Hepatitis B virus-associated hepatocellular carcinoma in South Africa: epidemiology and impact of HIV-1 co-infection and immune dysregulation

by

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Abstract

Co-infection with the human immunodeficiency virus (HIV) negatively impacts the natural progression of hepatitis B virus (HBV) infection, including causing rapid progression to liver fibrosis and hepatocellular carcinoma (HCC). In sub-Saharan Africa the overlap between high HIV and HBV prevalence may increase the incidence of HCC. The aim of this study was to investigate the effect of HIV co-infection on presentation of HCC among HBV-infected patients. Since HCC is thought to be driven by ongoing severe inflammation, the study also evaluated the association between the expression of markers of immune activation/exhaustion and liver inflammation in patients with chronic hepatitis B (CHB) to determine if the risk of hepatofibrosis is increased by exposure to gut microbial products and compared HIV-infected patients with HBV-infected and HIV-HBV co-infected patients.

Ethical approval was obtained to conduct two sub-studies. The first sub-study (HCC Epidemiology Study) involved recruitment of patients diagnosed with HCC at oncology units of selected teaching hospitals in South Africa. A total of 107 HCC cases were recruited between December 2012 and October 2015. Demographic, laboratory and clinical data together with blood specimens were collected. Patients were tested for HBV, hepatitis C virus (HCV) and HIV. Molecular characterization of HBV and HCV was also performed. For the second sub-study (Liver Fibrosis and Immune Markers Study), 46 HBV/HIV co-infected; 47 HBV monoinfected; 39 HIV monoinfected and; 39 HIV-/HBV-uninfected controls were recruited following informed consent. All HIV-infected patients had been on highly active antiretroviral therapy (HAART) for ≥3 months. Liver stiffness measurements were taken using the Fibroscan 402. Cell-based immunomarkers of activation/exhaustion were measured using flow cytometry of fresh whole blood. Soluble serum/plasma immunomarkers were measured using ELISA and Luminex. HIV and HBV viral loads and genotyping of HBV were performed.

Of 107 cases in the HCC Epidemiology study, 83 (78%) were male and 68/106 (64%, 95% CI: 59-77) were positive for HBsAg. HIV seropositivity was seen in 22/100 (22%, 95% CI: 14-30) of all HCC cases. Among HBsAg-positive HCC cases, 19/66 (29%, 95% CI: 18-40) were HIV-infected compared to only 3/34 (9%) among those that were HBsAg-negative, p=0.04. The proportion of females among the HBV/HIV co-infected HCC cases 6/18 (33%, 95% CI: 11-55) was significantly higher compared to those that were HBV-mono-infected 6/47 (13%, 95% CI: 3-23), p=0.005. HIV/HBV co-infected females presented younger, at

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mean age 36.8 years (95% CI: 32.2-41.5) compared to 50.5 years (95% CI: 30.2-70.8) in HBV-mono-infected women, $p=0.09$. Males continue to be disproportionally affected with HCC. There is a trend towards younger age at diagnosis of HCC among HIV-positive compared to HIV-negative women.

The Liver Fibrosis and Immune Markers Study showed a high percentage of CD8+ T lymphocytes from co-infected subjects expressing HLA-DR/CD38 and PD-1 ($p<0.05$). Soluble CD14 and IP-10 were also significantly elevated in plasma of co-infected patients. Co-infected subjects exhibited delayed immune recovery with lower CD4/CD8 T cell ratio; CD4 cell counts and frequent HIV viremia compared to HIV mono-infected participants ($p<0.05$). The HBV mono-infected group had the highest proportion of participants with moderate/advanced liver fibrosis measured by Fibroscan, together with highest plasma concentrations of most of the cytokines measured. The results showed positive correlation between HIV and HBV viral replication and liver fibrosis. The results suggest that there is persistent T-lymphocyte dysregulation and delayed immune recovery in ART-experienced HBV/HIV co-infected patients. However this does not appear to be associated with severity of liver fibrosis in this cohort. HAART used in HIV is also effective against HBV and may therefore have led to control of viral replication leading to better fibrosis scores compared to the HBV mono-infected patients. Moderate/advanced liver fibrosis in HBV-mono-infection may well be an indicator of poor access to HBV screening and treatment.

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Abbreviations

AFB_1	aflatoxin B1
AFP	alpha-fetoprotein
ALT	alanine transaminase
APC	Allophycocyanin
APRI	aspartate to platelet ratio index
ASR-W	weighted age-standardized rate
CCl_4	carbon tetrachloride
CD	cluster of differentiation
CT	computed tomography
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte antigen 4
DCs	dendritic cells
DNA	deoxyribonucleic acid
ECD	Phycoerythrin Texas Red-X
ECM	extracellular material
FIB-4	Fibrosis-4 score
FITC	Fluorescein isothiocyanate
FMO	fluorescence minus one
HAART	highly-active antiretroviral therapy
HBeAg	hepatitis B e antigen
HBsAg	Hepatitis B virus surface antigen
HBV	Hepatitis B virus
HBx	HBV X protein
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	human immunodeficiency virus
HSC	hepatic stellate cell
IARC	International Agency for Cancer Research
IFN	Interferon
IL-	interleukin
INR	international normalised ratio
IQR	interquartile range
kb	kilobases
KC	Kupffer cell
KO	Krome Orange
LPS	lipopolysaccharide
MMPs	matrix metalloproteinases
MRI	magnetic resonance imaging
NASH	non-alcoholic steatohepatitis
NKT	natural killer T cell
nm	nanometres

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ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PC7	Phycoerythrin Cyanin 7
PD-1	programmed cell death protein-1
PDGF	platelet derived growth factor
PD-L1	programmed cell death protein-ligand 1
PE	Phycoerythrin
Prop	proportion
RCF	relative centrifugal force
RNA	ribonucleic acid
RPM	rounds per minute
SNAEs	serious non-AIDS-related events
SSA	sub-Saharan Africa
TENOFOVIR	tenofovir disoproxil fumarate
TGF-β	transforming growth factor beta
TIMPs	tissue inhibitors of matrix metalloproteinases
TLRs	toll-like receptors
TNF	tumour necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
US	Ultrasound
VEGF	vascular endothelial growth factor
WHO	World Health Organization
CHB	chronic infection with hepatitis B virus
AIDS	Acquired Immunodeficiency Syndrome
AST	aspartate transaminase
HBc	hepatitis B virus core protein
DAMP	damage associated molecular patterns
Treg	regulatory T lymphocytes
FoxP3	forkhead/winged helix transcription factor
pDCs	plasmacytoid dendritic cells
mDCs	myeloid dendritic cells
NK cells	natural killer cells
FDC	fixed dose combination
mCMV	murine cytomegalovirus

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Chapter one

1 Introduction and problem identification

Chronic infection with hepatitis B virus (HBV) is endemic to sub-Saharan Africa where it is the predominant cause of hepatocellular carcinoma (HCC) (Ferlay et al., 2010). HCC is one of the leading causes of cancer deaths worldwide and has a very high incidence within Africa, second only to Asia (Bosch et al., 2004). Apart from HCC, chronic HBV infection (CHB) is also a cause of liver fibrosis which is an abnormality characterized by excessive deposition of fibrous extracellular material.

A safe and effective vaccine to prevent HBV infection has been available for more than 2 decades. Despite this, 240 million people are chronically infected with CHB, a significant proportion of who are at risk of progressing to end-stage liver disease including liver cirrhosis and HCC (Ott et al., 2012). Even more people will become infected with HBV if vaccination is not administered timeously and if coverage with all recommended doses is not complete. This study built on the work from a pilot epidemiological study performed at Tygerberg Hospital, South Africa which showed that up to 5.9% of human immunodeficiency virus (HIV)-infected pregnant women carried HBV surface antigen (HBsAg) as a marker of active hepatitis B infection (Andersson et al., 2012). Also, using a larger sample size, the prevalence of HBsAg was found to be 3.4% among HIV-infected and HIV-uninfected pregnant women from the Western Cape whose mean age was 26 years (Andersson et al., 2013). In addition, HBV/HIV co-infected pregnant women were found to have higher infectivity compared to those with HBV-mono-infection as shown by increased carriage of HBV e antigen (HBeAg) as a marker of highly active HBV infection and thus have a higher likelihood of transmitting infection to their infants (Oshitani et al., 1996, Rouet et al., 2004).

The likelihood of vertical transmission of HBV from an infected mother to her infant is about 70-90% when the mother is seropositive for HBsAg and HBeAg (Beasley et al., 1977, Shepard et al., 2006). These data showed that hepatitis B continues to be an important public health issue despite the HBV vaccine being included in the South African national infant immunisation programme. Achieving a 90% HBV vaccination coverage with the first dose being given at birth would result in 84% decrease in mortality arising from HBV-related liver disease, which includes HCC, worldwide (Goldstein et al., 2005). Unfortunately, this level of HBV vaccine coverage is not being met in South Africa and in many African countries. The

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number of African countries achieving >90% of third dose vaccine coverage of HBV is low, with South Africa consistently achieving <79% third dose coverage since launching HBV immunisation in 1996 (World Health Organisaion, 2015).

Untreated HBV infection among pregnant women coupled to the current schedule for HBV infant vaccination in South Africa leaves infants at risk of perinatal transmission particularly from highly infectious HBV-infected mothers because the first dose is only administered at 6 weeks of age and not at birth as recommended by the World Health Organisation (WHO). The HBV vaccination schedule in South Africa is based on the premise of very little perinatal transmission and was designed to prevent horizontal transmission early in life, but not perinatally (Guidozzi et al., 1993). However, available data shows that vertical transmission of HBV continues to occur in sub-Saharan countries especially from HIV- and HBV- co-infected mothers to their infants (Sangare et al., 2009, Hoffmann et al., 2014, Chotun et al., 2015). As long as some infants continue to acquire hepatitis B through vertical transmission due to the existing vaccination protocols, it also implies that there will always remain a pool of persons with CHB that are at risk of HBV-associated HCC within South Africa. Perinatally- and childhood-acquired hepatitis B infections are known to commonly result in chronic infection because of the inability to effectively control infection probably due to infants' immune systems being set towards general immune tolerance during development in-utero (Prendergast et al., 2012). Also, the liver as an "immune organ" is generally biased towards immune tolerance because of its constant exposure to antigens and neoantigenic metabolites delivered through the hepatic portal vein (Gao, 2016, Grakoui and Crispe, 2016).

Considering hepatitis B epidemiology in pregnant women, it is expected that HCC will continue to be a major cause of morbidity and mortality for decades to come, especially given the immune dysregulation that is associated with HIV co-infection despite the use of antiretroviral therapy. There are reports of an increase in liver disease and HCC among HIV-infected patients in the era of effective antiretroviral therapy (Brau et al., 2007, Rosenthal et al., 2009, Kew et al., 2010, Merchante et al., 2013). The likely explanation is the increased survival of HIV-infected patients is allowing the progression of underlying hepatitis B infections which was not happening when there was no treatment of HIV and patients died due to opportunistic infections seen in AIDS. An aging effect will therefore increase the incidence of malignancy among HIV-infected patients that have been shown to have rapid immunoaging due to chronic systemic immune activation associated with HIV infection

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(Appay and Kelleher, 2016). HCC cases are increasingly being seen presenting at a much younger age due to HBV/HIV co-infection but the precise mechanisms behind the change in age at presentation are unknown. Even though HCC is classified as a non-AIDS defining cancer, HIV infection seems to have an effect on the epidemiology of the malignancy.

Sub-Saharan Africa, including South Africa, represents an intersection point between the HIV epidemic and endemic CHB. The mechanism by which liver disease arises especially in HBV-infected patients in the context of HIV is complex and an under-investigated area with most research focussing on HCV/HIV co-infected patients in resource-rich settings. HCV is not as prevalent as HBV in Southern Africa and pathogenesis of the two viral hepatitis infections is not similar. Despite the limited data on HBV/HIV co-infection and its association with liver inflammation/fibrosis, it is known that the pathology surrounding HIV infection is immune driven. HIV infection, especially when untreated, is accompanied by elevated levels of inflammation and immune activation/dysregulation, a component of which is mediated by translocation of microbial products across the gastrointestinal tract into the systemic circulation (Crane et al., 2012). Microbial products such as lipopolysaccharide (LPS) may directly mediate liver fibrosis through stimulating Kupffer cells (KCs) and hepatic stellate cells (HSCs) to produce fibrogenic cytokines and fibrogenic extracellular matrix proteins. In addition, microbial translocation may also indirectly cause liver fibrosis through immune activation-induced apoptotic cell death of hepatocytes (Page et al., 2011). As more data emerges concerning liver inflammation and fibrosis, it is clear that the fibrogenesis process is complex and involves a number of cell types and biomarkers that were previously not recognized as crucial in the pathogenesis of HBV-driven liver disease. For example, recent data using mice models showed that interferon-gamma (IFN-γ) produced by HBV-specific CD4+ T cells is also responsible for tolerance in CHB and persistence of hepatitis B (Zeng et al., 2016). Even more data of the different role players in pathogenesis of liver fibrosis is expected to emerge as hepatocyte co-culture models to investigate hepatitis B become more widely available.

This study sought to provide information on the epidemiology and presentation of HCC in HBV/HIV co-infected patients. In addition to providing epidemiological evidence, the study also aimed to provide an immunological explanation for how HIV may contribute to HCC pathogenesis and liver disease by investigating the role of immune dysregulation (driven by microbial translocation) towards the pathogenesis of liver fibrosis which is a checkpoint along

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the CHB to HCC pathway. The HCC Epidemiology component of this study hypothesized that HIV co-infection increases the risk of HBV-associated HCC reflected by differences in the demographic and clinical characteristics between HIV-infected and HIV-uninfected HCC patients. The study found that HBV/HIV co-infected HCC patients presented at a younger age compared to HIV-uninfected HCC patients and that this difference was particularly evident among females.

The hypothesis of the Liver Fibrosis and Immune Markers component of this study was that HIV infection leads to higher systemic levels of microbial products, increased immune activation causing stimulation of key liver cells and the promotion of hepatic fibrosis. Although the data from this study observed an increased level of microbial translocation and systemic immune dysregulation, there seemed to be no increased liver fibrosis in HBV/HIV co-infected patients and antiretroviral therapy also effective against HBV might be the reason for these findings.

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Chapter two

2 Literature review

This literature review covers the epidemiology of hepatocellular carcinoma (HCC) with emphasis on sub-Saharan African (SSA) countries. This is followed by discussion of the risk factors associated with hepatocarcinogenesis with a focus on hepatitis B infection. Literature on liver fibrosis and its pathogenicity in hepatitis B infection together with the effect of HIV infection and microbial translocation is also reviewed followed by the link between HCC and liver fibrosis. Also covered are the diagnostic modalities for HCC and liver fibrosis and also treatment for chronic hepatitis B.

2.1 HCC epidemiology in Africa

HCC is the second most common cause of cancer mortality, following only after lung cancer, in all sexes combined worldwide (Ferlay et al., 2010). HCC contributes 85-90% of all primary liver cancers (Zhang and Cao, 2011). SSA has the second highest number of HCC cases worldwide after South East Asia (Bosch et al., 2004). It is estimated that there were over 37,000 deaths in 2012 from primary liver cancer within SSA, with the modal age group being 30-49 years old for both males and females (World Health Organization, 2014). In SSA, HCC has the second highest incidence and mortality among men and the third highest in women (Ferlay et al., 2013). The mortality rate of HCC matches the incidence rate, highlighting the fatality of the malignancy and also the inadequacy of current screening protocols for the malignancy (Cole and Morrison, 1980).

The distribution of HCC across Africa varies according to geographical region, as appears in Table 2.1. Western Africa has the highest incidence and mortality rates, closely followed by North Africa while East Africa has the lowest according to the 2012 estimates (Ferlay et al., 2013). There are also inter-country differences within the geographical regions as shown in Table 2.2. Among the top 20 countries in Africa with the highest incidence of HCC, all are found in SSA, except for Egypt which is in North Africa (Ferlay et al., 2013). It should be pointed out that even with these available statistics, the true incidence and mortality rates associated with HCC in SSA are not definitively known because most cancer registries in Africa are incomplete and are virtually non-existent in other countries (Kew, 2013). There are also challenges associated with diagnosis of the malignancy because of the scarcity of resources needed for the clinical investigation of HCC (Kew, 2013). The GLOBOCAN 2012

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report shows poor data quality for both incidence and mortality of different cancers for most African countries and this makes it difficult to obtain reliable estimates of HCC (Ferlay et al., 2013). The highest weighted age standardized rates (ASR) for HCC incidence of 10.1% was found in Swaziland within the Southern Africa region where a total of 75 incident cases were reported and these were matched by mortality of 71 cases.

Table 2.1 Regional age-standardised incidence and mortality rates of HCC. Data from the International Agency for Research on Cancer (IARC) GLOBOCAN 2012 tables (http://globocan.iarc.fr/)

	Incidence		Mortality	
	Number of people	**ASR (w)**	**Number of people**	**ASR (w)**
Global	554 369	15.3	521 031	14.3
Western Africa	23 040	12.1	22 181	11.5
North Africa	19 653	12.3	18 704	11.8
East Africa	7 947	4.0	7 530	3.8
Middle Africa	5 508	8.0	5 515	7.5
Southern Africa	2 232	4.8	2 127	4.7

Table 2.2 Age standardized rate (ASR) for incidence and mortality for selected African countries for 2012.

	Incidence			Mortality			5-year prevalence		
	Number	**(%)**	**ASR (W)**	**Number**	**(%)**	**ASR (W)**	**Number**	**(%)**	**Prop**
Western Africa									
Gambia	238	42.7	25.8	225	49.7	24	173	20.2	16.8
Guinea	1102	20.8	19.5	1050	24.8	18.5	733	6.8	12.2
Ivory Coast	2237	18.6	17	2141	23	16.1	1514	6.4	12.3
Burkina Faso	1254	16.2	14.4	1219	19.6	13.9	898	5.7	9.4
Ghana	1923	12.2	11.1	1856	17.4	10.7	1329	4	8.4
Nigeria	12047	11.8	11.5	11663	16.3	11	8477	3.7	8.9
Niger	516	8.7	6	489	10.5	5.6	355	2.8	4.2
Eastern Africa									
Uganda	1351	4.6	7.9	1292	6	7.4	921	1.6	5
Zimbabwe	548	3.5	6.9	520	4.6	6.5	280	0.9	3.5
Mozambique	658	3	4.5	606	3.6	4.2	441	1	3.2
Kenya	1120	2.7	5.4	1037	3.6	5	768	0.9	3.1
Zambia	230	2.2	3.2	221	2.9	3.1	147	0.7	2
Tanzania	595	1.8	2.1	565	2.4	2	406	0.6	1.5
Ethiopia	996	1.6	2	926	2.1	1.8	653	0.5	1.3
Southern Africa									
Botswana	75	4.6	5.4	73	7.1	5.3	54	1.7	3.9
Namibia	27	2	1.7	27	3.4	1.7	21	0.7	1.4
South Africa	1972	2.5	4.8	1877	4	4.6	1158	0.7	3.2
Swaziland	75	8.3	10.1	71	11.3	9	52	2.9	6
Central Africa									
Congo	235	10.7	8.6	225	15.1	8.1	163	3.3	6.5
Cameroon	727	5.3	4.8	716	7.6	4.6	506	1.7	4.2
Gabon	28	2.7	2.3	28	4.5	2.3	20	0.9	2

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2.2 Hepatitis viruses as risk factors for HCC in Africa

A major risk factor for HCC is chronic infection with hepatitis B virus (HBV) which has a high intermediate endemicity (5-7%) among adults in Southern SSA (Ott et al., 2012). The high incidence of HCC in SSA reflects the distribution of HBV and other risk factors such as chronic hepatitis C virus (HCV) infection, aflatoxin exposure, non-alcoholic steatohepatitis, hereditary hemochromatosis, autoimmune hepatitis and also excessive alcohol consumption (Kew, 2010). It is estimated that 60% of all HCC cases in developing countries are due to hepatitis B infection which is preventable through the administration of a safe and effective vaccine (Han et al., 2011). Global statistics suggest that the risk of developing HCC is about 20 times more in people that are seropositive for HBV surface antigen (HBsAg) compared to those that do not have the marker (Beasley, 1988). It is for this reason that HBV is classified as a Group 1 carcinogen by the International Agency for Cancer Research (IARC) (IARC, 1994). Group 1 carcinogens are those for which there is enough evidence showing them to be a cause of human cancer.

