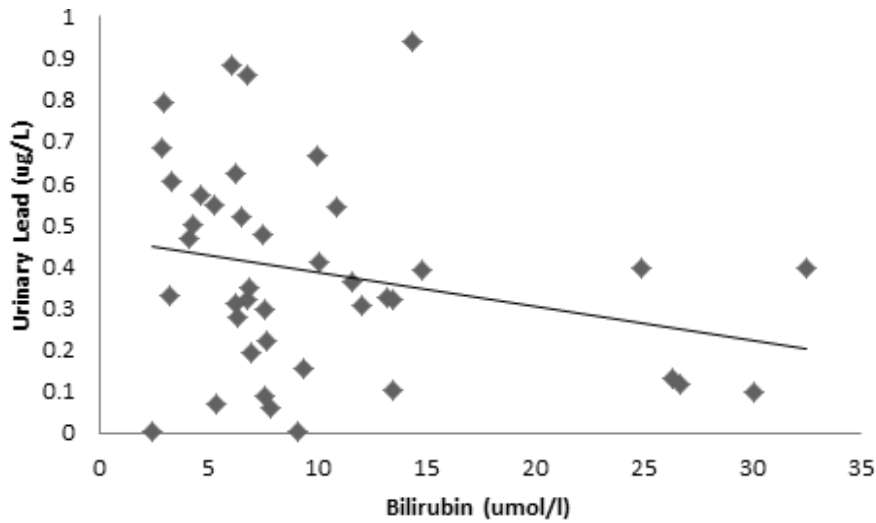


Figure 4.6c,  $r^2=0.063$ .

Figures 4.6b and 4.6c shows the correlation of urinary lead concentrations with ALT and Bilirubin respectively. A weak correlation was observed between the urinary lead levels and serum ALT ( $r^2 = 0.065$ ) and also between the urinary lead levels and serum total bilirubin ( $r^2 = 0.063$ ). Correlation coefficient,  $r = 0.00-0.25$  shows weak/little correlation,  $r = 0.25-0.50$  shows fair correlation,  $r = 0.50-0.75$  shows moderate to good correlation, whilst  $r = 0.75- 1.00$  shows a strong positive.

## CHAPTER FIVE

### 5.0 DISCUSSION

Chronic hepatitis B (CHB) is the persistence of hepatitis B surface antigen (HBsAg) in the blood of Hepatitis B Virus (HBV) infected persons for more than six months (WHO, 2015). Studies have shown links between factors and agents (such as toxic heavy metals) which enhance and speeds up the complications associated with CHB (Beth *et al.*, 2010; Bartoli *et al.*, 1983). In this study, levels of cadmium, arsenic and lead were measured to determine their association with liver function markers in persons with CHB in the Ejura-Sekyedumase district of the Ashanti region of Ghana. The gender distribution shows that, males had high prevalence of CHB, compared to females (Table 4.1) (Ott *et al.*, 2012). This is in agreement with the WHO's observation that the ratio of male to female CHB and hepatocellular carcinoma (HCC) is 4 to 1 , and that males are more likely to remain chronically infected with the virus than females (WHO, 2008). Though the reason for this observation is pathologically unclear, speculations exist around the role of genes, iron stores, and steroid hormones such as testosterone in the sex-specific susceptibility to infectious diseases (Klein, 2000).

The presence of HBeAg is a high risk factor for the development of cirrhosis and HCC (Liaw *et al.*, 2004). From this study, 7.3% of the CHB subjects were HBeAg – positive (Table 4.1). Similar study reported by Mahoney *et al.* recorded that 30% of CHB carriers are HBeAg – positive (Mahoney *et al.*, 1999). A 4-fold decreased as compared to this study. This decreased could be as a result of increasing public awareness and interventions available to hepatitis infected individuals today or the small sample size of

my studies. Moreover, development of active or passive CHB depends on sex, race, genotype of hepatitis B virus, host genetics and immune characteristics, age of first infection, nutritional and economic status (Livingstone, 2007).