In contrast to most sub-Saharan Africa countries, HCC in Egypt is mainly driven by infection with HCV, with hepatitis B having a less prominent yet still significant contribution (Darwish et al., 1997, Lehman and Wilson, 2009, Ferlay et al., 2013). Data suggests that the contribution of HCV infection towards HCC is also significant considering that the WHO Africa region has the second highest anti-HCV prevalence of 3.2% after South East Asia which has a population seroprevalence of 4.7% (Lavanchy, 2011). Within sub-Saharan Africa, countries such as Cameroon and Burundi have the highest prevalence of HCV at 13.8% and 11.3% respectively (Lavanchy, 2011). In total, it is estimated that there are just under thirty million Africans infected with HCV, a significant proportion of whom will develop HCC following chronic infection (Lavanchy, 2011). With regards to gender, HCC in SSA has a predilection for male patients who also have a higher prevalence of chronic hepatitis B infection (CHB) (Blumberg et al., 1966, Ryder et al., 1992). Indeed, according to the latest statistics there were almost 2-3 times as many deaths from HCC in males compared to females with the highest mortality seen in the 30-49 years age group (Parkin et al., 2001, World Health Organization, 2014). In contrast to the observations from SSA, HCC in the developed countries of Europe and North America is usually seen in patients above 50 years of age (Kew, 2013, World Health Organization, 2014).

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2.3 Non-viral risk factors for HCC

Apart from the oncogenic hepatitis viruses (HBV and HCV) which pose the most prevalent risk factors for HCC, other environmental and hereditary factors are also known to favour the development of hepatocellular malignancy. The non-viral factors for hepatocarcinogenesis include-

- Exposure to hepatotoxins such as aflatoxin and alcohol.
- Cirrhosis arising from alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), primary biliary cirrhosis, dietary iron-overload and autoimmune hepatitis (AIH).
- Hereditary diseases such as tyrosinemia, α1-antitrypsin deficiency and hereditary hemochromatosis.
- Obesity and diabetes mellitus.

2.3.1 NAFLD/NASH

NAFLD includes a wide spectrum of hepatic disorders that range from uncomplicated fatty liver (pure steatosis) to progressive NASH which may ultimately lead to cirrhosis and HCC (Bertolotti et al., 2014, Rinella, 2015). Fatty liver is histologically defined by increased hepatocellular storage of triglycerides and involves more than 50% of hepatocytes (Zoller and Tilg, 2016). Meanwhile, steatosis is a histological finding of lipid deposits in more than 5% of hepatocytes (Tannapfel et al., 2011, Zoller and Tilg, 2016). NASH is considered to be a progression of NAFLD which can be distinguished from the latter by the presence of hepatocellular injury with or without fibrosis (Rinella, 2015). HCC development in NAFLD is increased by the presence of obesity, diabetes, high iron and also alcohol consumption (Zoller and Tilg, 2016). The increasing incidence and prevalence of NAFLD-associated HCC mirrors the increasing prevalence of type-2 diabetes and obesity (Kew, 2015). A study on HCC risks from the United States reported that 14.1% of 4929 HCC cases were NAFLD-related (Younossi et al., 2015). These NAFLD-related HCC cases tended to be older but with a shorter survival time (Younossi et al., 2015). Obesity in men with a body mass index (BMI) greater than 35 has been described to increase the risk of death from liver cancer by 4.5 times relative to men with normal BMI (Calle et al., 2003). Lipid accumulation which is the hallmark of NAFLD results in increased free fatty acids and other lipid metabolites accumulation in hepatocytes which leads to hepatocyte injury, endoplasmic reticulum stress

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and inflammation (Page and Harrison, 2009). This results in hepatocyte death which triggers compensatory regeneration and proliferation that initiate hepatocarcinogenesis.

2.3.2 Aflatoxin exposure

The environmental hepatotoxin aflatoxin B1 (AFB_1) is a natural fungal metabolite that is hepatocarcinogenic and is produced by fungi of the *Aspergillus* species, specifically *A. flavus* and *A. parasiticus* (Kew, 2003). These fungal species are normally found in hot and humid climates that are characteristic of many sub-Saharan countries where they contaminate human and animal food during harvest time and storage (Henry et al., 1999). Aflatoxins may contaminate foods such as peanuts, maize, rice, dried fruit and tree nuts. They may also be found in milk and milk products of dairy cows that are fed with contaminated feed (Henry et al., 1999). The metabolism of AFB_1 after absorption is known to lead to the formation of AFB_1-guanine complexes in hepatic DNA leading to the development of mutations in the p53 gene, specifically a missense mutation at codon 249 (Hussain et al., 1994). Exposure to AFB_1 in combination with CHB has a synergistic effect toward the development of HCC (Kew, 2003). Within sub-Saharan Africa, exposure to aflatoxin is mostly seen in rural populations who are then placed at increased risk of HCC development. A report from China suggested that exposure to aflatoxin in HBV-infected males triples the risk of HCC development (Ming et al., 2002). Within sub-Saharan Africa, rural populations are at increased risk of HCC development due to the fact that the prevalence of CHB is also highest in these settings coupled with greater exposure to aflatoxin compared to urban dwellers (Kew et al., 1986). Earlier studies point to high aflatoxin exposure with concomitant CHB as being the greatest contributing factors of a high HCC prevalence in some African countries (Alpert et al., 1971, Van Rensburg et al., 1975, Wogan, 1975, Peers et al., 1976, Peers and Linsell, 1977).

2.3.3 Iron overload

Dietary iron overload is a risk factor that is common in rural Africa where people consume traditional beer that is brewed in iron pots or drums resulting in iron leaching into the brew (Walker and Arvidsson, 1953). Excess iron in the hepatocytes can lead to malignancy through a number of mechanisms which may include: oxidative stress of hepatocytes initiating an inflammatory cascade which progresses to cirrhosis and finally HCC and; increased lipid peroxidation causing formation of by-products that are cytotoxic and capable of impeding cellular function and protein synthesis (Gordeuk et al., 1996, Kew, 2009).

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2.3.4 Alcohol misuse

Ethanol found in alcoholic beverages in also listed as a Group 1 carcinogen by IARC and heavy consumption (≥80g/ day for 10 years or more) poses as an important risk factor for HCC (IARC, 1988, Vineis et al., 2004). Apart from exerting an independent effect, alcohol has an additive relationship with CHB towards HCC development (IARC, 1988, Mohamed et al., 1992, Morgan et al., 2004). The effect of alcohol misuse on HCC development in South Africa was found to be more pronounced amongst urban men over the age of 40 years compared to women that did not consume as much alcoholic beverages (Mohamed et al., 1992).

2.4 Virology of HBV

Globally, the incidence of HCC mirrors the prevalence of chronic hepatitis B (Beasley, 1988). The precise molecular mechanisms that lead to the development of HCC are incompletely understood and this is compounded by the lack of an HBV-encoded dominant oncogene (Neuveut et al., 2010).

2.4.1 Taxonomy of HBV

HBV is prototypic of the *Hepadnaviridae* family of hepatotropic DNA viruses that replicate through an RNA intermediate. HBV naturally infects humans and is a member of the genus that infects mammals (*Orthohepadnavirus*) while the *Avihepadnavirus* genus infects birds. Presently, there are at least 10 HBV genotypes (annotated A-J), with genotypes A, B, C, D and F having several subtypes (Lin and Kao, 2011). The genotypes differ from each other by 8% of the nucleotide sequence of the entire genotype. However, most genotyping algorithms use only the surface antigen region. Of the 10 genotypes, only 8 (A-H) are widely described in literature. Genotypes I and J are thought to have recently arisen from recombination events (Olinger et al., 2008, Lin and Kao, 2011). The different genotypes and subtypes have distinct geographical distribution and also clinical implications with regards to response to therapy and pathogenicity. HBV genotype A is the most abundant and is widely distributed within sub-Saharan Africa followed by genotype D while genotype E is restricted to West Africa (Kramvis et al., 2005). Genotype A is most hepatocarcinogenic among South African patients. It is the most frequently detected genotype among patients with HCC in much of Southern Africa (Kew et al., 2005). In Asian countries, genotype C is associated with a greater risk of HCC development compared to genotype B (Kew et al., 2005).

2.4.2 Structure of HBV

HBV has one of the smallest known viral genomes and has a 3.2 kilobases (kb) long circular DNA genome. HBV is the only hepatitis virus to possess a DNA genome (Ganem and Prince, 2004). The circular DNA of the HBV genome is partially double-stranded because of an incomplete positive sense strand (Howard, 1986). The complete, infectious hepatitis B virion, also known as the Dane particle has a diameter of 42 nm and is composed of an icosahedral nucleocapsid core of 27 nm diameter that is surrounded by a lipoprotein envelope composed of a lipid bilayer derived from host's internal membrane with embedded viral surface proteins (Howard, 1986).

In addition, HBV also exists in two other subviral forms which are the filamentous and spherical particles. The spherical subviral particles have a diameter of 22nm while the filamentous particles have variable sizes and can get up to a length of 200nm. The subviral particles are produced in much greater quantities than the Dane particle. A schematic diagram of the Dane particle and the associated non-infectious sub-viral particles is shown in Figure 2.1.

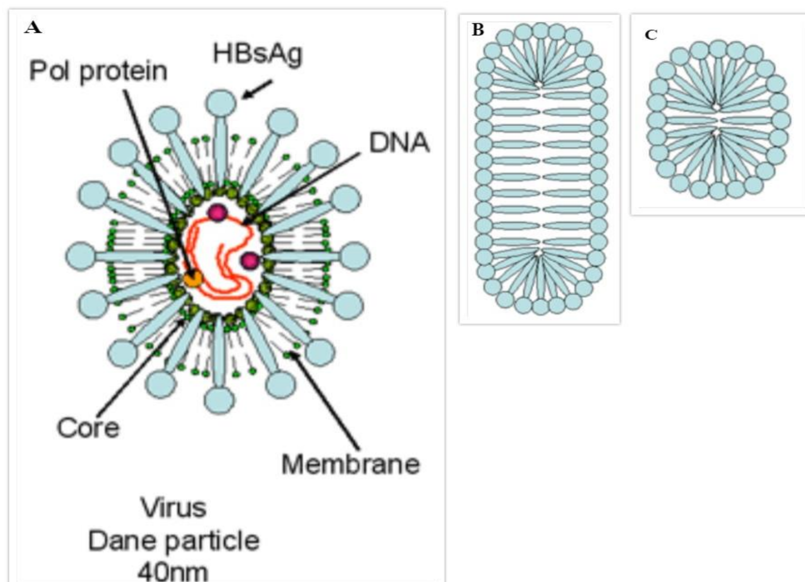

Figure 2.1 Morphology of HBV particles. The infectious particle (Dane particle) is shown in Figure A with the HBV surface proteins (HBsAg) surrounding a nucleocapsid core that encloses the relaxed circular DNA with an incomplete positive sense strand and complexed with the polymerase enzyme (Pol protein). The membrane of the infectious particle is derived from the host plasma membrane during budding from infected hepatocytes Figure B shows the filamentous particle while Figure C is a representation of the spherical particle. Both of these particles are aggregates of HBsAg. Used with permission from Richard Hunt (http://www.microbiologybook.org/virol/hepatitis-virus.htm).

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2.4.3 Genetic organization of HBV

The HBV genome contains four open reading frames (ORFs) that overlap each other in a frame-shifted manner to produce seven proteins. These four open reading frames are C, P, S and X (Lüsebrink, 2009). ORF-C (core/precore) codes for the HBV core protein (HBc) and the HBV e antigen (HBeAg). The core protein is necessary for viral assembly and makes the nucleocapsid that encapsulates the viral DNA and the polymerase. HBeAg has no role in viral assembly but is known to induce immunotolerance. Although HBeAg does not have a role in viral assembly and is a secreted protein, its detection in blood serves to show active replication of HBV and is a surrogate marker of high infectivity (Harrison et al., 2008). ORF-P codes for the polymerase enzyme that is involved in replication and also has transcriptase and RNAse H activity. ORF-S (preS-S) contains genetic information for the three polypeptides of the surface antigen (preS1, preS2 and S). Surface antigen proteins of different lengths are produced depending on which translation site has been read for initiation within the gene but all share the same C-terminus (Ganem and Prince, 2004, Harrison et al., 2008, Lüsebrink, 2009). ORF-X codes for the HBV X protein (HBx) which is a transactivator of viral transcription (Pawlotsky, 2006, Lüsebrink, 2009). HBx is non-specific and modulates expression of both viral and host cellular genes (Ganem and Prince, 2004). A schematic of the genetic organization of HBV is shown in Figure 2.2.

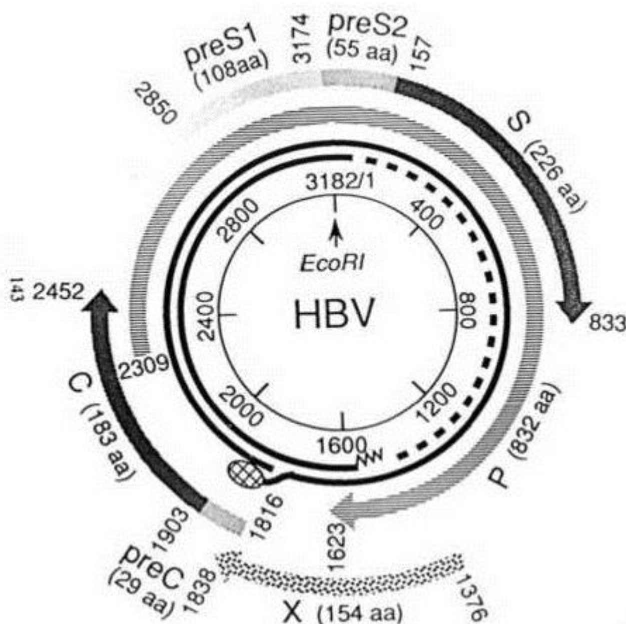

Figure 2.2 Genome organisation of HBV and its transcripts. The precore protein is the smallest of the HBV transcripts being only 29 amino acids long whilst the polymerase protein also containing the reverse transcriptase domain is the longest with 832 amino acids. The polymerase ORF completely overlaps the surface antigen ORF and this has implications for mutations that emerge due to treatment with anti-viral agents. Used with permission (Malik and Lee, 2000).

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2.5 Immunology of hepatitis B

Primary infection with HBV causes hepatic inflammation that may or may not be asymptomatic (Chang and Lewin, 2007). Asymptomatic hepatitis explains why many patients are undiagnosed because they have no need to seek medical care. Whether infection is cleared or becomes chronic is dependent on the age and immune status of the infected individual. CHB most commonly occurs in childhood-acquired infection following vertical or horizontal infection as well as in adults that are immunosuppressed as occurs in untreated HIV infection (Ganem and Prince, 2004). The likelihood of primary hepatitis B becoming chronic in immunocompetent adults is very low (<5%) (Hou et al., 2005). All arms of the immune response (innate, cellular and humoral) are important in determining the outcome of hepatitis B (Guidotti and Chisari, 2006, Chang and Lewin, 2007, Chisari et al., 2010).

2.5.1 Acute hepatitis B

Antiviral cytokines produced by innate and adaptive immune cells are important for the non-cytolytic control of HBV replication in the acute phase of infection of individuals that are able to clear infection (Chang and Lewin, 2007, Bertoletti et al., 2009). Interferon (IFN)-γ, tumor necrosis factor (TNF)-α and IFN- α/β secreted by CD8+ cytotoxic T lymphocytes (CTLs) have been described to lead to inhibition of HBV DNA replication without the cytolytic damage of infected cells using transgenic animal models (Guidotti et al., 1994, Protzer et al., 1999). The influx of HBV-specific and non-specific T-cells into the liver during the incubation/acute phase is associated with a further decrease of HBV DNA that eventually results in symptomatic hepatitis (Webster et al., 2000, Thimme et al., 2003). Clearance of HBV following acute infection is associated with resolution of inflammation that is punctuated by normalization of serum alanine transaminase (ALT) and also the appearance of antibodies to the core (HBc), precore (HBeAg) and surface antigen (HBsAg) proteins of HBV (Chang and Lewin, 2007) A schematic summary of the interplay between serological, immunological and molecular markers in acute hepatitis B is shown in Figure 2.5.

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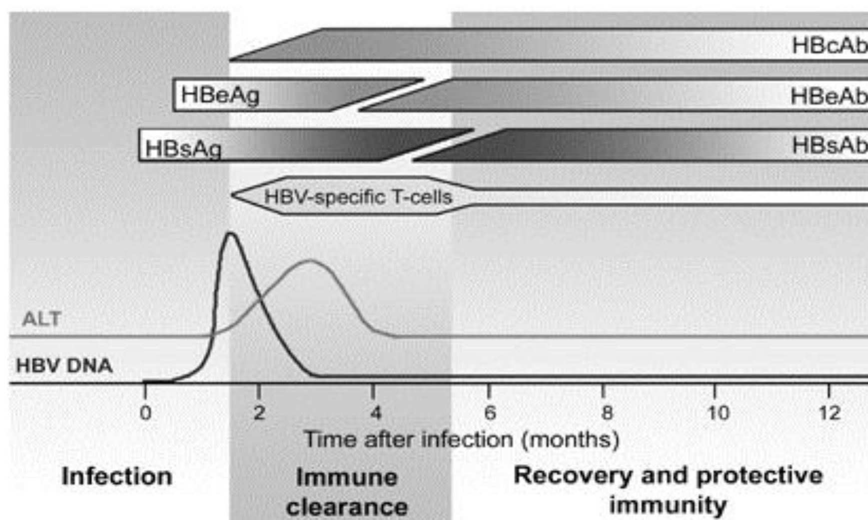

Figure 2.3 Serological and molecular changes in acute hepatitis B. Following acute infection, there is rapid replication of HBV DNA within hepatocytes. Before the influx of HBV-specific T-cells, there is control of HBV replication by non-cytolytic mechanisms from cytokines. The influx of the HBV-specific T cells eventually leads to symptomatic hepatitis shown by increased serum ALT (released from hepatocyte lysis). This leads to a further decrease of HBV DNA and the appearance of HBcAb, HBeAb and HBsAb. The HBc core protein is part of intact viral particles and is not freely detected in serum. Reprinted by permission from Macmillan Publishers Ltd: Immunology and Cell Biology (Chang and Lewin, 2007).

2.5.2 Chronic hepatitis B

CHB is defined as the detection of HBsAg in the serum of an infected individual for a period exceeding 6 months after acute infection. Unlike in resolved acute hepatitis B, antibodies to HBsAg do not become detectable and HBV DNA continues to be detected in CHB. Dependent on the phase during chronic infection, ALT levels fluctuate as shown in Figure 2.6. Childhood-acquired hepatitis B infections tend to become chronic perhaps as a reflection of the immaturity of a child's immune systems (Pungpapong et al., 2007). It is thought that the immune system in infants is set to be tolerant to hepatitis B, failing to mount the typical antibody and T cell response resulting in high levels of HBV DNA with no attendant liver inflammation (Prendergast et al., 2012). However, tolerance does not completely explain the propensity for infants to develop CHB as some infants have been reported to develop early clinical hepatitis B disease that is probably associated with variants of HBV that do not produce HBeAg and also the fact that infants are able to respond to HBV vaccine after birth (Beasley et al., 1983, Prendergast et al., 2012, Hong et al., 2015). HBeAg has been described to cross the placenta rendering HBsAg-positive infants borne to HBV-infected mothers tolerant to HBV in-utero by inducing HBV specific T cell tolerance (Zhu et al., 2003, Heathcote, 2008, Trehanpati et al., 2013). It has been described that HBsAg-positive neonates have higher levels of immunosuppressive T regulatory cells and CD8+ T cell dysfunction that

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signify the established chronic and immune tolerant state of hepatitis B at birth during vertical transmission (Trehanpati et al., 2013).

Viral factors also contribute to the establishment of chronicity following acute hepatitis B. HBV has been described as a stealth virus in the sense that it does not induce the host hepatocytes (which are the primary targets of infection) to express innate immune response genes, specifically the type 1 interferons, primarily IFN-α/β (Wieland and Chisari, 2005, Vierling, 2007). The fact that the virus is non-cytopathic enables it to form some sort of "non-harmful" symbiotic-type relationship with the host hepatocyte such that damage associated molecular patterns (DAMPs) may fail to be displayed, shielding the virus from the host immune system. HBV inhibits the expression of toll-like receptor-2 (TLR2) and TLR9 on innate immune cells, especially plasmacytoid dendritic cells (pDCs), leading to reduced induction of inflammatory cytokines, primarily the type 1 IFNs following TLR stimulation (Ma et al., 2015). TLR-9 is a pattern recognition receptor (PRR) that recognises viral and bacterial DNA motifs and leads to the secretion of type 1 IFN by pDCs via IRF7 activation (Ma et al., 2015). HBV proteins also suppress TLR signalling in hepatocytes from producing IFN-α which would normally cause induction of innate immune cells (Zhang and Lu, 2015).

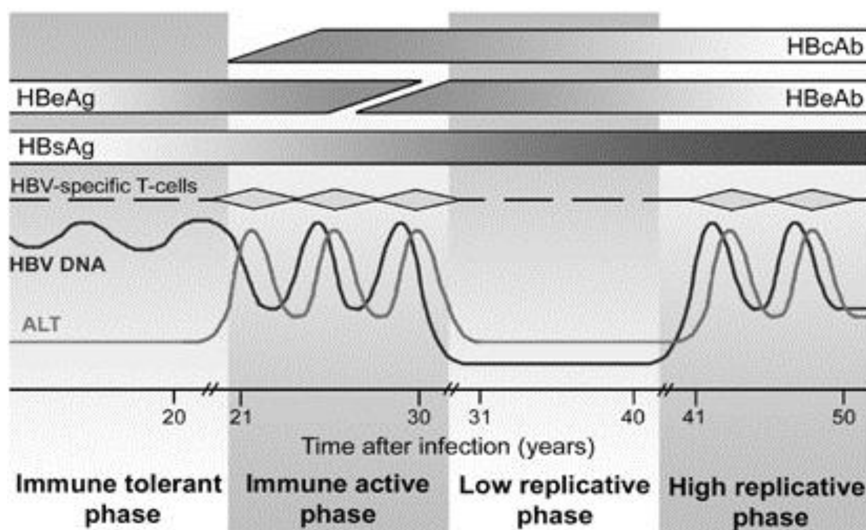

Figure 2.4 Serology of chronic hepatitis B. HBV replication is high yet ALT levels remain normal in the immune tolerant phase. During the immune active phase of CHB, the frequency and activity of HBV-specific CTLs increase and there is also recruitment of other non-specific immune cells into the hepatic environment with lysis of infected hepatocytes and an increase in serum ALT activity. The immune active phase is accompanied by a change in seroconversion from HBeAg with subsequent detection of HBeAg and anti-HBc. HBeAg negativity may arise from the establishment of mutants with a precore change that eliminates HBeAg synthesis. However, replication of HBV does not cease and HBsAg continues to be produced in excess of HBsAb production. Reprinted by permission from Macmillan Publishers Ltd: Immunology and Cell Biology (Chang and Lewin, 2007) copyright 2007.

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Furthermore HBV has a very high rate of replication with production of 10^{10}-10^{12} virions/day (Rehermann and Bertoletti, 2015). A high replication rate permits infection of almost all hepatocytes thereby overwhelming of the immune system. The production of high quantities of HBsAg is also thought to subvert the immune response from responding to the actual infectious virions. In addition, the high rate of replication and the use of non-proofing reverse transcriptase also lead to the introduction of mutations, some of which may be responsible for immune escape, analogous to the scenario which is well-described in HIV infection (Rehermann and Bertoletti, 2015). Indeed it has been shown that many of the mutations occur in regions coding for epitopes that are recognized by HBV specific T cells (Vierling, 2007). HBV also produces tolerogenic HBeAg which aids in the establishment of chronicity (Harrison et al., 2008).

Much work has been done in investigating the role of the host, primarily the immune system, towards the establishment of CHB. Different cells of the immune system including DCs, cytotoxic T lymphocytes (CTLs), regulatory T cells (Tregs) and natural killers (NK) cells have been described to contribute to the development of chronicity. T cell clonal anergy leads to hyporesponsiveness to HBV (Vierling, 2007). During CHB, HBV-specific CTLs exhibit a decreased capacity to secrete IFN-γ and express immune exhaustion markers such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death protein-1 (PD-1). The binding of PD-1 to its ligands PD-L1 or PD-L2 transmits a negative signal that causes a reduction of cytokine production (starting with IL-2) and cell proliferation (Li et al., 2014, Zhou et al., 2014). HBV-specific T cells of patients with CHB have been shown to express high levels of PD-1 and PD-L1 and these cells were hyporesponsive when stimulated using a wide range of HBV-specific T cell epitopes (Boni et al., 2007). Despite the hyporesponsiveness, these HBV-specific T cells are found in the liver where they are responsible for a persistent inflammatory response that is ineffective for viral clearance yet may lead to fibrosis (Maini et al., 2000). However, blocking of PD-1 restores functional responses and may lead to clearance of infection.

In addition, patients with CHB were described to have a high percentage of CD4+ FoxP3+ regulatory T-cells (CD4+ Tregs). CD4+ Tregs suppress the function of effector HBV-specific T cells, perhaps as a way of protecting against immune-driven liver disease. Patients with CHB were shown to have high circulating CD4+CD25+ Treg frequency that significantly correlated with serum viral load (Xu et al., 2006). Similar association between CD4+ Tregs and chronicity were also shown in a study that described a higher percentage of CD4+ Tregs

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that stain positively for CD25, CD45RO, and CTLA-4 in the circulating blood of CHB patients compared to healthy controls and individuals with resolved hepatitis B (Stoop et al., 2005). The CHB patients also had higher levels of forkhead/winged helix transcription factor (FoxP3) messenger ribonucleic acid (mRNA) per CD4+ cell compared to controls. Tregs supress the ability of HBV-specific T-cells to proliferate upon stimulation with HBV antigens and their depletion leads to stronger responses as demonstrated by production of IFN-γ (Stoop et al., 2005). As in vertically-infected infants, the presence of HBeAg was associated with a higher percentage of Tregs compared to healthy subjects (Stoop et al., 2005). This may also explain the mechanisms through which perinatally infected infants tend to develop CHB. About 15% of the CD4+ T cell population during foetal life is composed of Tregs which declines to about 5% by the time of birth and finally to around 2% in teenage/adulthood (Prendergast et al., 2012). In the context of hepatitis B, this leads to suppression of HBV-specific CTL responses by Tregs. This means that the infected hepatocytes are not targeted and killed and virus is not eliminated even as the frequency of Tregs is reduced later on in life.

Apart from T-cells, circulating blood DCs (total DC population) from CHB patients have been reported to have decreased maturation and frequency compared to normal individuals and that this may be associated with disease progression (Balmasova et al., 2014). However, there is some controversy with regards to frequency and function of DCs of CHB patients due to conflicting findings by different research groups, partly as a result of the assays used. A review by Gehring and Ann D'Angelo suggests that the only distinction between the DCs from CHB patients and those from healthy donors is that the former's plasmacytoid DC (pDCs) subset has reduced IFN-α production (Gehring and Ann D'Angelo, 2015).

NK cells are the main effector cells that are involved in innate immune responses against intracellular microbes, such as viruses, and also tumour cells through cytolysis and cytokine production. In CHB, NK cells assume a negative effect on the host by inducing exhaustion of HBV-specific CD8+ T cells through production of immunosuppressive cytokines, upregulation of apoptosis and reducing the capacity of antigen presenting cells function. The depletion of NK cells leads to control of the persistent viral infection (Wang et al., 2015).

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The contribution of B cells in CHB is not as clear as that of T cells, mostly as a result of the limited techniques for studying B cells (Xu et al., 2015). Despite the limited amount of data, it is accepted that B cells are important for the clearance of hepatitis B and preventing re-infection. Anti-HBs neutralizes circulating HBsAg and clears infectious HBV particles in vivo and the detection of serum anti-HBS is considered to indicate resolution of CHB signifying the importance of anti-HBs in the clearance of HBV infection (Xu et al., 2015). Recent reports have shown that in CHB there is increased total B-cell activation and decreased HBsAg-specific B-cell responses (Oliviero et al., 2011, Xu et al., 2015). Figure 2.7 shows the different cells of the immune system and the roles that they play in hepatitis B being cleared or establishing chronicity.

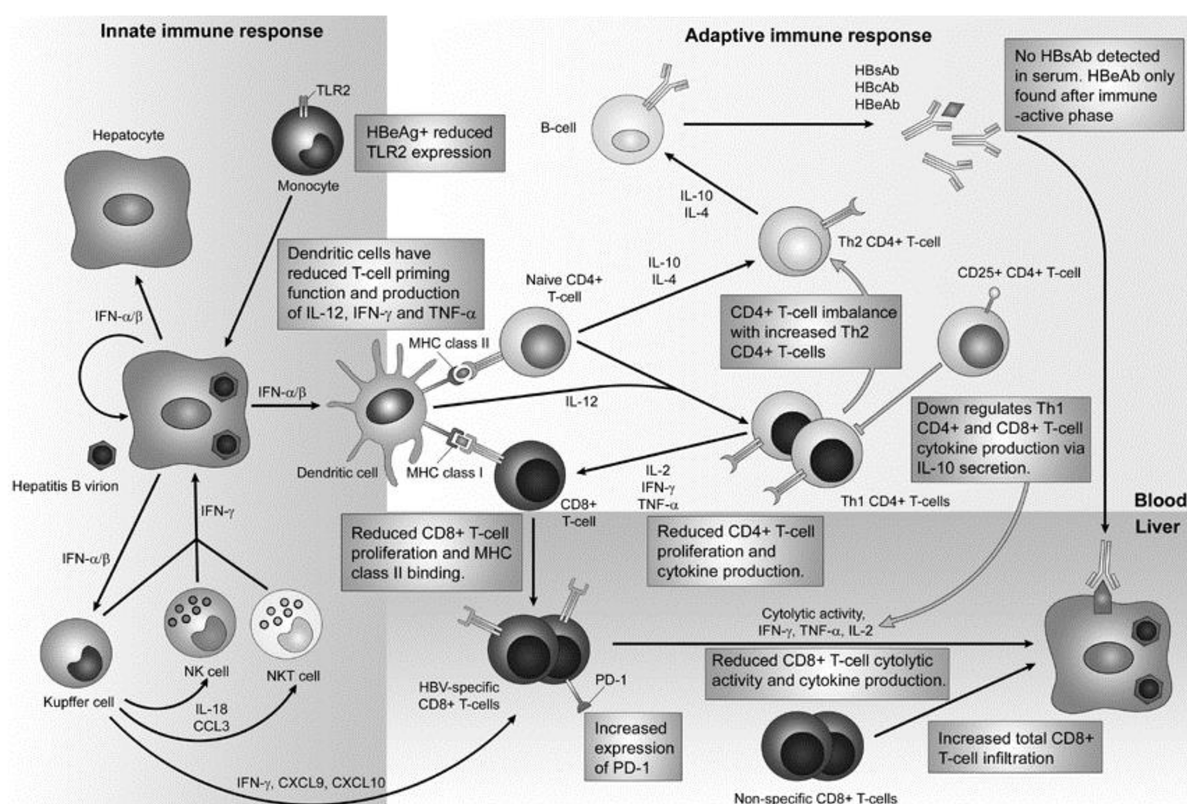

Figure 2.5 HBV infection and the immune system. The figure which highlights several important pathways (but is not comprehensive) shows that the innate and adaptive (cellular/humoral) all contribute to the clearance of HBV infection or its establishment of chronicity. The boxes within the figure highlight potential mechanisms by which persistence of infection is established. The role of the KCs and the chemokines that they produce following induction by IFN-γ is indicated. When HBV loses its stealth ability, infection of hepatocytes is followed by production of IFN-α/β which activates immune cells that are responsible for clearance and/or immune-mediated damage. Modified with permission from Macmillan Publishers Ltd: Immunology and Cell Biology, (Chang and Lewin, 2007)

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2.6 Pathogenesis of HBV-driven HCC

HBV is not directly cytopathogenic and this allows it to survive within host cells without overt pathology. Instead, host immune responses induced by viral persistence are responsible for liver pathology in HBV infections (Zhang et al., 2012). It is known that HBV-specific T cell impairment leads to failure of HBV clearance, while the infiltration of non-HBV-specific inflammatory cells into the liver may actively participate in pathogenesis (Zhang et al., 2012). Persistent inflammation due to hepatitis B is known to lead to fibrosis and cirrhosis which may progress to HCC (Brechot, 2004). The direct ways in which HBV may lead to HCC include integration of its DNA into the host genome, the activities of HBx and truncated HBsAg proteins that possess trans activating properties (Lai et al., 1997, Ganem and Prince, 2004, Kim et al., 2005, Kew, 2011). The integration of HBV DNA into host chromosomal DNA may lead to chromosomal instability. Also, integration may occur within or near functional cellular genes that are involved in signal transduction pathways associated with cell cycling and proliferation, thereby increasing the chances of hepatocarcinogenesis (Brechot, 2004). HBx is a non-specific transcriptional transactivator and may influence expression of both viral and host promoter genes (Kim et al., 2005). Specifically, HBx has been reported to interfere with host cellular signalling processes that are responsible for cell proliferation, apoptosis and responses to cell damage (Brechot, 2004). Truncated HBsAg proteins are thought to be retained in the endoplasmic reticulum causing cellular stress, leading to oxidative damage of chromosomal DNA and subsequently in oncogenesis (Hsieh et al., 2004, Lin et al., 2007).

Whether HBV plays a direct or indirect role towards hepatocarcinogenesis, the common underlying theme is that there is always presence of unresolved inflammation (that may present as fibrosis/cirrhosis) that promotes clonal expansion of hepatocytes. It should be pointed out that a significant proportion of African patients develop HCC without detectable underlying cirrhosis (Paterson et al., 1985, Kew, 1989, Yoshida et al., 1994). Viral agents have been shown to be responsible for initiating carcinogenesis in many cancers because of, amongst other mechanisms, the associated immune-mediated persistent “smouldering” inflammation that results in cell proliferation and death (Grivennikov et al., 2011). Inflammation per se is not pathogenic but rather useful for containment and clearance of pathogens. However, failure to regulate the immune system appropriately can have unintended consequences, one of which is an increased risk of malignancy. Hepatocyte damage and compensatory regeneration due to chronic inflammation induced by viral

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infections such as HBV and HCV provides a potential environment for initiation of HCC through the introduction of genetic and epigenetic mutations that lead to the transformation of normal cells to cancer cells. In addition, some products of inflammation, such as reactive oxygen species and nitrous species, may induce DNA damage that precedes cancer development (Hussain et al., 2003). Figure 2.3 summarises the mechanisms of HBV-induced HCC as well as hepatocarcinogenesis arising from other risk factors.

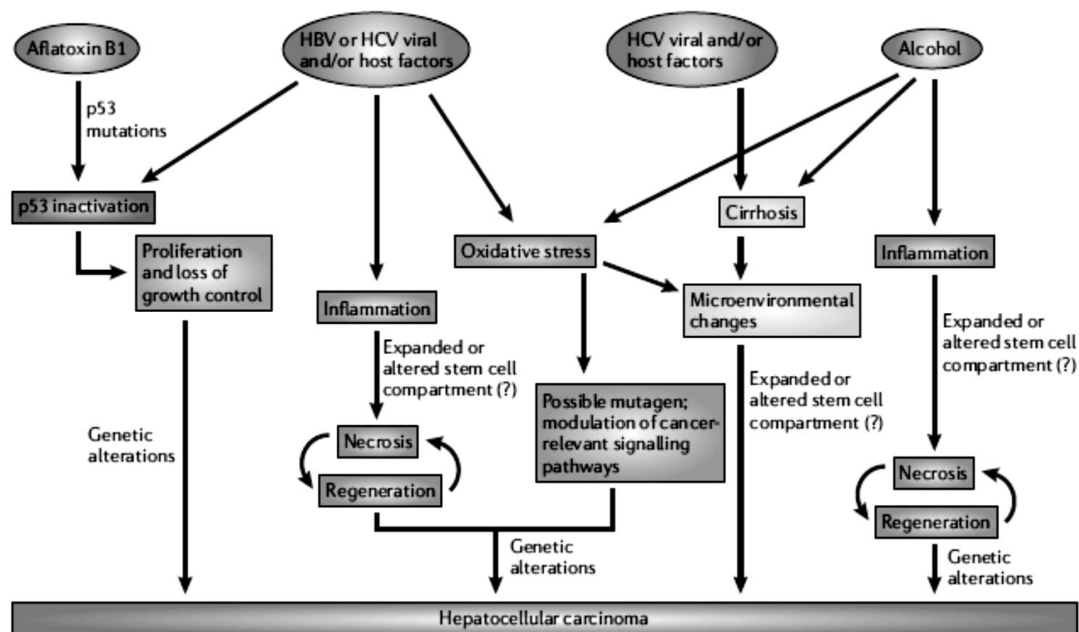

Figure 2.6 Mechanisms of hepatocarcinogenesis. HBV-induced hepatocarcinogenesis arises from a number of possible mechanisms, including the activities of HBV proteins (HBx, truncated HBsAg), integration into the host genome and host immunity-driven inflammation. Reprinted by permission from Nature Publishing Group: Nature Reviews Cancer (Farazi and DePinho, 2006).

2.7 Liver fibrosis in hepatitis B

Chronic viral hepatitis due to infection with HBV, which is highly endemic in SSA, may cause liver fibrosis which may lead to cirrhosis and ultimately to the development of HCC (Pellicoro et al., 2012). Liver fibrosis refers to the excessive deposition of extracellular proteins including collagen, and this usually occurs in the setting of a chronic liver disease (Bataller and Brenner, 2005). Apart from viral causes, liver fibrosis may also be caused by chronic alcohol abuse, NASH and autoimmune hepatitis (Pellicoro et al., 2012). HCV is also a major viral cause for development of hepatic fibrosis, cirrhosis and HCC. However, the prevalence of HCV in sub-Saharan Africa is lower than that of HBV which has intermediate-high prevalence in sub-Saharan Africa (Sitas et al., 2008, Kew, 2010). In order to understand the pathogenesis of liver fibrosis, there is need to first appreciate the pathogenesis and

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immunology of HBV with regard to correlates that permit establishment of chronicity of infection.

2.8 Pathogenesis of liver fibrosis in hepatitis B

Clearance of HBV and disease pathogenesis is mediated by the adaptive immune response (Guidotti and Chisari, 2006). Patients that are able to clear the infection have a vigorous, polyclonal T-cell response to multiple antigenic epitopes (Chisari et al., 2010). However, the adaptive immune response can only be strong following an effective innate immune response (Boltjes et al., 2014). Meanwhile, patients that develop active chronic infection with HBV have a weak adaptive immune response that causes chronic hepatocyte injury, regeneration, non-resolving inflammation, DNA damage and dysregulation of cell growth genes that may lead to liver cirrhosis and HCC (Chisari et al., 2010). Liver fibrosis is reversible but may progress to cirrhosis and eventually to hepatocellular carcinoma if the underlying stimuli are not eliminated (Crane et al., 2012). The majority of cases of HBV-related HCC take place after many years of chronic immune-mediated hepatitis with continuous cycles of low-level hepatocyte destruction and regeneration (Guidotti and Chisari, 2006).

In essence, liver fibrosis occurs as a scarring response to liver damage, usually following either acute or chronic liver insult by both infectious and non-infectious agents. The process of liver fibrosis is driven by a particular cell type found in the hepatic environment called hepatic stellate cells (HSCs) (Friedman, 2008a). The HSCs are situated within the *space of Disse* in the normal liver where they are usually restive, their principle function being the storage of vitamin A within cytoplasmic droplets (Friedman, 2008a). However, following injury to hepatocytes and biliary cells, the usually quiescent HSCs are activated and acquire a contractile, proinflammatory and fibrogenic phenotype following their activation through inflammatory and injury-associated signals that are released by the damaged cells (Friedman, 2008b). The inflammatory and injury signals that are secreted causing the activation of HSCs include fibrogenic cytokines such as transforming growth factor beta (TGF-β) and angiotensin II and angiogenic factors such vascular endothelial growth factor (VEGF) and angiopoetin. In addition to being secreted by liver parenchymal cells, HSC-stimulating factors are also produced by Kupffer cells (KCs) that are resident liver macrophages, monocyte-derived liver-infiltrating macrophages, B and T lymphocytes, natural killer T (NKT) cells and liver sinusoidal endothelial cells (LSECs) (Gao and Radaeva, 2013). Literature suggests that liver-infiltrating macrophages play a greater role in chronic inflammation and liver fibrosis than

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resident KCs that are usually tolerogenic but are essential for the initial inflammatory response and sensing of tissue injury (Tacke and Zimmermann, 2014, Ju and Tacke, 2016).

There are also anti-fibrogenic factors that are produced by platelets and NK cells and these include nitric oxide, FasL and TNF-related apoptosis inducing ligand (TRAIL) that result in the death of activated HSCs and subsequent reversal of the fibrotic process (Seki et al., 2007). In hepatic fibrosis, activated HSCs trans-differentiate into myofibroblast-like cells that produce excess fibrillar extracellular material (ECM), primarily type 1 collagen, which is the hallmark of liver fibrosis (Seki et al., 2007). Also upregulated during development of liver fibrosis are tissue inhibitors of matrix metalloproteinases (TIMPs) which as the name suggests are inhibitors of matrix metalloproteinases (MMPs). MMPs are fibrolytic enzymes whose function is to degrade ECM (Han and Lang, 2003, Lebensztejn et al., 2007). As a result of the inhibition of MMPs accumulation of ECM ensues. Underlying the importance in the balance between MMPs and TIMPs in CHB is the finding that administration of lamivudine or IFN-α2b for treatment of hepatitis B was associated with a significant decrease of TIMP-2 and increase of MMP-9 level accompanied by an improvement of liver histology (Han and Lang, 2003, Lebensztejn et al., 2007). A schematic of the interactions between immune cells and HSCs and the development and maintenance of liver fibrosis is shown in Figure 2.8.