In hepatitis B liver disease, liver function markers differ from acute, through chronic to liver failure. Bilirubin concentrations of the CHB subjects were greater than that of the controls (Table 4.2), though the levels for both groups were within reference range ( $\leq 21\mu\text{mol/l}$ ). The result from this study concurs with a research by Gitlin (1997). It also agrees with Rozen *et al* (1970), who observed that there is elevation of total and direct bilirubin in subjects with acute HBV infection above Upper Limit Normal (ULN), but insignificant elevation in persons with CHB (Rozen *et al.*, 1970). This is attributed to the rapid inflammation and destruction of hepatocytes associated with acute HBV infection, compared with the often slow hepatocyte destruction in CHB (Stewart *et al.*, 1978). There was no significant difference between the albumin of CHB subjects and controls (Table 4.2). Albumin is a protein made specifically by the liver and forms the main constituents of the total plasma protein (Farrugia, 2010). When the liver function is severely impaired by toxicants, it is unable to produce adequate albumin. There is usually no significant reduction in serum albumin levels in persons with CHB, except when complications such as cirrhosis sets in or a significant part of the liver is destroyed by chemotherapy (Hui *et al.*, 2002).

Significant association between toxic heavy metal levels and chronic liver diseases has been reported in viral hepatitis endemic areas (Hanan *et al.*, 2005; Gurjar and Mohan, 2003). In this study, cadmium was found to be significantly higher in persons with CHB, (Table 4.3). Cadmium, one of the extremely toxic heavy metals is commonly found in

industrial workplaces and agricultural inputs (Taylor, 1997). People in occupations such as cadmium plating, scrap and e-waste collection and agriculture are highly exposed (Taylor, 1997). In agriculture, cadmium, lead and arsenic are found in polluted water, soil and are used in different inorganic fertilizers and pesticides usually in higher concentrations (Gimeno-Garcia *et al.*, 1996). For example, mono-ammonium phosphate fertilizers have cadmium content of between 0.14mg/kg and 50.9mg/kg (Lugon-Moulin *et al.*, 2006). In this study, there was a significant difference in the levels of cadmium between the CHB subjects and the controls (Table 4.3). However, Hanan *et al.* (2005) found no significant difference in the levels of cadmium in persons with hepatitis B and persons without hepatitis B. The parallel rise in cadmium and Alanine aminotransferases (ALT) concentrations in persons with CHB, is probably due to a synergistic pathological effect of cadmium and the hepatitis B virus on the liver; and inability of the liver to detoxify cadmium in the presence of hepatitis by HBV (Thijissen *et al.*, 2007). Various studies have clearly indicated that hepatotoxic exposure of cadmium is associated with hepatocyte swelling, focal necrosis, hepatocyte degeneration, and deranged liver function (Stohs *et al.*, 2011; Thijissen *et al.*, 2007).

There was no significant difference in concentrations of lead and arsenic between the CHB subjects and the controls (Table 4.3). Ghazali *et al.* (2012), has reported that levels of arsenic, lead and cadmium are in normal reference ranges among farmers of Muda in Malaysia (Ghazali *et al.*, 2012).

In this study, ALT level was found to be significantly higher in persons with CHB than controls (Table 4.2), as was reported by Anna and McMahon (2005). Unlike acute HBV, in CHB (especially in the inactive state, which constitute over 90% of this study

population) (Table 4.1), there is decreased replication of the HBV, decreased HBeAg reduced destruction of hepatocytes, and decreased release of ALT into circulation (Gitlin, 1997). Therefore, levels of ALT in CHB ranges from normal to high (Dufour *et al.*, 2000). However, Acute HBV infection is characterized by transient elevation of ALT to about times 5 of the upper reference limit (Rozen *et al.*, 1970). This acute increase in ALT is due to the rapid replication of the HBV and destruction of hepatocytes, releasing ALT and other liver enzymes into the blood

There was no significant difference in the levels of aspartate aminotransferase (AST), between the CHB subjects and controls, however the level was slightly elevated in the CHB subjects compared with controls (Table 4.2). AST, unlike ALT, is distributed in other tissues such as red blood cells (RBC's), heart and skeletal muscles (Nyblom *et al.*, 2006; Nyblon *et al.*, 2004). Moreover the ALT/AST ratio  $> 1$  (1.021), indicates that the rise in the hepatic enzymes in the CHB group is more associated with the liver (Nyblon *et al.*, 2004).

There was a rather weak correlation between urinary cadmium levels and liver function markers (ALT, AST and bilirubin) (Figures 4.4a-c). Hyder *et al* (2013), have however reported increased risk of hepatic inflammation in persons with high urinary cadmium levels. Kelishadi *et al* also reported positive, but insignificant correlation between cadmium and liver function enzymes concentration (Kelishadi *et al.*, 2013). This study found that cadmium level was significantly higher in CHB subjects than in the controls ( $p = 0.004$ ). It is possible that the hepatitis B infection impaired the normal functioning of the liver in a way that it could not produce the threshold antioxidant levels as well as other biomolecules necessary in cadmium detoxification, hepatic healing and

regeneration (Adikwu *et al.*, 2013). There was a weak correlation between the urinary arsenic levels and liver injury markers ALT and AST, but no correlation with bilirubin (Figures 4.5a-c). This finding is consistent with the observation of Nandana *et al* (2012), who reported that levels of ALT, AST, and other hepatic markers are raised in arsenic exposure. This may be due to exposure to arsenic causes inflammation of hepatocytes leading to release of ALT, AST into the blood (Lu *et al.*, 2001), liver dysfunction and eventually other liver complications (Zhang *et al.*, 2000).