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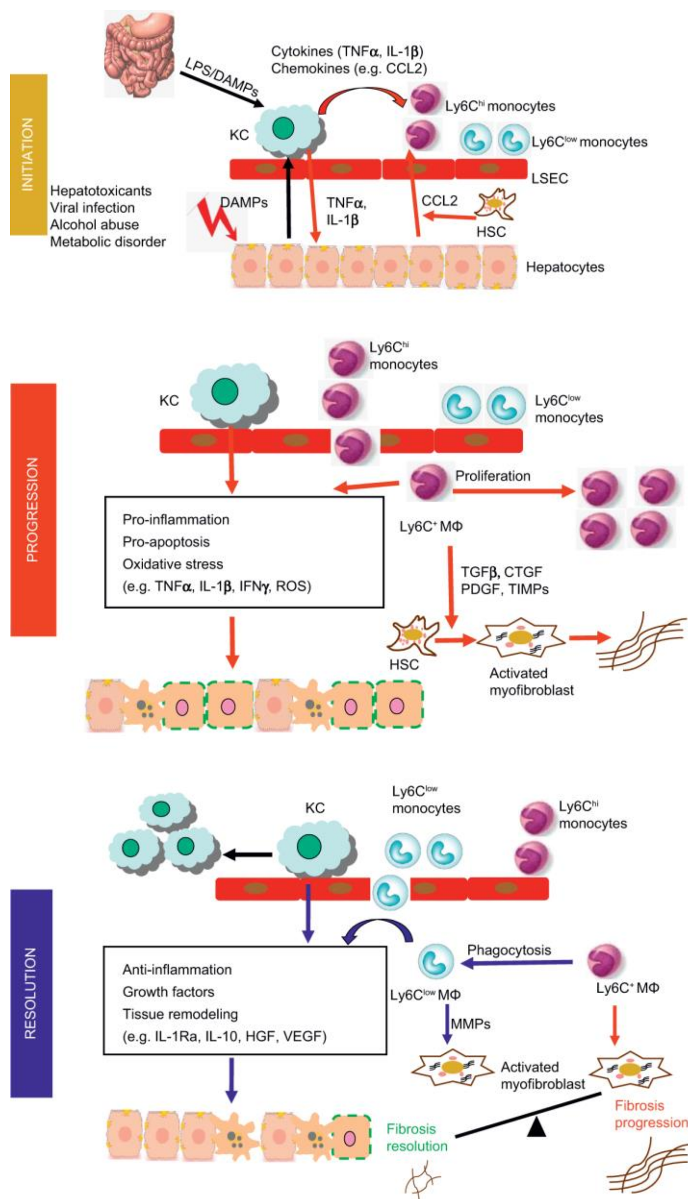

Figure 2.7 Development of liver fibrosis in hepatitis B (mouse model). Following infection with hepatitis B, hepatocytes display damage associated molecular patterns (DAMPs) which stimulate Kupffer cells (KCs) and dendritic cells (DCs). KCs release chemokines that result in chemotaxis of bone-marrow derived monocytes into the liver where they differentiate into infiltrating liver-infiltrating Ly-6C^{+} macrophages that have a pro-inflammatory phenotype. These liver-infiltrating macrophages release cytokines that result in quiescent hepatic stellate cells (HSCs) transforming into activated HSCs that differentiate to become myofibroblast producing fibrogenic collagen. Meanwhile, LSECs lose their differentiation and become capillarised causing further activation of HSCs and development of fibrosis. In addition, there is upregulation of tissue inhibitors of matrix metalloproteinases (TIMPs) (TIMP-1 and TIMP-2) that inhibit matrix metalloproteinases (MMPs) (1, 8/13) further promoting extracellular matrix (ECM) accumulation resulting in liver fibrosis. Ly-6C^{high} monocytes correspond to the CD14^{high} monocytes in humans while the Ly-6C^{low} monocytes are the equivalent of CD14^{dim}CD16^{++} monocytes in humans. LPS, lipopolysaccharide; LSEC, liver sinusoid endothelial cells. Modified with permission from Macmillan Publishers Ltd: Cellular & Molecular Immunology (Ju and Tacke, 2016).

2.8.1 Immune cells in the pathogenesis of liver fibrosis

As HBV-driven pathogenesis is mostly immune-mediated, different immune cell types and associated molecules have a role in the development of liver fibrosis in HBV infection (Sun et

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al., 2012). The virus, host immunity and hepatocytes all interact in the development of liver disease. The increased range of immune cells that are seen in livers exhibiting inflammatory activity are thought to play critical roles in the genesis of liver injury (Wang, 2007). KCs which are resident liver macrophages that have been described to play a role in development of liver fibrosis through secretion of pro-inflammatory and fibrogenic mediators such as TGF-β during the injury phase of chemically-induced liver fibrosis in a mouse model (Duffield et al., 2005). However, KCs are equally important for the resolution of liver fibrosis as their depletion during recovery in the murine model is associated with persistent accumulation of ECM because of the absence of TRAIL that would otherwise result in apoptosis of activated HSCs thereby inhibiting the production of TIMP-1 (Duffield et al., 2005).

NK cells also play a role in liver fibrosis by the killing of fibrogenic HSCs. Depletion of NK cells was reported to enhance hepatic fibrosis in a murine model of carbon tetrachloride (CCl_4)-induced liver fibrosis, implying that these cells play an anti-fibrogenic role mediated by the cytokine IFN-γ (Radaeva et al., 2006). NKT cells have been described to play contrasting roles in liver fibrosis whereby they exhibit a pro-fibrogenic role through the secretion of type 2 pro-fibrogenic cytokines such as interlekin-4 (IL-4) and IL-13 while the anti-fibrotic capabilities are evidenced by their secretion of IFN-γ in certain conditions (Gao and Radaeva, 2013). T lymphocytes, depending on their polarity, have diverse contributions in liver fibrosis. T-helper-1 (Th1) lymphocytes have been described as anti-fibrotic due to their secretion of IL-2 and IFN-γ while Th2 are described as pro-fibrogenic secreting IL-4, IL-5, IL-6 and IL-10 (Xu et al., 2012b). Th17 lymphocytes have also been described to play a role in the development of liver fibrosis, particularly in CHB where positive correlation exists between the number of liver infiltrating as well as circulating Th17 cells and their associated cytokines and the amount of liver damage (Zhang et al., 2010). Dendritic cells have complex roles in liver fibrosis which are not well-understood although it has been reported that there is a change in the quality and quantity of DCs during liver fibrosis (Xu et al., 2012b).

2.8.2 Soluble immune mediators and pathogenesis of liver fibrosis

Cytokines are the mediators through which the effects of each cell type involved in liver fibrosis are carried out. Cytokines are a component of the immune system that determine the characteristics of the response to any pathogen or injury and as such are an integral component in the development of a chronic inflammatory state. They may either potentiate or downregulate the inflammatory response. Several pro-inflammatory cytokines such as TNF-α,

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IL-1α, IL-1β, IL-6, and IL-8 have been associated with chronic inflammation in the liver environment and may be important in the relationship between inflammation and liver disease, including fibrosis and hepatocellular carcinoma (Budhu and Wang, 2006). The expression of these pro-inflammatory cytokines has been shown to increase with exposure to lipopolysaccharide (LPS) while secretion of potentially protective anti-inflammatory cytokines is reduced (Jirillo et al., 1991). Cytokines that are involved in the development or reversal of liver fibrosis are produced by immune cells as well as hepatic parenchymal and non-parenchymal cells. The most important cytokines produced in the liver during the pathogenesis of liver fibrosis are produced by liver-resident macrophages, mainly KCs. The effects of some cytokines towards the development or resolution of liver fibrosis are variable and are thought to depend upon the responding cell and also the signalling pathway that is triggered within the particular cell type. An example is IL-6 which is pro-fibrotic when acting on HSCs but is anti-fibrotic through its effect on hepatocytes (Kong et al., 2012). TGF-β which is predominantly recognized as a pro-fibrotic cytokine has been described to exhibit anti-fibrotic properties when secreted by Tregs (Kitani et al., 2003). It is apparent that the net effect of each cytokine is dependent on the net difference between the pro-fibrotic and anti-fibrotic signals that it elicits. Despite the contributions of many of these cytokines towards liver fibrosis being known, most data has come primarily from murine models of carbon tetrachloride (CCl_4)-induced liver disease and also HCV-associated liver disease with very little data known about HBV-driven liver fibrosis. A summary of the effect of some cytokines and their effect in the pathophysiology of liver fibrosis is shown in Figure 2.9.

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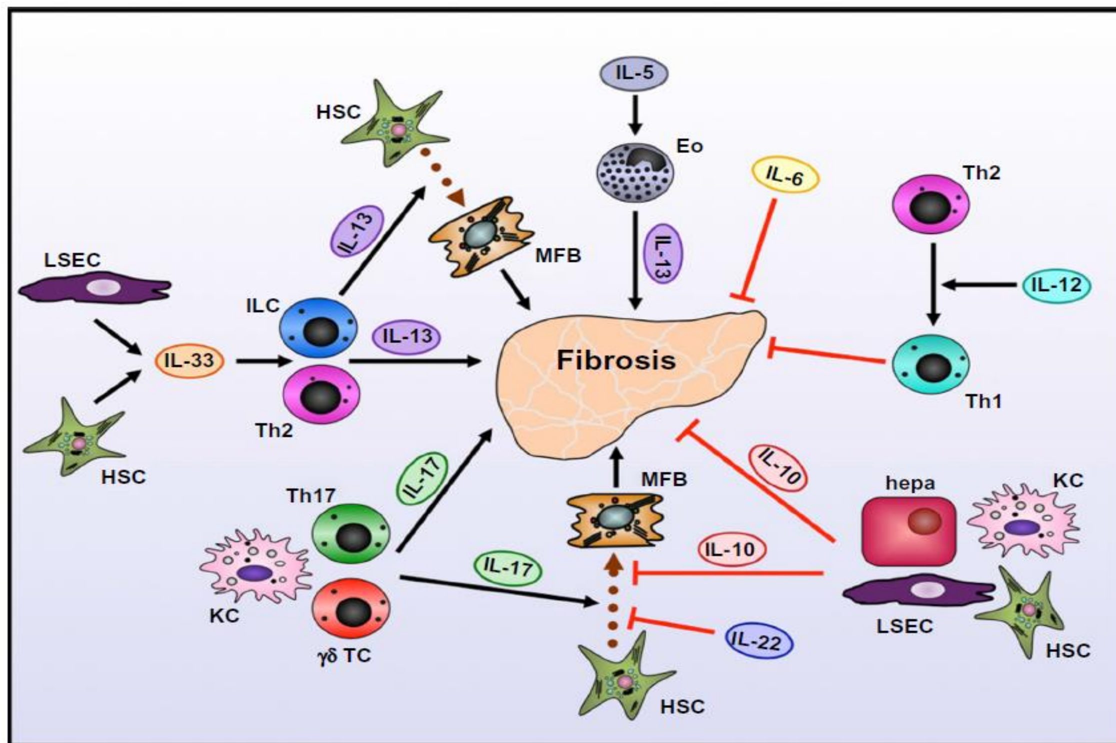

Figure 2.8 Role of cytokines in liver fibrosis. The figure shows the role of some selected cytokines and their roles in the pathogenesis of liver fibrosis. Cytokines such as IL-10 are anti-inflammatory and hepatoprotective against liver fibrosis. CXCL10 (IP-10) whose expression is stimulated by IFN-γ (not shown in the figure) are pro-inflammatory and their secretion in the hepatic environment is associated with increased liver fibrosis. The roles of the different cytokines shown in the figure were determined from experiments using different mouse models. Eo, eosinophilic granulocyte; hepa, hepatocyte; ILC, innate lymphoid cell; KC, Kupffer cell; LSEC, liver sinusoidal endothelial cell; MFB, myofibroblast; TC, T cell; Th, T helper. Republished with permission of Dove Medical Press, from Clinical and Experimental Gastroenterology, Hammerich and Tacke, Volume 2014:7 (Hammerich and Tacke, 2014).

2.8.3 HIV infection in the pathogenesis of liver fibrosis

The central feature of HIV infection pathogenesis is chronic inflammation (Utay and Hunt, 2016). Markers of inflammation and immune activation such as CD38 and HLA-DR expression by CD8+ T cells are elevated in chronic HIV infection and are not normalized to the same state as in HIV-uninfected persons even with HAART (Giorgi et al., 1993, Deeks, 2012, Utay and Hunt, 2016). The increase in activated cells and inflammation/activation-associated cytokines has a profound effect on multiple cells and organ systems such as the cardiovascular system and the central nervous systems. Among the important contributing factors in the continuous immune activation process is translocation of GIT-associated components into the systemic circulation, persistence of HIV especially in lymphoid tissue and presence of co-infecting viruses (Brenchley et al., 2006). Early HIV infection is characterized by dramatic loss of gut-associated memory CD4+ T cells. The events leading to this loss also lead to damage of gut epithelium, particularly destruction of tight junctions

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(Brenchley et al., 2004). This initiates leakage of gut components into the circulation (Sandler and Douek, 2012). Apart from stimulating innate cells via toll-like receptors (TLRs), which results in a pro-inflammatory milieu, the increased levels of GIT components are bound to have a profound effect in the liver.

The liver is linked to the gut via the hepatic portal vein and as such the liver is the first organ to be exposed to high levels of LPS and other gut components. Kupffer cells which are resident liver macrophages are located in liver sinusoids and sample the blood for pathogenic bacteria or bacterial components. On detection of these components the Kupffer cells are capable of inducing both immunogenic and tolerogenic responses. In cases of liver injury (as occurring in active HBV infection), there is a shift from Kupffer cell predominance to monocyte-derived macrophage predominance indicating an influx of monocytes from the peripheral blood (Pellicoro et al., 2012). Pro-inflammatory monocyte/macrophages are highly phagocytic and capable of producing large amounts of pro-inflammatory and pro-fibrotic cytokines. These include myofibroblast activators such as TGF-β and galectin-3 which activate HSCs to become myofibroblasts (Brenner et al., 2012). Pro-survival cytokines such as TNF-α and IL-1β, and PDGF work together to maintain myofibroblast activity. The net outcome of this process is deposition of collagen and scar formation. Under normal conditions, removal of the injury stimulus would lead to homeostatic shift back to Kupffer cell predominance and removal of factors driving HSCs transformation into myofibroblasts (Bilzer et al., 2006, Boltjes et al., 2014). Chronic viral infection in the liver precludes complete homeostatic reversion.

Apart from increased microbial translocation, other factors such liver toxicity associated with some highly-active antiretroviral therapy regimens and also the associated metabolic changes may lead to an increased propensity to develop liver fibrosis in HIV/HBV co-infected patients. A study on Asian patients co-infected with HBV and HIV has suggested that increased apoptosis rather than inflammation is the driver of progressive liver disease, at least in therapy-naïve patients with severe immunosuppression (Iser et al., 2011). The question of whether the extent of hepatocyte apoptosis remains the same when retroviral multiplication is controlled by the administration of HAART remains unanswered.

It has also been shown that the Gp120 protein of HIV induces apoptosis of human hepatocytes through the HIV co-receptor chemokine receptor type 4 (CXCR4) that is also expressed on the cell surfaces of hepatocytes. It is postulated that HIV-induced hepatocyte

apoptosis could then lead to development of hepatic fibrosis and/or cancer (Vlahakis et al., 2003). In addition, HIV directly infects activated HSCs stimulating the production of type 1 collagen and monocyte chemoattractant protein-1 (MCP-1), a chemokine which promotes lymphocyte infiltration into the liver (Tuyama et al., 2010). A schematic of the different mechanisms by which HIV infection may cause liver fibrosis is shown in Figure 2.10.

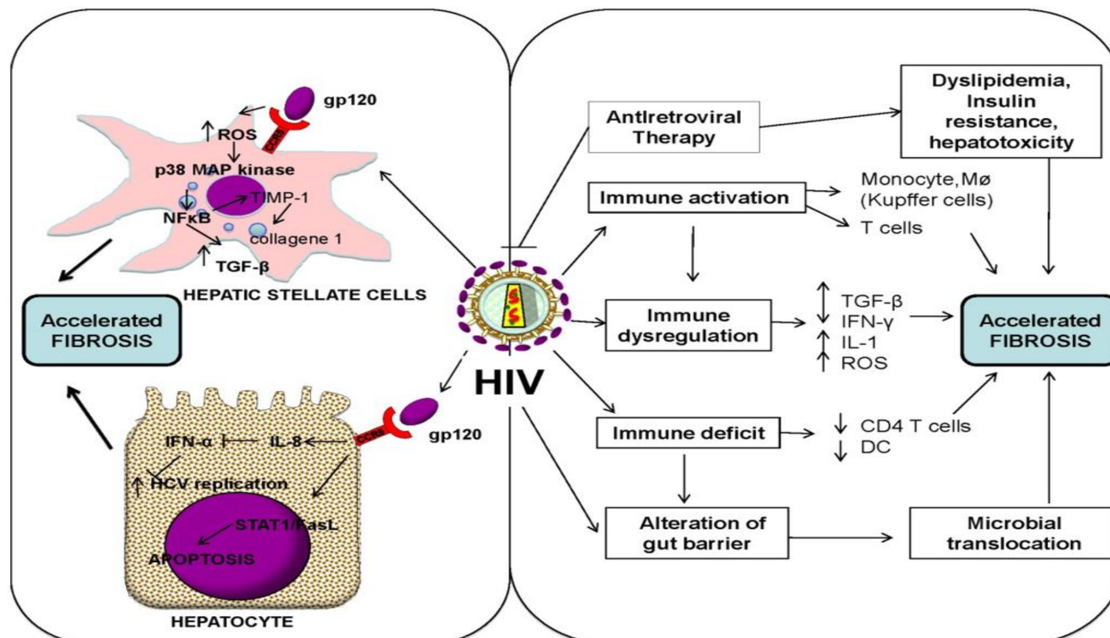

Figure 2.9 Mechanisms of HIV-induced liver fibrosis. HIV infection causes disruption of the gut barrier and a profound loss of CD4+ T cells resulting in the immune deficit that contributes to accelerated fibrosis. Use of HAART may also give rise to dyslipidaemia as well as insulin resistance. Some of the antiretrovirals may also be hepatotoxic in some individuals leading to development of hepatic fibrosis. Figure used with permission (Mastroianni et al., 2014).

2.8.4 HBV/HIV co-infection and pathogenesis of liver fibrosis

Chronic HBV mono-infection causes localised immune suppression in the liver. Patients with CHB have decreased expression of IFNα/β from pDCs (Xu et al., 2012a). The secretion of IFNα/β from pDCs is important for inhibiting replication of virus-infected cells and activation of other immune cells to control hepatitis B (Vincent et al., 2011). However, HBV/HIV co-infection may lead to the loss of localized immune tolerance in the liver because of the non-specific immune activation that drives HIV pathology. HIV infection may also lead to reactivation of previous HBV infections (Hoffmann and Thio, 2007). Altered gut permeability and increased levels of serum LPS have been associated with progression of liver disease of various aetiologies such as chronic misuse of alcohol and non-alcoholic steatohepatitis and is thought to contribute to liver pathology (Crane et al., 2012). However, the question arises as to whether LPS is the cause or the result of a diseased liver since this is the organ responsible for the clearance of endotoxins. Within the liver environment, LPS binds to TLRs,

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specifically to TLR-4 on Kupffer cells and hepatocytes (Chow et al., 1999). The interaction of LPS with TLR-4 initiates secretion of proinflammatory and fibrogenic cytokines culminating in hepatic stellate cell activation and collagen deposition (Chow et al., 1999, Tilton et al., 2006, Crane et al., 2012). It has been proposed that chronic exposure to elevated LPS in individuals with HBV/HIV co-infection could contribute to a loss of tolerance in TLR4 signalling in the liver leading to an inflammatory response that is punctuated by upregulation of pro-fibrotic cytokines. This includes increased levels of TGF-β as well as chemotactic and adhesive cytokines (Seki et al., 2007). The ensuing chronic inflammation causes liver fibrosis which may in turn increase the risk of progression to HCC. Another potential mechanism by which tolerance could be lost in HBV/HIV co-infection is through the loss of CD4+ lymphocytes. A recent study using transgenic HBV-carrier mice and IFN-γ–deficient (GKO) mice showed that the carrier mice failed to respond to vaccination as evidence of systemic tolerance toward HBsAg. Meanwhile, the GKO mice had robust responses against peripheral HBsAg vaccination. These findings are taken as evidence that IFN-γ produced by HBV-specific CD4+ T cells in CHB is essential for the maintenance of tolerance to hepatitis B (Zeng et al., 2016).

2.9 HIV and HBV co-infection and pathogenesis of HCC

In addition to being highly endemic for hepatitis B, sub-Saharan Africa has the highest number of people living with the human immunodeficiency virus (HIV) infection (UNAIDS, 2015). Figure 2.4 shows the number of deaths due to hepatitis B as well as the prevalence of HIV on a global scale. The role of HIV infection has been clearly shown in other virus-driven malignancies such as Kaposi's sarcoma (due to Human Herpes Virus-8 infection), human papilloma virus-associated anal and cervical cancer and also non-Hodgkin’s lymphoma which are all classified as being AIDS-defining cancers and usually develop in untreated HIV infection (Deeks and Phillips, 2009, Tanon et al., 2012b). However, with the availability of highly-active antiretroviral therapy (HAART), HIV infection has become a manageable chronic infection but there is now development of serious non-AIDS-related events (SNAEs) that are normally seen with aging (Deeks and Phillips, 2009, Pathai et al., 2013, Appay and Kelleher, 2016). HCC is included among the list of SNAES (Weber et al., 2006, Monforte et al., 2008, Salmon-Ceron et al., 2009).

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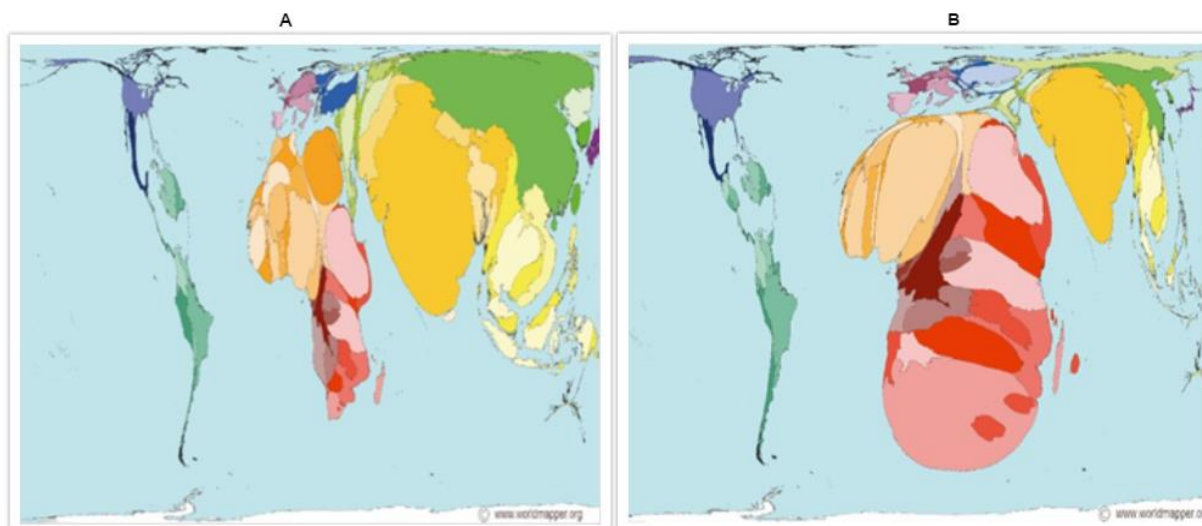

Figure 2.10 Population maps of HBV-related deaths and HIV prevalence. Map A shows the proportion of deaths attributed to HBV infection per year for each geographical region while Map B shows the prevalence of HIV infection. The maps show that Africa has the second highest number of HBV deaths and the greatest prevalence of HIV infection. (Source: http://www.worldmapper.org/)

HIV infection may also have an effect of HBV-driven HCC but this is not well demonstrated. Existing data from HBV/HIV co-infected patients suggests that HIV hastens the progression of CHB to HCC (Brau et al., 2007). It has been shown that HIV causes higher HBV replication rates, leading to higher HBV viral loads, and also delays seroconversion from HBeAg antigen positivity to anti-HBe antibody positivity (Thio, 2009). The important question of whether HIV alters the risk of HCC in patients already infected with HBV or in those that are HIV-mono-infected remains largely unanswered. A case-controlled study conducted within the Swiss HIV Cohort Study reported that decreased CD4+ cell counts were associated with an increased risk of HCC development among HIV-infected patients that were also co-infected with HBV (Clifford et al., 2008). There is very limited data on whether the incidence of HCC is increased among HIV-infected patients within sub-Saharan Africa. A meta-analysis study reported an increased standardised incidence ratio (SIR) of HCC among HIV-infected patients of 5.6 (95% confidence interval: 4.0 to 7.0) compared to the general population (Shiels et al., 2009). The SIR was only slightly alleviated in patients on HAART compared to those not on HAART but still higher than the general population. Perhaps telling of the lack of African data on HCC incidence in HIV-infected patients is the fact that only one African study was incorporated into the meta-analysis by Shiels et al. Earlier data from a retrospective study conducted in USA and Canada suggested patients infected with HIV presented at a younger age but with similar tumour stage of HCC compared to those without

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HIV infection (Brau et al., 2007). Among the few sub-Saharan Africa studies investigating the differences in epidemiology of HCC between HIV-infected and HIV-uninfected cases, Tanon et al. reported a significant difference in median age of 32 years (interquartile range (IQR): 31 to 44) in those with HIV compared to 49 years (IQR: 44 to 59) among those without HIV infection (Tanon et al., 2012a). However, the results must be interpreted with caution because the study had a very limited number of HIV-infected individuals (only 7/60 of HCC cases) and there was no indication of their viral hepatitis status.

2.10 HBV/HIV co-infection and hepatocarcinogenesis

A direct effect of HIV on hepatocarcinogenesis is yet to be established although a few case studies have reported HCC in HIV-infected patients without any other known risk factors (Tanaka et al., 1996, Murillas et al., 2005, Venkataramani et al., 2010, Parikh et al., 2012). It has been suggested that HIV may directly facilitate the process of malignant transformation (Wistuba et al., 1999, Kew et al., 2010). HIV is known to cause persistent systemic immune activation which results in fibrosis of the lymph nodes, but its role in immune activation and fibrosis in the liver environment in humans is not known (Mehandru et al., 2005). In HIV infection, the persistent stimulation of T lymphocytes and their destruction leads to recruitment of other immune cells including monocytes (which become phagocytic inflammatory macrophages) to the lymph nodes. These cells, in attempting to remove dead cells and repair tissue damage in a chronic non-resolving disease, actually cause collagen deposition and fibrosis. Localised production of pro-fibrotic cytokines such as TGF-β is elevated in lymph nodes in HIV mono-infection (Estes et al., 2008). The production of TGF-β is also elevated in the liver in HBV mono-infection and probably amplified in HBV/HIV co-infection. The development of fibrosis seen in the lymph nodes of HIV-infected individuals may well be analogous to the fibrotic process that occurs in the liver due to HBV mono-infection where antigen non-specific cells drive pro-inflammatory activity which leads to liver pathogenesis. Regardless of the initial stimuli to chronic liver disease, hepatic fibrosis is the common endpoint and may consequently and directly lead to cancer development.

An epidemiologic study from Uganda reported a statistically significant higher prevalence of liver fibrosis of 17% in HIV-infected patients compared to 11% in HIV-uninfected patients using transient elastography (Stabinski et al., 2011). Other previous studies have shown that patients co-infected with HIV and either HBV or HCV present with a more rapidly progressive liver disease and when they reach cirrhosis are also at increased risk of HCC

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(Benhamou et al., 1999, Pineda et al., 2005). Furthermore, it appears that HBV/HIV co-infected individuals are more likely to develop HCC compared to those with HBV mono-infection. A retrospective study using a limited number of samples from South Africa reported a higher prevalence of HIV infection in patients with HBV-related HCC compared to healthy HBV carriers (Kew et al., 2010). Since HBV-associated inflammation leads to liver fibrosis and the pathology surrounding HIV infection is also inflammation-driven, co-infection with both viruses may lead to exacerbated inflammation and increase the risk of liver fibrosis eventually leading to HCC.

2.11 Diagnosis of liver fibrosis

It seems beneficial that before HCC develops and is subsequently diagnosed, it would be beneficial to be able to diagnose liver fibrosis and cirrhosis using non-invasive markers. These have an advantage in that they can be performed serially and thus it will be easier to track changes over time compared to using an invasive procedure. There is much interest in the drive to identify non-invasive markers of liver fibrosis. Currently available methods are able to identify severe fibrosis and minimal fibrosis from normal tissue, but the specificity required to differentiate between intermediate grades of fibrosis is still lacking (Bataller and Brenner, 2005).

2.11.1 Liver biopsy

Biopsy remains the gold standard, although it has considerable limitations (Ratziu et al., 2006). It is invasive and may cause complications such as severe bleeding and hypotension in some patients (Ratziu et al., 2006). Liver biopsy also has a significant limitation of sampling error that may lead to misdiagnosis and erroneous classification of the stage of fibrosis and the interpretation is subjective and is highly dependent on the experience of the histopathologist (Kruger et al., 2011). Traditionally, a raised ALT has been used to screen patients for active liver disease but newer results suggest that this may result in individuals with significant liver disease being missed as they may not even considered for liver biopsy or treatment for HBV (Martinot-Peignoux et al., 2002, Mohamadnejad et al., 2006, Kumar et al., 2008). It has been described that a needle biopsy will only avail 1/50 000 of the entire liver thereby posing a serious risk of sampling error (Manning and Afdhal, 2008). The invasive nature of the liver biopsy makes it difficult to be serially repeated for liver disease surveillance purposes (Ratziu et al., 2006). As a result there is a push towards non-invasive tests for liver fibrosis.

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2.11.2 Non-invasive tests for liver fibrosis

The use of non-invasive markers for liver fibrosis such as transient elastography (Fibroscan) and serum-based markers such as the Fibrotest, aspartate to platelet ratio index (APRI) and Fibrosis-4 (FIB-4) test should also be considered for SSA in the monitoring of patients with HBV/HIV co-infection which may assist in early detection of liver disease and help in HCC screening efforts. By being able to first diagnose liver disease including HBV infection, decisions can be taken on whom to place under HCC surveillance and also the frequency of surveillance testing. The Fibrotest, APRI and FIB-4 are among a group of serum biomarkers that are considered to be indirect markers of liver fibrosis (Manning and Afdhal, 2008). These tests are classified as indirect markers of liver fibrosis because they reflect changes in hepatic function but do not directly inform on the accumulation or degradation of ECM in the hepatic environment. The APRI and FIB-4 indices make use of widely available routine clinical laboratory tests and are thus easy to employ. Direct serum markers of liver fibrosis such as hyaluronic acid, procollagen type III amino-terminal peptide (PIIINP) and laminin reflect the deposition or removal of ECM (Afdhal and Nunes, 2004, Manning and Afdhal, 2008). However, the direct markers of liver fibrosis are mostly used for research and have rarely been used in the routine clinical setting.

There are currently efforts to combine both the direct and indirect markers of liver fibrosis to improve the non-invasive diagnosis of hepatic fibrosis. One such combination is the Fibro-check which was shown to have higher accuracy for the staging of liver fibrosis and superior efficiency compared to the APRI and Fibrosis index (a test using a combination of platelet count and serum albumin) among Egyptian patients infected with HCV (Attallah et al., 2015). Transient elastography is one of the newer imaging modalities for liver fibrosis. Other newer imaging modalities include magnetic resonance elastography, acoustic radiation force impulse imaging and real-time shear wave elastography (Nightingale et al., 2002, Manning and Afdhal, 2008, Ferraioli et al., 2012). In order for these non-invasive tests of liver fibrosis to be considered as reliable alternatives to the current gold standard, liver biopsy, further validation needs to be carried out especially among HBV/HIV co-infected patients because most of the available data on the sensitivity and specificity of these tests has mostly been derived from HCV mono-infected patients in resource-rich settings. The fact that little data is available for African patients with hepatitis B and by extension those with HBV/HIV co-infection, reflects the lack of information with regards to true estimates of the burden disease, its diagnosis, prognosis and care.

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2.12 Diagnosis of HCC

The diagnosis of HCC is made using histological examination of liver tissue and detecting malignant cells. Alternatively, the diagnosis may also be made using dynamic contrast-enhanced radiological studies and/or serological tests. Frequently, a diagnosis of HCC is made using a combination of clinical history, physical examination and an imaging modality. An elevated serum alpha fetoprotein (>400 ng/ml) may also be taken into account (Jelic et al., 2010).

2.12.1 Radiological examinations for HCC

A number of options are available for the radiological diagnosis of HCC which include ultrasound (US), computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET). Although there have been technological advancements, the capacity of these tests to detect tumorous lesions <2 cm still remains challenging (Patel et al., 2012).

2.12.1.1 Ultrasound

Ultrasound is the first line diagnostic modality for the detection of HCC lesions, primarily due to its low cost and ease of use although it has limitations (Patel et al., 2012). The sensitivity of US varies with the size of the tumour with rates of 70% for tumours <1 cm and 90% for those >5 cm (Patel et al., 2012). The sensitivity of the test is also subject to the experience of the operator while reproducibility is poor (Saar and Kellner-Weldon, 2008). The diagnostic sensitivity and specificity is greatly improved when US is used in conjunction with CT or MRI although this comes at a cost and also increases the radiation dose to the patient. Hyper-vascularized nodular HCC lesions can be identified using contrast-enhanced US (CEUS) as their blood supply is predominantly arterial rather than portal venous (Saar and Kellner-Weldon, 2008, Patel et al., 2012). An early, intense and homogenous enhancement followed by 'washout' in the portal venous or delayed phases is considered characteristic of HCC tumours. It is recommended that all focal liver lesions suspected on US should be verified using: CT and/or MRI (Stefaniuk et al., 2010).

2.12.1.2 Computed tomography

Similar to CEUS, CT using contrast agents identifies HCC tumours, based on their early arterial enhancement and late portal venous 'washout'. The grade and stage of the tumour as well as metastasis of HCC can also be determined using CT (Patel et al., 2012). Despite the

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advantages of being able to grade and stage the tumour, CT, has limited resolution for tumours <2 cm and contrast agents are potentially nephrotoxic (Patel et al., 2012). MRI has a better safety profile than CT and can equally be used for HCC staging.

2.12.1.3 Magnetic resonance imaging

MRI is useful for the detection and characterization of regenerating and dysplastic nodules and HCC (Digumarthy et al., 2005). Regenerating liver nodules are non-malignant nodules that are seen in cirrhotic patients while dysplastic nodules are focal nodular regions that are over 1mm in size but have no definitive evidence of malignancy. MRI has been shown to be superior to CT for detection and characterisation of HCC lesions (Yamashita et al., 1996, Semelka et al., 2001).

2.12.2 Serological diagnosis of HCC

In addition to the use of serum alpha-fetoprotein (AFP), newer biomarkers such as des-gamma carboxyprothrombin, glypican-3, Golgi protein 73 and human hepatocyte growth factor have been proposed for the diagnosis of HCC (Liebman et al., 1984, Marrero and Lok, 2004, Spangenberg et al., 2006). The majority of these newer biomarkers have mostly been investigated in HCV-driven HCC and among non-African patients. However, AFP remains the widely used serum biomarker in the diagnosis of HCC. Serum AFP is a glycoprotein that has a molecular weight of about 70 kDa and is produced by cells of the yolk sac and the liver during foetal development. AFP is usually absent or detected at very low levels in the serum of adult patients (Spangenberg et al., 2006). It is the most commonly used biomarkers for HCC surveillance. AFP has been used for decades in the diagnosis of HCC although it is known to have low sensitivity with ranges between 40 to 65%, at least among those with HCV-related malignancy (Gupta et al., 2003). Furthermore, the levels of serum AFP are raised in viral hepatitis infections even in the absence of HCC (Spangenberg et al., 2006). AFP levels greater than 400 ng/ml are used as a cut-off for diagnosis of HCC in combination with a radiological examination suggestive of HCC. However, not all HCC cases will have an elevated AFP and it has been reported that as much as 35% of small tumours will have normal serum AFP concentration (Chen et al., 1984, Lok et al., 2010). AFP has three variants, AFP-L1, AFP-L2 and AFP-L3, based on the sugar chain. Of these three variants, AFP-L3 has been described as a “more reliable and better HCC marker than total AFP and an excellent prognostic parameter in patients with HCC” (Spangenberg et al., 2006).

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2.13 Treatment of chronic hepatitis B

Apart from using vaccination to prevent hepatitis B, slowing the progression of CHB and prevention of HCC development is also achievable through the use of potent therapy that suppresses HBV replication without causing development of drug resistance. The primary goals of CHB therapy are to prevent cirrhosis and HCC (Khokhar and Afdhal, 2008). However, current guidelines are that not all HBV-infected patients need therapy unlike in HIV infection. The benefits and risks of therapy for each patient must be evaluated before initiation of treatment through balancing the risk of untreated hepatitis B (morbidity and mortality) against the likelihood of achieving sustained viral control. The decision to treat is primarily based on HBeAg status, serum ALT and the HBV viral load. Other factors that may be taken into consideration include the histological activity, age and also family history of HCC. The long-term desired outcomes for initiating hepatitis B therapy include the clearance of HBsAg, delaying/preventing the development of HCC and improving survival of infected patients (Khokhar and Afdhal, 2008).

A number of agents are currently approved and in use for CHB therapy in adults and these include: interferon (IFN) α-2b (also known as standard IFN), pegylated IFN α-2a (PEG-IFN), lamivudine, adefovir, telbivudine, emtricitabine, entecavir and tenofovir disoproxil fumarate (Khokhar and Afdhal, 2008, Bhattacharya and Thio, 2010). Standard IFN and PEG-IFN are cytokines and act as immune modulators that also have antiviral activity (Khokhar and Afdhal, 2008, Bhattacharya and Thio, 2010). The IFNs have the advantages of having a finite duration, do not cause emergence of drug-resistance mutations and achieve HBsAg seroconversion rates in about 10% of CHB patients (Brahmania et al., 2016). The disadvantages of IFN-based therapy include: need for subcutaneous injections, low sustained response rates, adverse events and high cost. Lamivudine, emtricitabine and telbivudine are L-nucleosides that are phosphorylated intracellularly to form active nucleotides and cause premature termination of HBV DNA synthesis by competing with natural substrates for binding to HBV DNA polymerase (Bhattacharya and Thio, 2010). Tenofovir and adefovir are acyclic disphosphonates (nucleotide analogues) that are intracellularly prosphorylated to form active metabolites that inhibit HBV DNA polymerase through competitive inhibition causing premature DNA chain termination (Bhattacharya and Thio, 2010). Tenofovir is one of the most potent agents against HBV while adefovir is the least potent. The major limitation to use of tenofovir is its nephrotoxicity but this has since been rectified by the development of a newer tenofovir alafenamide fumarate (TAF) (Ray et al., 2016). Entecavir is a guanosine

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analogue that inhibits HBV DNA polymerase in three ways: inhibits the HBV priming reaction, inhibits reverse transcription of negative strand from pregenomic mRNA and also replication of the HBV DNA positive-strand (Seifer et al., 1998, Levine et al., 2002). Despite the several options for treating CHB, the three most prominent international liver organisations American Association for the Study of Liver Diseases, European Association for the Study of the Liver, and Asian Pacific Association for the Study of the Liver recommend only PEG-IFN, entecavir and tenofovir as first-line agents for treatment of therapy-naïve patients (Brahmania et al., 2016).

In addition to anti-HBV activity, lamivudine, emtricitabine, tenofovir, adefovir and entecavir are also active against HIV and can thus be used in HBV/HIV co-infected patients. Lamivudine is potent against HBV but its use particularly as monotherapy for HBV is associated with rapid emergence of viral drug-resistance mutations (Bhattacharya and Thio, 2010). Although active against HIV, entecavir is not used in HBV/HIV co-infected patients that have uncontrolled viraemia because it leads to the development of HIV lamivudine resistance mutations (Hirsch, 2007, McMahon et al., 2007, Sasadeusz, 2007, Bhattacharya and Thio, 2010). Also, entecavir has potential drug-drug interaction with other antiretrovirals used against HIV such as abacavir which is also a guanosine analogue with potential for inhibitory competition (Soriano et al., 2008a, Soriano et al., 2008c). Therefore, entecavir is rarely used in HBV/HIV co-infected patients. A combination of tenofovir and emtricitabine as the nucleotide reverse transcriptase inhibitors is the preferred choice of treatment for HBV/HIV co-infected patients although lamivudine can be used in place of emtricitabine (Soriano et al., 2008b).

2.13.1 Development of HBV drug-resistance mutations

Development of drug-resistance due to the error-prone nature of HBV reverse transcriptase is a major limitation in treatment of CHB and should always be borne in mind when selecting the appropriate treatment modality (Khokhar and Afdhal, 2008). Primary antiviral failure is defined as less than one $\log_{10}$ reduction in serum HBV-DNA within the first 3 months of anti-HBV therapy and this may be a result of poor pharmacokinetics or infection with an already drug-resistant strain of HBV. Secondary anti-HBV therapy failure arises from poor drug adherence or drug resistance or both, and is defined by an increase of greater than one $\log_{10}$ in HBV DNA from nadir in patients who initially responded to therapy (Soriano et al., 2008b). HBV drug-resistance mutations are classified as being either primary or secondary. Primary

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resistance mutations are those that render the virus less susceptible to therapy but these may come at a cost of loss of fitness for the virus leading to the development of secondary mutations that are compensatory by improving or restoring fitness of the mutated virus (Soriano et al., 2008b). The rate of resistance to anti-HBV agents develops rapidly, first to the L-nucleosides (lamivudine> emtricitabine > telbivudine) followed by adefovir > entecavir > tenofovir. Development of the single mutation methionine to isoleucine or valine change at amino acid 204 of the reverse transcriptase region (M204I/V) renders HBV resistant to lamivudine, emtricitabine and telbivudine (Minuk, 2002, Soriano et al., 2008b). In contrast, entecavir and tenofovir require a number of mutations to be present in the HBV genome before they lose potency hence their classification as being drugs with a high genetic barrier to resistance. For example the mutations L180M+M204V+T250V must be present in order for HBV to be resistant to entecavir (Soriano et al., 2008b).