The correlation between urinary lead levels and ALT, AST and bilirubin was weak and insignificant (Figure 4.6a-c). This finding is in disagreement with a research by Won-Joon *et al* (2013) who reported raised ALT and AST levels in persons exposed to lead.

## **CHAPTER SIX**

### **6.0 CONCLUSION, LIMITATIONS AND RECOMMENDATIONS**

#### **6.1 CONCLUSION**

The results of this study shows a significantly increased cadmium levels in persons with Chronic Hepatitis B (CHB) compared with persons without CHB. Cadmium acts synergistically with HBV to cause the significantly high levels in CHB subjects and the HBV also impairs and burden liver function, making it difficult for the liver to detoxify cadmium. There were no significant increase in the levels of arsenic and lead in persons with CHB compared with persons without CHB. There were weak correlation associations between urinary cadmium, arsenic and lead with ALT, AST and total bilirubin.

#### **6.2 LIMITATIONS**

Time and logistic constraints restricted the selection of subjects with only CHB. It is therefore recommended for a similar study to be carried out in persons with Chronic Hepatitis C and acute hepatitis B.

### **6.3 RECOMMENDATIONS**

- ▶ The study should be replicated in other agricultural communities and also in other regions of the country (especially in mining towns with high prevalence of CHB), with larger sample population.
  
- ▶ Levels and effect of dietary micro minerals such as selenium and zinc should be studied in persons with CHB and also persons exposed to cadmium, arsenic and lead, since adequate selenium and zinc nutrition has been observed to reduce the levels and toxic effects of cadmium, arsenic and lead.

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## APPENDICES

### **Informed Consent form:**

I, **Eric Nyarko** of the Department of Chemical Pathology, School of Biomedical and Allied Health Sciences (SBAHS), University of Ghana wish to embark on a research study titled **Cadmium, Arsenic and Lead Levels in Persons with Chronic Hepatitis B in the Ejura-Sekyedumase district, Ghana.**

The purpose of this study is to find out the levels of toxic heavy metals Cadmium, Arsenic and Lead in the study subjects, investigate if there is any association between such levels, the function of their liver and development of Chronic Hepatitis B. The benefits of the study are to educate you on how your lifestyles and occupation affect your health, and to get understanding in the role of Cadmium, Arsenic and Lead in CHB. I humbly implore you to volunteer, though participation is entirely voluntary and strictly confidential. You may choose to withdraw from the study whenever you wish. The amount of blood to be taken by phlebotomists and used for this research study will not exceed 8mls (less than two tea spoonful).

You are assured of the strictest confidentiality of your personal information. This study will adhere to all applicable protocol and maintain quality assurance in accordance with good laboratory practices. The blood samples collected will bear an identification code to ensure anonymity, confidentiality and ease of identification. In addition, the information obtained and conclusions drawn will be applied in the adoption of relevant health policies as well as the appropriate supplementation, care and management of the disease. All abnormal test results will be explained to the persons concerned and referred for the appropriate medical care. All data will be entered onto a lock/word-protected Microsoft Excel spreadsheet. My contact numbers are **0246963470/0277827123**. You may call me for any further clarification.

Thank you for the cooperation and anticipated compliance to the study requirements.

Signature: ..... Date: .....

**PARTICIPANT CONSENT RESPONSE**

**Research title: Cadmium, Arsenic and Lead Levels in Persons with Chronic Hepatitis B in the Ejura-Sekyedumase District, Ghana.**

I,.....have been invited to take part in this research. I have been told of the purpose and procedure of this study which is to answer the questions raised about the hepatic risk associated with the exposure of persons with Chronic Hepatitis B to toxic heavy metals. I will not be reimbursed monetarily for participating in this research study.

The risk or dangers and discomforts might involve the pain of blood collection. The study team will try to reduce the chances of those risks happening by employing trained phlebotomist. The arm will be sanitized before blood collection, and new sterile needles and gloves will be used for each participant. I promise to comply with this research study and I consent accordingly.

Signature..... or Thumbprint.....

Date: .....