The best strategy to prevent or delay the emergence of drug-resistant HBV is by achieving complete viral suppression and adherence to therapy. A combination of anti-HBV agents has also been described to reduce the risk of developing drug-resistance mutations (Soriano et al., 2008b). Due to the overlapping nature of the reverse transcriptase/polymerase ORF with the surface antigen ORF, there are also valid concerns that the emergence of drug-resistance mutations may also lead to simultaneous development of HBV strains that are able to escape detection in diagnostic tests and also lead to vaccine escape thereby posing a risk of infection to already-vaccinated individuals (Minuk, 2002, Colson et al., 2007, Sheldon and Soriano, 2008, Soriano et al., 2008b, Clements et al., 2010).

2.14 Treatment of HIV

South Africa recently adopted the "universal test and treat" (UTT) programme for HIV according to the 2015 guidelines of the WHO (WHO, 2015, Health_e-News, 2016). Under the UTT programme, all patients that test positive for HIV infection are placed on therapy regardless of their CD4+ cell count. The aim of highly active antiretroviral therapy (HAART) (UTT in particular) is to ensure that HIV-infected patients achieve viral suppression to levels below detection and also to ensure that the chances of transmission of the infection are significantly lowered. The current first line HAART regimen in South Africa for adolescents and adults including pregnant and breastfeeding women is a fixed dose combination (FDC) of tenofovir, emtricitabine and efavirenz (SADoH, 2015). The FDC was adopted to strengthen adherence and retention of patients to HAART. This regimen is also particularly beneficial for

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HBV/HIV co-infected patients because it contains two NRTIs that are also effective against HBV. The South African Department of Health recommends that patients that shows evidence of virological failure, defined as HIV viral load >1000 copies/mL on at least two occasions two months apart despite good adherence, be switched to a combination of zidovudine + lamivudine + ritonavir-boosted lopinavir (LPV/r). Co-infected patients should be placed on second-line combination of zidovudine+ tenofovir + lamivudine + LPV/r to suppress the emergence of lamivudine-resistant HBV and prevention of fatal hepatitis flares that may occur upon withdrawal of tenofovir in patients with CHB (SADoH, 2015).

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2.15 Aims and objectives

This PhD research project is presented as two studies with the first study focussing on the epidemiology of HCC designated the HCC Epidemiology Study (HCC Study). The second study investigated the association between liver fibrosis, inflammation and microbial translocation and will from hereon be referred to as the Liver Fibrosis and Immune Markers Study (Immune Study). The two studies, although interlinked, had independent Study Hypotheses, Aims and Objectives.

2.15.1 HCC Study Hypothesis

- HIV co-infection increases the risk of HBV-associated HCC.

2.15.2 HCC Study Aim

- To describe the occurrence of HIV and/or HBV infection in patients presenting with HCC at oncology departments of selected teaching hospitals in South Africa.

2.15.3 HCC Study Objectives

- To determine the prevalence of HBV infection in HIV-infected and HIV-uninfected patients with HCC.
- To determine the HBV genotypes and mutations in HIV-infected and HIV-uninfected patients with HBV-related HCC.

The HCC Study was performed as a cross-sectional study using data collected from a prospective study in which patients with a diagnosis of HCC were recruited from the oncology units of selected teaching hospitals in the Western Cape and Gauteng provinces of South Africa.

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2.15.4 Immune Study Hypothesis

- HIV/HBV co-infection is associated with increased liver fibrosis compared to HBV mono-infected through increased immune activation.

2.15.5 Immune Study Aim

- To measure and compare levels of serum and cell-associated markers of immune activation/suppression associated with fibrosis in HBV mono-infected patients (HBV), HBV/HIV co-infected patients (HBV/HIV), HIV mono-infected patients (HIV) and HBV- and HIV-uninfected individuals (controls).

2.15.6 Immune Study Objectives

- To measure and compare levels of serum markers of translocated gut microbial products in HBV, HBV/HIV, HIV and HBV- and controls.
- To measure the presence and degree of hepatic fibrosis using non-invasive methods in HBV, HBV/HIV, HIV and HBV- and controls.

The Immune Study was performed as a multi-group, cross-sectional study in which patients were recruited from the Infectious Diseases Clinic and the Gastroenterology Clinic at Tygerberg Hospital.

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Chapter three

3 Methods and materials

3.1 Ethical Approval and participant recruitment

3.1.1 HCC study

Patients diagnosed with HCC were recruited from oncology units at Tygerberg Hospital and Groote Schuur Hospital in the Western Cape Province and from Chris Hani-Baragwanath Hospital, Charlotte Maxeke Hospital and Steve Biko Academic Hospital in Gauteng Province. Recruitment took place between December 2012 and August 2015. Diagnosis of HCC was based on serum AFP results in combination with radiological imaging examinations, these being any one or a combination of contrast-enhanced computed tomography (CT), abdominal ultrasound scan (USS) or magnetic resonance imaging (MRI). For some of the HCC cases, diagnosis of malignancy was also aided by results of histological examination. Participants were recruited after informed written consent according to the Declaration of Helsinki 2008. Consent forms were provided in English, Afrikaans and isiXhosa which are the three official languages of the Western Cape Province (see Appendix 1). Consent forms were also provided in the appropriate languages spoken in Gauteng Province The recruiting healthcare workers interviewed each participant according to a written questionnaire attached in Appendix 2. Ethical approval was obtained from the Health Research Ethics Committees (HREC) at University of Stellenbosch, University of Cape Town, University of Witwatersrand and the University of Pretoria (see Appendices 3-6).

3.1.2 Immune study

Ethical approval to conduct the study was obtained from the HREC at Stellenbosch University (ethics clearance number S13/04/072) (Appendix 7). HBV/HIV co-infected and HIV-mono-infected patients were recruited from the Infectious Diseases Clinic while HBV-mono-infected and HBV-/HIV-uninfected patients (hospital-based controls) were enrolled from the Gastroenterology Clinic at Tygerberg Hospital, following informed written consent according to the Declaration of Helsinki 2008. Controls were patients attending the Gastroenterology Clinic for other conditions that were not liver-related such as gastrointestinal reflux disease. Patients with other known liver diseases or HCV infection were excluded from the study. The consent form for the immune study was provided in three languages. Following informed consent, participants were interviewed according to a

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questionnaire designed for the study (Appendix 8). Study participants had their anthropometric measurements taken for weight (in kilograms) and height (in centimetres) using the MDW-300L Person Scale (Adam Equipment Inc., Danbury, CT, USA) that provides measurements for both parameters. Following anthropometric measurement, participants had liver stiffness measurements taken using the Fibroscan 402 instrument (Echosens, Paris, France) employing a medium probe. (Refer to section 3.10.1.1 for principle and operation of the instrument).

3.1.3 Blood collection and processing for the HCC study

HCC cases had blood drawn for laboratory testing into a single 5 ml EDTA-anticoagulated tube (Becton Dickinson Biosciences, San Jose, CA, USA) and two 7 ml BD Vacutainer SST II tubes with clot activator (Becton Dickinson Biosciences). In addition, urine specimens were also collected in a clean, sterile 50 ml container. The blood and urine specimens were transported to the laboratory within 4 hours of collection. Following delivery of the samples to the laboratory, both SST tubes were centrifuged at 2,310 rcf (3,000 rpm) for 10 minutes using the Rotanta 460R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) in order to separate the serum from the blood cells. The EDTA tube was centrifuged for 10 minutes at a speed of 1,020 rcf (2,000 rpm) using the Rotanta 460R centrifuge in order to separate the plasma from the cells. Upon separation of plasma from cells, peripheral blood mononuclear cells (PBMCs) were harvested using density gradient centrifugation using Ficoll Histopaque-1077 (Sigma, St Louis, MO, USA) for possible future studies. Plasma and serum samples for the HCC study were stored at -70°C until testing.

3.1.4 Blood collection and processing for the Immune study

Following informed consent, study participants had venous blood drawn into one 10 ml BD Vacutainer EDTA-anticoagulated tube one 10 ml sodium heparinized tube and one 8.5 ml BD Vacutainer SST II tube that contains silicone and micronized silica particles that act as clot activators and cell-separating gel to separate serum from cells during centrifugation. In addition to the three mentioned tubes, additional blood was drawn into a 5 ml EDTA-anticoagulated tube for platelet count determination and also into a 5 ml SST II Advance tube for serum AST and ALT measurements. The platelet counts as well as AST and ALT activity values were used to calculate the APRI and FIB-4 indices.

Participants' blood specimens were processed within 3 hours. Aliquots of EDTA whole blood were prepared for flow cytometry experiments with the rest of the blood being used to extract

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plasma and PBMCs by centrifugation and density gradient centrifugation respectively. PBMCs were collected and stored for possible future studies. SST tubes were centrifuged at 3,000 rpm for 10 minutes on the Rotanta 460R centrifuge to separate the serum from the blood cells. Serum was aliquoted in labelled 2 ml cryovials and frozen at -70°C until testing. Whole blood remaining after aliquots for flow cytometry had been removed was centrifuged at 2,000 rpm for 10 minutes using the Rotanta 460R centrifuge to separate plasma from cells.

3.2 Serological tests for HBV

Serological tests were performed for HBsAg, HBeAg, and anti-HBe using manual ELISA kits (Murex Diasorin) as previously described (Maponga, 2012).

3.2.1 HBsAg

The HBsAg test was performed using the Diasorin Murex HBsAg Version 3 immunoassay kit (Diasorin, Sallugia, Italy). Each well is pre-coated with mouse monoclonal antibody to HBsAg that capture any HBsAg in a sample/control (Abbott Murex version 3 kit insert). A volume of 25 µL of sample diluent was added to each well of the microplate, followed by addition of 75 µL of the patient's sample. The plate was covered with a lid and left to incubate for 60 minutes at 37°C.

After the one hour incubation, 50 µL of conjugate was added to each well. The conjugate is composed of horseradish-peroxidase labelled goat antibody to HBsAg. The sides of the microplate were tapped gently for 10 seconds to release any air bubbles from the wells. The plates were covered with a lid again and incubated for 30 minutes at 37°C. At the end of the incubation time, the plate was washed five times on a Mindray MW-IZA microplate washer (Shenshen Mindray Biomedical Electronics, Hamburg, Germany) using a wash fluid volume of 500 µL in each well. The wash step serves to remove excess of unbound HBsAg and conjugate from the well.

After washing was completed, the plate was inverted and tapped onto absorbent paper to remove any residual wash fluid. Substrate solution of 100 µL was immediately added to each microwell, the plate was covered with a lid and incubated for 30 minutes at 37°C to allow for colour development. The substrate solution contains hydrogen peroxide and TMB. The TMB turns a purple colour when oxidised by the breakdown of hydrogen peroxide catalysed by the horseradish-peroxidase and antibody conjugate in the positive samples. Stop solution made of 50 µL of 1M sulphuric acid was then added to each well. Colour intensity for each well was

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measured on a microplate reader at 450 nm using 650 nm as the reference wavelength on the ELx800 universal microplate reader (Bio-Tek Instruments, VT, USA). The HBsAg reactivity in each well was then measured by comparing its absorbance to the cut-off value. The cut-off value for each run was calculated using the following formula that is provided by the kit manufacturer (Abbott Murex version 3 kit insert): Cut-off value = Mean of the Negative Control replicates + 0.05.

3.2.2 HBeAg Testing

The DiaSorin ETI-EBK PLUS assay is a direct, non-competitive assay and is based on the use of polystyrene microwells coated with mouse monoclonal antibodies to HBeAg (DiaSorin ETI-EBK PLUS kit insert). A volume of 50 µL of patient specimen/controls/calibrator was incubated with 50 µL of incubation buffer in antibody-coated microwells. The plates were sealed using a cardboard cover and left to incubate for two hours in a 37°C incubator. If HBeAg is present in a specimen or control, it binds to the anti-HBe antibody coated on the microwell. Excess sample was removed by a wash step on Mindray MW-IZA microplate washer, composed of five wash cycles using 400 µL of wash buffer.

A volume of 100 µL of enzyme tracer was then added to the microwells and allowed to incubate for one hour in a thermostatically-controlled 37°C incubator. The enzyme tracer contains antibodies to HBeAg conjugated to horseradish peroxidase and binds to any antigen-antibody complexes present in the microwells. Excess enzyme tracer was removed by a wash step as previously described above. A 100 µL volume of TMB/hydrogen peroxide (chromogen/substrate) solution was added to the microwells and allowed to incubate for 30 minutes at ambient temperature in the dark. Wells containing HBeAg bind to the antibody-enzyme conjugate whose enzyme then reduces the hydrogen peroxide, which then oxidizes the chromogen to a blue colour (DiaSorin ETI-EBK PLUS kit insert). The blue colour of oxidised TMB was converted to a more stable yellow by adding 100 µL of 0.4N aqueous sulphuric acid stop solution into all wells maintaining the order and rate in which chromogen/substrate had been added. The wells of samples without HBeAg remained colourless after addition of both the hydrogen peroxide/TMB solution and aqueous sulphuric acid (stop solution).

Colour intensity of each well was measured spectrophotometrically using the ELx800 universal microplate reader at 450 nm, using 650 nm as the reference wavelength, within 15 minutes of the addition of stop solution. The intensity of the yellow colour quantified using

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the spectrophotometer indicates carriage of HBeAg in the patient's sample (DiaSorin ETI-EBK PLUS kit insert). Optical density values for study samples were compared to a cut-off value derived from the average optical density of the calibrator. The cut-off value was calculated by adding 0.060 to the average absorbance for the calibrator values after subtraction of the substrate blank absorbance value (DiaSorin ETI-EBK PLUS kit insert).

3.2.3 Anti-HBe Testing

The Diasorin ETI-AB-EBK PLUS assay is a competitive test based on the use of polystyrene microwells coated with mouse monoclonal antibodies to HBeAg (Diasorin ETI-AB-EBK PLUS kit insert). In the procedure, 50 µL of incubation buffer was added into all wells except for the blank well. Calibrator, negative and positive controls and samples at volume of 50 µL were pipetted into their respective wells followed by addition of 50 µL of neutralizing solution into all wells except for the blank well. The neutralization solution has, among other components, recombinant HBeAg (produced in transfected *Escherichia coli* bacteria) that provides the basis for the competitive assay (Diasorin ETI-AB-EBK PLUS kit insert). A cardboard sealer was then used to cover the plate in order to prevent evaporation followed by gentle tapping of the reaction wells to release any air bubbles trapped in the liquid. The plates were incubated for two hours in a 37°C thermostatically-controlled incubator. Excess sample was removed by a wash step on the Mindray MW-IZA microplate washer, composed of five wash cycles using 400 µL of wash buffer for each well.

Following the wash step, 100 µL of working enzyme tracer solution was added into all wells except for the blank well. The enzyme tracer solution is a conjugate of antibody to HBeAg (mouse monoclonal) and horseradish peroxidase (HRP) and attaches to unbound HBeAg that is coated on the microwell plate (Diasorin ETI-AB-EBK PLUS kit insert). A cardboard sealer was applied onto the plate to prevent evaporation followed by gentle tapping of the reaction wells to release any air bubbles trapped in the liquid. The plate was incubated for one hour at 37°C in a thermostatically-controlled incubator. After lapsing of the incubation period, the plates were washed again as previously described.

Thereafter, 100 µL of TMB/H_2O_2 substrate solution was pipetted into all wells followed by incubation of the plate for 30 minutes at room temperature, away from direct or intense light. This was followed by addition of 100 µL stop solution into all wells. The absorbance of each well was measured using the ELx800 universal microplate reader at 450 nm, using 650 nm as the reference wavelength within 15 minutes of adding the stop solution. The presence or

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absence of anti-HBe antibodies in each sample was determined by comparing the absorbance value of unknown samples to that of the cut-off value (Diasorin ETI-AB-EBK PLUS kit insert). The cut-off value was calculated using the manufacturer's provided formula which is determined by multiplying the mean absorbance of the calibrator values by 0.500 after subtraction of the substrate blank absorbance value (Diasorin ETI-AB-EBK PLUS kit insert).

3.3 Molecular tests for HBV

Extraction of HBV DNA, performance of viral loads and genotyping for both HCC- and Immune Study were performed as previously described (Garson et al., 2005, Maponga, 2012).

3.3.1 HBV DNA extraction

HBV DNA was extracted from the serum specimens using the QIAamp MinElute Virus Spin kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The kit allows purification of nucleic acids and removal of PCR inhibitors.

A volume of 3.5 µL of murine CMV internal control at a concentration of 400 copies/µL was added to each 200 µL of lysis buffer. A 200 µL aliquot of serum was added to 25 µL QIAGEN protease solution in a sterile 1.5 ml microcentrifuge tube. Two-hundred microlitres of the mCMV and buffer AL (lysis buffer) mixture was then added to the tube and mixed by pulse-vortexing for 15 seconds. The sample was then incubated at 56°C for 15 minutes in order to release a maximum yield of DNA from the degraded virions. The tube was then briefly centrifuged to remove liquid droplets from the cap. A volume of 250 µL of 99.99% ethanol (Sigma-Aldrich, St Louis, MO) was then added to the tube and the mixture was mixed by pulse-vortexing. The mixture was incubated for five minutes at room temperature.

The lysate was applied to a QIAamp MinElute column in a 2 ml collection tube and centrifuged at 5,900 rcf (8,000 rpm) for one minute using an Eppendorf 5415D microcentrifuge (Eppendorf, Hamburg, Germany). This enabled the adsorption of the DNA onto the silica gel membrane of the spin column while allowing the rest of the liquid to pass through the column. The tube containing the filtrate was discarded and replaced with a clean 2 ml collection tube, and the QIAamp MinElute column was then washed with 500 µL Buffer AW1 and centrifuged at 8,000 rpm for one minute. The wash step was repeated using 500 µL Buffer AW2 and centrifugation at 8,000 rpm for one minute in order to remove any residual contaminants. Again, the collection tube with the flow-through was discarded and replaced with a new collection tube and the column was subsequently washed using 500 µL of 99.99%

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ethanol. Since residual ethanol in the eluate may inhibit downstream applications, the spin column was centrifuged again at full speed 16,100 rcf (14,000 rpm) for three minutes in a clean 2 ml collection tube. To ensure complete removal of any residual ethanol, the columns were placed onto clean collection tubes and incubated on a dry heat block at 56°C for three minutes to evaporate the ethanol completely. The MinElute columns were then transferred to sterile, labelled 1.5 ml microcentrifuge tubes followed by application of 60 µL elution buffer (buffer AVE). The columns were allowed to incubate at room temperature for five minutes followed by centrifugation at 14,000 rpm for one minute to elute the DNA from the columns. The eluted HBV DNA was stored at -20°C. Known HBV positive samples and normal human plasma that was negative for HBV markers were used as positive and negative controls for the extraction procedure.

3.3.2 HBV viral load

Quantitative detection of HBV DNA was performed using real-time PCR on the Rotor Gene 6000 real-time PCR machine (Corbett Sciences, Australia) according to the protocol developed by Garson et al. (Garson et al., 2005). Serial dilutions of a HBV standard (1 x 10^8 IU/ml) were made in HBV DNA negative human plasma to give a range from 1 x 10^2 – 1 x 10^7 IU/ml. The HBV standard was calibrated against the WHO HBV DNA standard (NIBSC, UK) with a viral load of 1 x 10^6 IU/ml before use. Table 3.1 shows the primer and probe sequences that were used to amplify a highly conserved segment of the surface antigen gene as well the primer and probe sequences that were employed to amplify and detect the mCMV internal control. All primers and TaqMan probes were used at a concentration of 100µM supplied by Applied Biosystems. A reaction volume of 25 µL was used. The PCR master mix reagents and their volumes are shown in the Table 3.2.

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Table 3.1: Oligonucleotide primers used for quantitative detection of HBV DNA

Primer/Probe	**Sequence**
HBV Primer 1	5'- GTGTCTGCGGCGTTTTATCA-3'
HBV Primer 2	5'- GACAAACGGGCAACATACCTT-3'
HBV Probe	5'-FAM-CCTCTTCATCCTGCTGCTATGCCTCATC-TAMRA
mCMV Primer 1	5’-AACCCGGCAAGATTTCTAACG-3’
mCMV Primer 2	5’-ATTCTGTGGGTCTGCGACTCA -3’
mCMV Probe	5’-VIC-CTA GTC ATC GAC GGT GCA CAT CGG C-3'TAMRA

Table 3.2 Reagent components of the quantitative HBV PCR reaction mix

Reagent	**Concentration (pmol/µL)**	**Volume/sample (µL)**
HBV primer 1	100	0.1
HBV primer 2	100	0.1
HBV probe	100	0.05
mCMV primer 1	100	0.1
mCMV primer 2	100	0.1
mCMV probe	100	0.05
2X QuantiTect QPCR mastermix	N/A	12.5
Nuclease-free water	N/A	2
Template	Variable	5

The 2X QuantiTect qPCR master mix was sourced from QIAGEN (QIAGEN, GmbH, Hilden, Germany). Amplification and detection was carried out under the following cycling conditions: 1 cycle of 95°C for 15 minutes and 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. After completion of each run, results were analysed using the Rotor-Gene 6000 Series Software 1.7 (Corbett Life Sciences, Australia). For quality control of each run, negative and positive working controls were used. The positive control was supplied by NIBSC (NIBSC, UK). Nuclease-free water (QIAGEN, Hilden, Germany) was also used as a non-template control.

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3.3.3 HBV polymerase and X/PreCore

All samples that had detectable HBV DNA during quantitative detection of HBV DNA were subjected to direct sequencing using dideoxynucleic acid chain termination technology. The remaining extract from the quantitative detection assay was used for sequencing of the polymerase/surface antigen and X/PreCore regions of the HBV genome. The list of reagents used for HBV molecular assays and their suppliers are shown in Table 3.3. Pre-nested and nested PCR were performed using the primer sequences shown in the Table 3.4.

Table 3.3: List of reagents used for HBV sequencing and their suppliers

Reagent	Supplier
Taq Polymerase (includes $MgCl_2$, 10x PCR buffer)	Invitrogen, CA, USA
10mM dNTP mix	Bioline, London, United Kingdom
Primers	IDT, Iowa, USA
Seakem agarose powder	Lonza, Rockland, ME, USA
6X Novel Juice	GeneDirex, USA
Wizard® SV Gel and PCR Clean-Up System	Promega, WI, USA
Big-Dye Terminator kit, v3.1	Applied Biosystems, CA, USA
Big-Dye Xterminator Purification kit	Applied Biosystems, CA, USA
O'GeneRuler 1 kb molecular markers	Fermentas, Lithuania

Table 3.4: Oligonucleotide primers used for pre-nested PCR amplification of the HBV polymerase gene

Region	Primer	Sequence	Nucleotide position on HBV genome
Polymerase	HBV Z	5' AGCCCTCAGGCTCAGGGCATA	1179 - 1199
	HBV 3	5' CGTTGCCKDGCAACSGGGTAAAGG	2478 - 2455
	HBV M	5' GACACA CTTTCCAATCAATNGG	2306 - 2287
	HBV P	5' TCATCCTCAGGCCATGCAGT	1292 - 1311
X/PreCore	H4072	5'- TCTTGCCCAAGGTCTTACAT	1602 - 1621
	Inner core	5'- CAGCGAGGCGAGGGAGTTCTTCTT	2422 - 2445
	Outer core	5'- TCCCACCTTATGAGTCCAAG	2468 - 2449

Nucleotide positions of primers are numbered according to Pugh et al. (Pugh et al., 1986).

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For the polymerase region, first round PCR was performed as previously described using primers HBV3 and HBVZ. The first round PCR master mix for the polymerase/surface antigen region was constituted as shown in Table 3.5. Five microliters of template was added to the master mix. The cycling conditions were 95°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds 72°C for 60 seconds followed by a final extension cycle at 72°C for two minutes. All PCR reactions were performed on ABI 9700 thermocyclers (Applied Biosystems, CA, USA). PCR reaction mix for first round PCR of the PreCore/Core region was constituted as shown in Table 3.6.

Table 3.5: PCR master mix components for first round for polymerase region

Reagent	**Volume (µL)**
10X PCR Buffer	2.5
50mM Mg Cl_2	0.75
10mM dNTP mix	0.5
Taq Polymerase	0.1
HBV 3 (20pmol/µL)	0.5
HBV Z (20pmol/µL)	0.5
dH2O	15.15
Total Volume	20

Table 3.6. PCR Master mix for First Round PCR for the X and Precore region

Reagent	**Volume (µL)**
10X PCR Buffer	2.5
50mM Mg Cl_2	0.75
10mM dNTP mix	0.5
Taq Polymerase	0.1
H4072 (20pmol/µL)	0.5
Outer Core (20pmol/µL)	0.5
dH2O	15.15
Total Volume	20

The cycling conditions for the first round PCR for PreCore/Core were similar to those of the polymerase region with a hold at 95°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds 72°C for 60 seconds followed by a final extension cycle at 72°C for two minutes and an indefinite hold at 4°C. A second round PCR was performed for the

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polymerase region and also for the PreCore/Core. The master mix for the second round PCR of the polymerase region is as shown in Table 3.7 while that for the PreCore/Core is shown in Table 3.8. A volume of 1 µl from the first round PCR was added to the master mix.

The cycling conditions for the second round PCR of the polymerase region were as follows: denaturation at 95°C for 5 minutes, 34 cycles of 94°C for 30 Seconds, 50°C for 30 Seconds, 72°C for 60 seconds followed by a final extension at 72°C for 2 minutes. The PreCore/Core region used an annealing temperature of 55°C instead of 50°C .The other temperatures and times were identical.

Table 3.7. Polymerase second round master mix

Reagent	**Volume (µL)**
10X PCR Buffer	5
50mM Mg Cl_2	1.5
10mM dNTP mix	1
Taq Polymerase	0.2
HBV P (20pmol/µL)	1
Outer Core (20pmol/µL)	1
dH2O	39.3
Total Volume	49

Table 3.8. X and PreCore second round master mix

Reagent	**Volume (µL)**
10X PCR Buffer	5
50mM Mg Cl_2	1.5
10mM dNTP mix	1
Taq Polymerase	0.2
H4072 (20pmol/µL)	1
Inner Core (20pmol/µL)	1
dH2O	39.3
Total Volume	49

PCR products were visualized using 2% Seakem agarose gel (Lonza, Switzerland) using Tris-acetate-ethylene diamine tetra acetic acid (TAE) as running buffer. A 2% gel was prepared by adding 2 g of Seakem agarose to 100 ml of deionized water. The suspension was heated in a microwave oven on high power for five minutes to completely dissolve the agarose powder and then allowed to cool at room temperature while sitting on a magnetic stirrer. The cooled

gel was poured onto an assembled electrophoresis tank with combs to make the loading wells. Samples were wet-loaded onto the cast gel wells using 6X Novel Juice (GeneDirex, USA) which also stains the DNA for visualisation under UV illumination. For loading PCR products onto the gel wells, 5 µL of the PCR product was mixed with 1 µL of the Novel Juice solution before pipetting onto the wells. O'GeneRuler 1 kb DNA ladder (Fermentas, Lithuania) was used as the molecular marker for determining the size of amplicons. TAE buffer was used as the running buffer. Electrophoresis was then performed using a voltage of 60V for 45 minutes. The gels were then viewed on the UVI Prochemi (UVItec, Cambridge, UK) image acquisition system. Images captured were enhanced and edited using UVIband software (UVItec). For the polymerase gene region, a 1 kb fragment was expected on samples positive for PCR and an example of the pictures obtained is shown in Figure 3.1. For the X/PreCore region, PCR products for positive samples had an approximately 840 bp fragment.

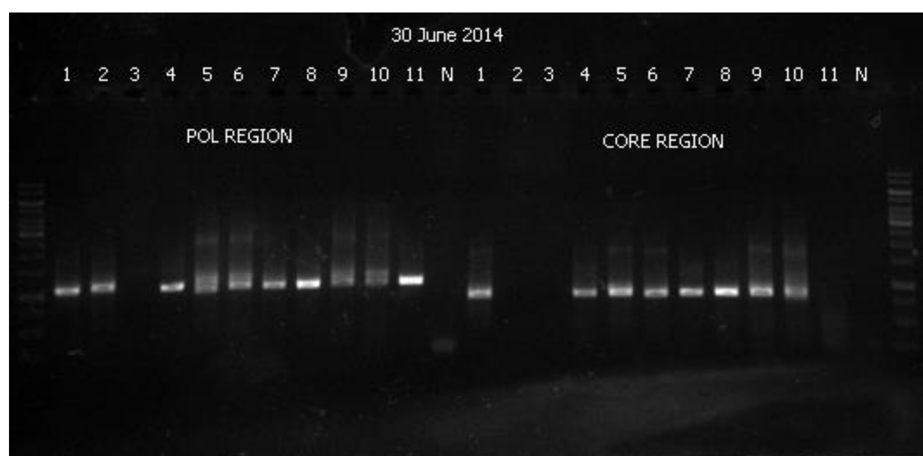

Figure 3.1 Gel electrophoregram for polymerase and X/PreCore regions. The polymerase product being larger (~1kb) compared to the X/PreCore (~840kb) travelled a shorter distance on the electrophoresis gel

3.3.3.1 PCR product clean-up

PCR reactions with visible bands on gel electrophoresis were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, WI, USA). To 45 µL of the PCR product 45 µL of membrane binding solution was added and mixed by pipetting up and down for 4-5 times. Thereafter, the mixture was loaded onto an assembled mini-column and allowed to incubate for a single minute so that the DNA could be adsorbed onto the silica membrane. Following incubation, the mini-column assembly was centrifuged for 1 minute. The mini-column was initially washed using 700 µL of Membrane Wash Solution followed by a final wash with 500 µL of the same wash solution. The DNA was then eluted using 50 µL of nuclease free water into a clean 1.5 ml microcentrifuge tube.

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3.3.3.2 Measurement of purified PCR product

The purity and concentration of the cleaned PCR product was determined spectrophotometrically on the NanoDrop 1000 spectrophotometer (Thermofisher Scientific, USA). Briefly 1 µL of the purified product was loaded onto the instrument pedestal and the concentration of DNA in each sample was determined. Pedestals were wiped clean with tissue paper between each measurement.

3.3.3.3 Nucleotide sequencing of HBV DNA from purified DNA

All purified samples for the three HBV regions were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). The primers used to sequence the three gene regions and their sequences are shown in Table 3.9.

Table 3.9: Primers employed for the direct sequencing reactions

Region	Primer	Sequence	Nucleotide positions on HBV genome
Polymerase	HBV P	5' TCA TCC TCA GGC CAT GCA GT	1292 - 1311
	HBV M	5' GAC ACA CTT TCC AAT CAA TNG G	2306 - 2287
	HBV H	5’ TAT CAA GGA ATT CTG CCC GTT TGT CCT	1767 - 1793
	HBV N	5’ ACTGAGCCAGGAGAAACGGACTGAGGC	1991 - 1965
X/PreCore	H4072	5'- TCTTGCCCAAGGTCTTACAT	1602 - 1621
	Inner core	5'- CAGCGAGGCGAGGGAGTTCTTCTT	2422 - 2445
	CSeqR	5’-GGAGGAGTGCGAATCCACACT	2314 - 2334
	RSP	5’- GTTCAAGCCTCCAAG	1830 - 1844

A reagent mix was prepared for the sequencing reaction according to Table 3.10. All sequencing primers were used at a concentration of 2 pmol/µL. Each PCR template was used at a concentration between 10-30 ng/µL in order to obtain optimal peaks on the chromatograms.

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Table 3.10: Components and volumes for sequencing PCR.

Reagent	Volume/reaction (µL)
Terminator ready mix	1
5X Sequencing buffer	3
Primer	1
Water	4
Template	1

Cycling conditions for the sequencing PCR were 30 cycles of denaturation at 96°C for 20 seconds; annealing at 50°C for 20 seconds and elongation at 60°C for 4 minutes. After completion of the sequencing reaction, products underwent cleaning with the Big-Dye Xterminator Purification kit to separate excess unincorporated dye terminators, salt ions and dNTPs from the dye-labelled extension products before loading on the ABI 3130xL genetic analyser (Applied BioSystems, CA, USA). The Big-Dye Xterminator Purification mixture was made by adding together 49.5 µL of SAM solution and 11 µL of Xterminator suspension followed by thorough mixing with vortexing. A total of volume of 55 µL of the reconstituted solution was then added to each well. The plates with the reactions were placed on a Genie Multi-Microplate shaker (Scientific Industries Inc., NY, USA) for 30 minutes at maximum speed after which the plate was centrifuged for 3 minutes at 1,000 rpm using the Rotanta 460R centrifuge before loading onto the ABI 3130xl genetic analyser.

3.3.3.4 Sequencing data and phylogenetic analysis

Sequence analysis and assembly of contiguous sequences was performed using Sequencher software, version 5 (GeneCodes USA). Trace files were exported from the ABI3130xl Genetic Analyzer and imported into Sequencher software, v5. The consensus sequences were exported from Sequencher. Genotypes and sub genotypes were obtained using online genotyping databases of Stanford University (http://hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html), the Max Planck Institute for Informatics (http://www.geno2pheno.org) and National Library of Medicine HBV genotyping tool (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi).

For detection of amino acid mutations in the polymerase/surface antigen and the X/PreCore regions, sequences were aligned with references sequences using Clustal W in MEGA 5.2 software (Institute of Molecular Evolutionary Genetics. PA, USA). Translation of sequences

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and detection of the presence of amino acids that are not known to be natural polymorphisms for that position were recorded as mutations. Analysis of mutations was performed in BioEdit software (Ibis Biosciences, CA). The frequency of mutations on a particular codon was recorded. Phylogenetic trees were constructed using MEGA 5.1 software. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Bootstrap testing was performed using 1000 replicates (Felsenstein, 1985). The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site.

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3.4 HCV testing of HCC samples

HCC samples were tested for antibodies against HCV and for HCV RNA in the event of a positive anti-HCV result. Samples that had detectable HCV RNA underwent sequencing to determine the HCV genotype using previously described primers targeted at the NS5B region (Mellor et al., 1995).

3.4.1 HCV serology

Antibodies to HCV were tested using the ARCHITECT Anti-HCV test on the Abbott Architect i2000SR immunoanalyzer (Abbott Laboratories, Abbott Park, Illinois, USA). The ARCHITECT Anti-HCV assay is a two-step chemiluminescent microparticle immunoassay that is based on the general principles of an enzyme immunoassay principle. The reactions of the ARCHITECT Anti-HCV assay occur in the following sequence: in the first step, patient sample, assay diluent and HCV antigen coated paramagnetic microparticles are combined. The HCV antigens used in this assay are recombinants, derived from NS3, NS4 and the core region of the HCV genome. Anti-HCV present in the sample binds to the HCV coated microparticles. After washing, anti-human acridinium-labeled conjugate is added in the second step. Following another wash cycle, pre-trigger and trigger solutions are added to the reaction mixture. The resulting chemiluminescent reaction is measured as relative light units (RLUs). ARCHITECT Anti-HCV has been designed to detect antibodies to structural and non-structural proteins of the HCV genome (NS3, NS4, core). The ARCHITECT Anti-HCV assay cut-off value is calculated by the following formula: calibrator 1 mean relative light units (RLU) value $\times$ 0.074 = cut-off RLU. ARCHITECT HCV delivers specimen results as a ratio of the specimen signal (in RLU) to the cut-off value (S/CO). S/CO ratios of greater than or equal to 1.0 are considered reactive for anti-HCV; ratios less than 1.0 are nonreactive for anti-HCV.

3.4.2 HCV RNA detection and quantification

Samples testing positive for anti-HCV underwent testing for HCV viral load using the COBAS AmpliPrep/COBAS TaqMan HCV Quantitative Test, version 2.0 test in the Division of Medical Virology NHLS Laboratory at Tygerberg Campus. The assay uses reverse transcription of HCV RNA to complementary DNA (cDNA) and simultaneous PCR amplification of cDNA and detection of cleaved dual-labelled oligonucleotide probes that are targeted to the highly conserved region of the 5′-untranslated region of the HCV genome. The

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lower limit of detection for the assay is 15 IU/ml and it is able to detect HCV genotypes 1-6. The HCV viral load assay is South African National Accreditation System (SANAS)-accredited.

3.4.3 HCV sequencing

HCV sequencing was performed on samples with detectable HCV RNA. The same nucleic acid extract from HBV viral load experiments was used for HCV sequencing. Complementary DNA (cDNA) synthesis and the first round PCR were performed using SuperScript™ III RT/ Platinum® Taq High Fidelity Enzyme Mix (Life Technologies, CA, USA) and the primers P1203 and P1204 (20 pmol/µL) that are directed at the NS5B region of the HCV genome. The PCR mastermix was made up as shown in Table 3.11. The cycling conditions for the one-step reverse transcription and first round PCR are shown in Table 3.12.

Table 3.11: PCR components for HCV first round PCR

Component	**Volume (µL)**
2X Reaction Mix	25.0
Template	10
P1203 (20pmol/µL)	0.5
P1204 (20pmol/µL)	0.5
SuperScript™ III RT/ Platinum® Taq High Fidelity Enzyme Mix	1.0
Nuclease Free Water	13

Table 3.12. Cycling conditions for reverse transcription and first round PCR

A: cDNA synthesis	**B: PCR amplification**	**C: Final extension**
1 cycle : 45°C for 20 minutes 94°C for 2 minutes	40 cycles Denature, 94°C for 15 seconds Anneal, 55°C for 30 seconds Extend, 68°C for 1 minute	1 cycle 68°C for 5 minutes

Products from the first round PCR were then put through a second round PCR using the primers NS5B2 and P1204 and MyTaq DNA Polymerase (Bioline, Germany). The reaction master mix for the second round PCR was made according to Table 3.13 and the PCR cycling conditions are shown in Table 3.14.

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Table 3.13: Mastermix preparation for second round PCR for HCV sequencing

Reagent	Volume/reaction (µL)
5x MyTaq Reaction Buffer	10
P1204 (20 pmol/µL)	1
NS5Bn2 (20 pmol/µL)	1
MyTaq DNA Polymerase	0.5
Water (ddH2O)	35.5
Template	2

Table 3.14. Cycling conditions for second round PCR for HCV

A: cDNA synthesis	B: PCR amplification	C: Final extension
1 cycle : 95°C for 1 minutes	30 cycles Denature, 95°C for 15 seconds Anneal, 60°C for 15 seconds Extend, 72°C for 10 seconds	1 cycle 72°C for 3 minutes 4°C hold

PCR products were visualized on 2% Seakem agarose gel (Lonza) as previously described for HBV (refer to section 3.3.3). Samples with a visible band on gel electrophoresis as shown in Figure 3.2 were cleaned up using the Wizard® SV Gel and PCR Clean-Up System (Promega). DNA content and purity in cleaned up PCR products was measured using the NanoDrop 1000 spectrophotometer (Thermofisher Scientific) as previously for HBV sequencing (refer to section 3.3.3.2).

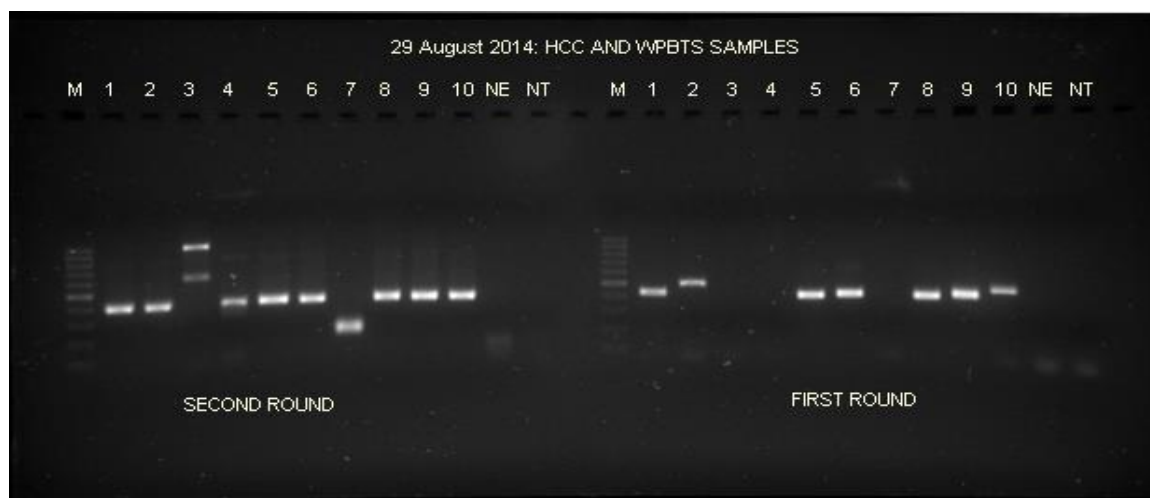

Figure 3.2: Electrophoresis picture of HCV PCR. A band size of around 450 bp was observed for the first round PCR product while the second round product was about 400 bp long.

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Cleaned-up PCR products were sequenced bidirectionally using the second round PCR primers and Big Dye Terminator v3.1 sequencing kit as shown in Table 3.15. The cycling conditions for the sequencing reaction were 25 cycles of denaturation at 96°C for 20 seconds, annealing at 55°C for 15 minutes followed by elongation at 60°C for 4 minutes.

Table 3.15: HCV sequencing PCR with Big Dye Terminator.

Reagent	Volume/reaction (µL)
Terminator ready mix	1
5X Sequencing buffer	3
Primer (P1204 or NS5Bn2)	1
Water	4
Template	1

Following the sequencing PCR, products were cleaned up using the BigDye Xterminator Purification kit as previously described for HBV sequencing. The plate was loaded for sequence analysis on the ABI 3130xl genetic analyser as described previously. Contiguous sequences were generated using Sequencher 5.0 software (GeneCodes Corp, MI, USA) and exported as FASTA files. The genotypes were determined from the online NCBI genotyping tool for HCV (NIH, USA) and also Geno2pheno (Max Planck Institute for Informatics, Germany).

3.5 HIV serology

Serology was performed on serum specimens of HCC cases whose HIV status was not known at the time of recruitment into the study. Testing was performed using the ARCHITECT HIV Ag/Ab Combo assay on the Abbott Architect i2000SR immunoanalyzer (Abbott Laboratories, Abbott Park, Illinois, USA). The ARCHITECT HIV Ag/Ab Combo test is based on a two-step immunoassay that determines the presence/absence of HIV p24 antigen and antibodies to HIV-1 and HIV-2 in human serum or plasma specimens using chemiluminescent microparticle assay technology. Samples testing positive on the ARCHITECT HIV Ag/Ab Combo had a repeat test done on the Mini Vidas (BioMérieux, Marcy l'Etoile, France) to confirm the result.

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3.6 HIV-1 Viral Load

Viral loads were performed only for the Immune Study subjects. EDTA blood was centrifuged at 2,000 rpm for 10 minutes using the Rotanta 460R centrifuge. Thereafter, 1ml of plasma was decanted into a clean, sterile 2 ml microcentrifuge tube and the sample was submitted for viral load testing using the COBAS/Ampliprep TaqMan HIV-1 version 2.0 on the COBAS TaqMan Analyser (Roche Molecular Systems, CA, USA). This is an HIV-1 RNA quantitative assay that monitors viral load by automated detection of amplified complementary DNA (cDNA) using TaqMan probes. The cDNA is generated from reverse transcription of HIV-1 RNA contained in patient's plasma. This is the routine method employed for HIV-1 viral load testing in the Division of Medical Virology, Faculty of Health Sciences, Stellenbosch University, and is SANAS-accredited. It has a linear range of 40-10^7 copies/ml.

3.7 CD4 T-cell count

CD4 cell counts were performed on EDTA-anticoagulated blood of Immune Study participants using the BD TruCount™ Assay on the BD FACSCalibur instrument (Becton Dickenson Biosciences, San Jose, CA, USA). Briefly, the 10 ml EDTA tube was inverted gently for 8 times to thoroughly mix blood. Thereafter, 1 ml of blood was transferred using a sterile transfer pipette into a clean and sterile 2ml microcentrifuge tube and submitted for CD4 T cell counting. CD4 T cell counts were performed in the Division of Medical Virology at the Faculty of Medicine and Health Sciences, Stellenbosch University. The CD4 T cell counting assay in the Division of Medical Virology is South African National Accreditation System (SANAS)-accredited.

3.8 AST and ALT measurements for Immune Study

BD Vacutainer SST II Advance tubes with Immune Study participants' blood were submitted to the Chemical Pathology Department of the National Health Laboratories Service (NHLS) at Tygerberg Hospital for determination of serum aspartate transaminase (AST) and ALT. AST and ALT levels were measured on the Siemens Advia 1800 clinical chemistry analyser (Siemens AG., Munich, Germany). Measurement of serum AST and ALT is based on the spectrophotometric detection of the rate of change of NADH concentration as it is broken down due to the activity of activated enzymes. The NHLS Chemical Pathology Laboratory at

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Tygerberg Hospital is SANAS accredited. AST and ALT measurements were used in the calculation of APRI and FIB-4 test score.

3.9 Platelet measurement

Platelet counts were measured in the Haematopathology Division of the NHLS at Tygerberg Hospital on EDTA whole blood using the Siemens ADVIA 2120 haematology analyser (Siemens AG., Munich, Germany). The measurement of platelet count on the Siemens ADVIA 2120 system is based on the principle of flow cell technology. The platelet count test performed in the Haematopathology Division of the NHLS at Tygerberg Hospital is SANAS-accredited. Platelet count was used for the calculation of APRI and FIB-4 scores (refer to sections 3.8.1.2 and 3.8.1.3.

3.10 Assessment of liver fibrosis

Liver fibrosis was assessed using transient elastometry on the Fibroscan 402 and also using the aspartate-to-platelet ratio index (APRI) test, and Fibrosis-4 (FIB-4) test.

3.10.1.1 Transient elastography using Fibroscan® 402

Liver stiffness measurements were taken on study participants using the Fibroscan 402 instrument (Echosens, Paris France) employing a medium transducer probe. The Fibroscan operates on the principle of measuring the velocity of a shear wave as it travels through the liver. In the procedure, a 50-MHz wave is passed into the right lobe of the liver from a small transducer on the end of an ultrasound probe that is held on an intercostal space of the patient lying on their back with the right arm behind the head in order to enlarge the intercostal space as much as possible (see Figure 3.3). The shear wave is generated from a mechanical impulse derived from a servo-controlled vibrator which is initiated by gently pressing the transducer probe against the patient's skin and then pressing the trigger button on the handheld probe. The shear wave then propagates through the sub-cutaneous tissue and into the liver. Ultrafast ultrasounds are then also generated from the probe and passed onto the liver to measure the velocity at which the shear wave is propagated through the liver.

An inbuilt algorithm within the instrument receives the ultrasonic signals measuring propagation of the shear wave through the liver and converts these into kilo Pascal (kPa). The volume of liver tissue measured using the Fibroscan can be as much as 3 cm^3 which is more than 100 times the volume that would normally be provided in a liver biopsy. The more

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fibrosis there is in the liver, the greater the speed that the shear wave travels. The velocity of the shear wave is thus reported by the instrument in kPa as a function of the stiffness of the liver. Figure 3.3 shows the correct positioning of the patient and the probe during measurement of liver stiffness using the Fibroscan.

Figure 3.3 Positioning of patient during the Fibroscan procedure. A minimum of 10 valid measurements were taken for each patient from which a median liver stiffness value is calculated by the instrument. For quality control, the interquartile range/median for each patient had to be less than 30% for the result to be considered valid and at least 70% of the reading taken had to be valid.

3.10.1.2 APRI score

The APRI score was calculated using the formula: APRI= [(serum AST concentration ÷ upper normal limit)/platelet counts (10^9/L)] × 100. The upper normal limit used was 40 U/L as is reported by the Chemical Pathology Division of the NHLS at Tygerberg Hospital. The APRI score has previously been used in African patients with HBV and HIV co-infection (Bonnard et al., 2010, Vinikoor et al., 2015).

3.10.1.3 FIB-4 score

The FIB-4 score employs a combination of 4 parameters, these being patient's age, AST levels, ALT levels and also platelet count in a mathematical formula expressed as

FIB-4 score = [(Age years x AST) / (PLT x √ALT)].

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The FIB-4 score test was originally developed for assessing liver fibrosis in patients co-infected with HCV and HIV but it has also been used in patients co-infected with HBV and HIV (Sterling et al., 2006, Bonnard et al., 2010, Ive et al., 2013).

3.11 Calculation of Child-Pugh scores

Child-Pugh scores which are used to predict the prognosis of cirrhosis were calculated using online tools freely available from the University of Washington (www.hepatitisc.uw.edu). These were calculated for all the HCC study cases. The Child-Pugh calculator uses a combination of 5 clinical measures of disease, these being total bilirubin, serum albumin, INR, presence/severity of ascites and presence/severity of encephalopathy. Each parameter is assigned between 1-3 points depending on the severity of derangement with 3 being the worst score. The sum of the points assigned to each of the 5 clinical measures is used to classify into one of the three Child–Pugh scores A, B or C. The parameters and the scores assigned are as shown in Table 3.16. HCC cases were assigned to Child-Pugh A if the sum of the 5 parameters was 5-6, B when 7-9 and C for sum score in the range of 10-15.

Table 3.16 Calculation of Child-Pugh scores

Parameter	**1 point**	**2 points**	**3 points**
Total bilirubin (μmol/L)	<34	34-50	≥50
Serum albumin (g/L)	>35	28-35	<28
INR	<1.7	1.7-2.3	>2.3
Ascites	none	mild-moderate	severe
Hepatic encephalopathy	none	mild-moderate	severe

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3.12 Six Colour Flow Cytometry for expression of markers of activation (38 and HLA-DR) and exhaustion (PD-1 and CTLA-4) expression on CD8+ T-cells

3.12.1.1 Flow Cytometry panels design

Two panels were designed and designated as Immune Activation Panel and Immune Exhaustion Panel. The Immune Activation Panel consisted of the following markers and fluorochromes: CD3-Fluorescein isothiocyanate (FITC); CD4-PhycoerythrinTexas Red-X (ECD); CD8-PhycoerythrinCyanin-7 (PC-7); CD45-Krome Orange (KO); CD38-Phycoerythrin (PE) and; HLA-DR-Allophycocyanin (APC). The Immune Exhaustion Panel was made of the following markers and fluorochromes: CD3-FITC; CD4-ECD; CD8-PC7; CD45-KO; CTLA-4-PE and; PD-1-APC. All antibodies used for the study were procured from Beckman Coulter (Beckman Coulter, MI, USA).

3.12.1.2 Instrument set-up

Flow cytometry data acquisition was performed using a Navios flow cytometer (Beckman Coulter, MI, USA). Optimal voltages for acquisition of flow cytometry events were established before beginning experiments on processed patient samples. In addition, compensation parameters were established and fluorescence minus one (FMO) analyses was performed.

3.12.1.2.1 Voltage set-up

Flow-Set Fluorospheres (Beckman Coulter, MI, USA) that are fluorescent microbeads were used to standardize light scatter, fluorescence intensity and ensure optimal hydrodynamic focusing of the instrument. An unstained fresh blood sample was used to set the voltages for the forward scatter (FSC) and side scatter (SSC). Flow-Check Pro fluorescent beads (Beckman Coulter, MI, USA) of known size were used to verify the optics and fluidics of the cytometer.

3.12.1.2.2 Compensation

The use of multiple fluorescent antibodies is associated with spectral overlap where the signal from one fluorochrome is read together with the emission of another. To counter this phenomenon of spectral overlap, a process of fluorescence compensation is used to ensure that only the emission of the fluorescent marker of interest is reported (i.e. overlap

eliminated). Essentially, compensation is performed by subtracting a portion of one detector's signal from another, leaving only the desired signal. Initial compensation settings were established using VersaComp beads (Beckman Coulter, MI, USA). These were then further adjusted/optimised using stained and unstained EDTA whole blood.

3.12.1.2.3 FMO analyses

FMO analyses were performed to correctly gate cells in the context of data spread due to the multiple fluorochromes in the designed panels and also to precisely distinguish between positive and negative marker expression. Whole blood from a healthy donor were used as FMO controls and stained with all the fluorochromes minus each of the fluorochromes of interest (CTLA-4-PE, PD-1-APC, CD38-PE, and HLA-DR-APC). By excluding each of the markers under investigation, gates were placed in the correct places to distinguish between positive staining cells from the negative cells. An example of the FMO analysis for PD-1 on CD8+ T cells is shown in Figure 3.4.

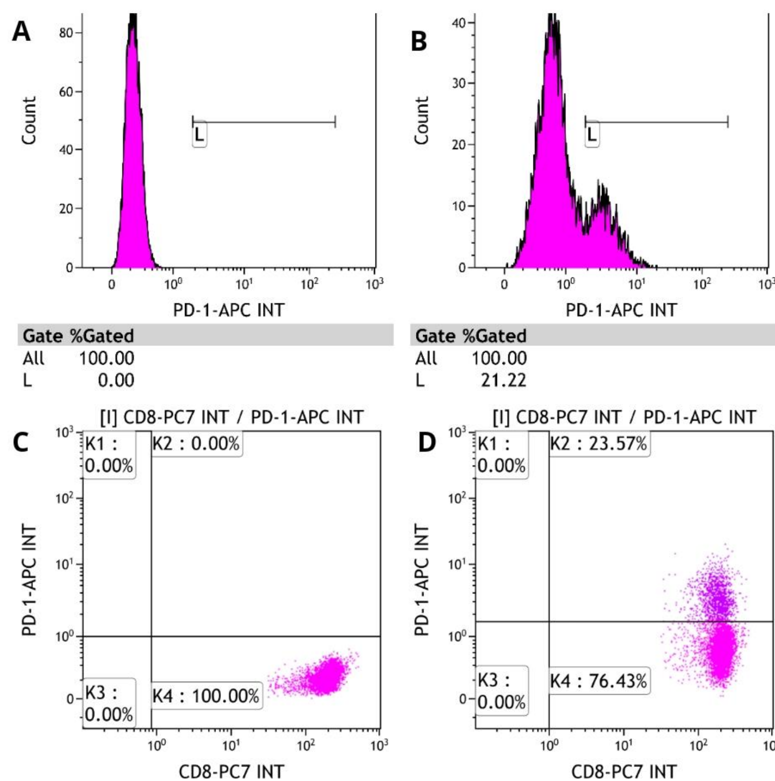

Figure 3.4 FMO analysis for PD-1. Figure A is a histogram of CD8+ T cells without PD-1-APC in the staining cocktail and showing the gate limits with no cells staining for the antigen of interest (0% of CD8+/PD-1+ in gate L). Figure B shows the same sample but stained with an antibody cocktail that included PD-1-APC. The percentage of CD8+ T cells staining for PD-1 APC appear in gate L and these were 21% in this example. Figure 3.3 C shows CD8+ cells without PD-1 staining while Fig 3.3 D shows the same cells staining positive for PD-1.

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3.12.1.3 Monoclonal antibody titration

A titration series of the backbone antibodies was performed in order to have the optimal concentration of each antibody for staining thereby minimising wastage of unbound antibody (Lamoreaux, Roederer, & Koup, 2006). To a volume of 100 µL of whole blood in a 12 x 7.5 mm sample preparation tube (Beckman Coulter, MI, USA) was added the amount of antibody as recommended by the manufacturer. This was mixed by vortexing and incubated at room temperature for 15 minutes in a dark environment. The procedure was repeated using doubling dilutions of the recommended antibody volume (i.e. 1/2 recommended volume, 1/4 recommended volume, 1/8 recommended volume). The tubes with the different antibody titrations were then processed on the TQ-Prep and acquired on the Beckman Coulter Navios (Beckman Coulter, MI, USA). The highest dilution which provided comparable signal intensity to the recommended volume was used in the final experiment. The final volumes that were chosen for the experiments are shown in Table 3.17.

Table 3.17: Antibody and fluorochrome combinations used for flow cytometry experiments. The Activation and Exhaustion Panels all contained the backbone markers.

Panel	Antigen	Fluorochrome	Clone	Antibody Type	Host	Final titrated Vol (µL)
Backbone Markers	CD3	FITC	UCHT1	IgG1	Mouse	5
	CD4	ECD	SFCI12T4D11	IgG1	Mouse	2.5
	CD8	PC7	SFCI21Thy2D3	IgG1	Mouse	5
	CD45	KO	J.33	IgG1K	Mouse	5
Immune Activation Panel Markers	CD38	PE	LS198-4-3	IgG1	Mouse	10
	HLA-DR	APC	Immu-357	IgG1	Mouse	5
Immune Exhaustion Panel Markers	CTLA-4	PE	BN13	IgG2a	Mouse	10
	PD-1	APC	PD1.3	IgG2b	Mouse	10

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3.12.1.4 Staining and acquisition protocol

After determining the optimal monoclonal antibody volume, antibody cocktails were prepared for each staining panel according to the final volumes shown in Table 3.17. Two 100 µL aliquots of EDTA whole blood underwent a stain-lyse-no wash protocol for determination of CD38 and HLA-DR expression on CD8+ T-cells in one tube and PD-1 and CTLA-4 on CD8+ T-cells in the second tube. Pre-optimised cocktails for the Immune Activation Panel and Immune Exhaustion Panel were added to a 7.5 mm sample preparation tube (Beckman Coulter, MI, USA) containing 100 µL EDTA whole blood and allowed to incubate for 15 minutes at room temperature in the dark. Following this, the tube underwent automated processing on the TQ Prep (Beckman Coulter, MI, USA) which makes use of the ImmunoPrep Reagent System (Beckman Coulter, MI, USA). On the TQ Prep, red blood cells in the sample are lysed by formic acid contained in Bottle A followed by dispensing of sodium bicarbonate to neutralize the acid. Finally, paraformaldehyde is added from Bottle C to fix the antibody-antigen binding in preparation for acquisition. After processing on the TQ-Prep, 500 µL of EPICS sheath fluid (Beckman Coulter, MI, USA) was added to the processed sample in order to dilute out the cells and reduce the likelihood of coincidental events being interrogated by the flow cytometer.

3.12.1.5 Acquisition parameters

Samples underwent acquisition on the Beckman Coulter Navios using medium pressure flow rate. Navios software (Beckman Coulter, MI, USA) was used during acquisition stages of the study. A total of 100,000 events were acquired or a maximum acquisition of 600 seconds depending on whichever occurred first. Since all HIV patients were on therapy, a total of 100,000 events generally resulted in approximately 20,000 CD3+ cells being gated in most samples within the 600 seconds.

3.12.1.6 Daily quality controls

Flow Check-Pro Fluorospheres (Beckman Coulter, MI, USA) which are beads of known size and fluorescence intensity were run on each day that an experiment was performed to verify that the optics and fluidics systems on of the instrument were working within expected ranges. Compensation was also performed following instrument service and when new antibody vials were opened.

3.12.1.7 Gating strategy

A plot of Forward Scatter (FS) integral against FS peak was made and a gate placed to include only the singlet cells and exclude doublets and larger multiplets that are caused by clumping (Fig 3.5A) (Wood, 2001). A SS INT vs. FS INT plot was also constructed to visualize the location of the CD45 gated events according to standard size and granularity spread (Fig 3.5B). A plot of SS versus CD45-KO was constructed and a gate was placed on the population that corresponds to lymphocytes based on their expected size (refer to Fig 3.5C). The CD45 gate was used to construct of a plot of SS against CD3-FITC in order to show the T lymphocyte subset (Fig 3.5D). A plot of CD4-ECD versus CD8-PC7 was constructed from the CD3-FITC positive events in order to separate between the CD4+ and CD8+ T lymphocytes (Fig 3.5E). Quadrants were placed for HLA-DR-APC and CD38-PE (PD-1-APC and CTLA-4-PE for the Immune Exhaustion panel using the previously defined FMO regions on the CD4+ and CD8+ T lymphocyte populations (Fig 3.5F). The gating strategy is shown graphically on Figure 3.5.

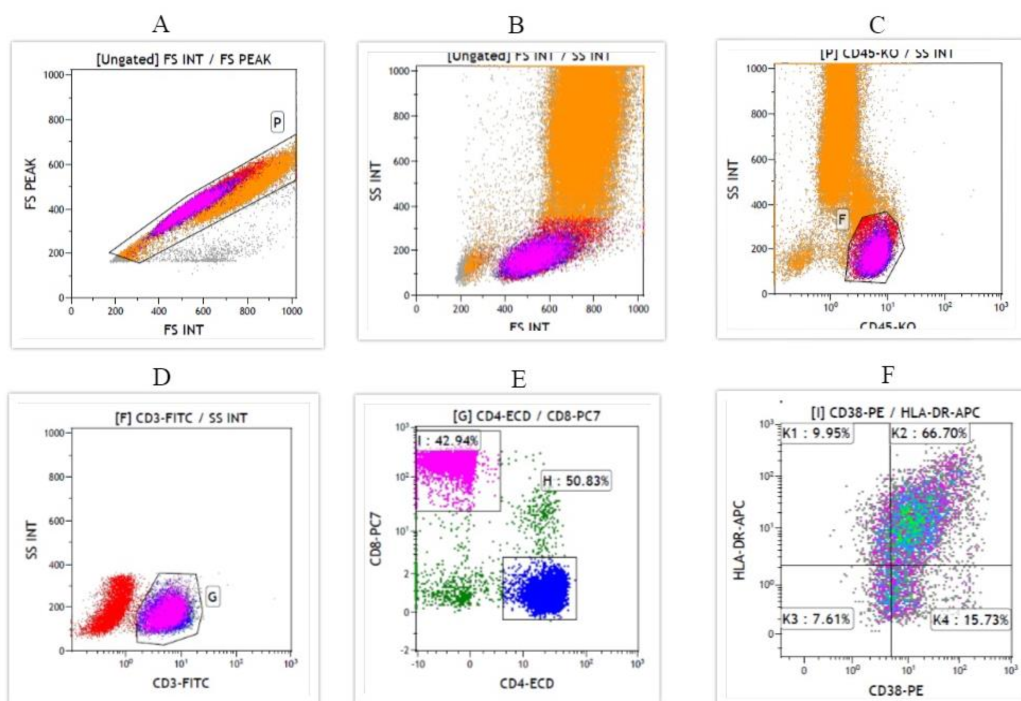

Figure 3.5 Gating strategy for Immune Activation panel. Plot A shows the singlet population gating while Plot B represents the SSC against FSC plot indicating the position of the singlet cells of the lymphocyte population. Plot C shows the lymphocyte population as shown by less complexity (SS) and intense staining for CD45-KO in a plot of side scatter against CD45-KO. Plot D shows the CD3+ population (Gate G) as gated from Gate F shown in Plot C. Using colour precedence and back gating, Plot D also shows the non-lymphocyte population (red colour) that is included within gate F based on use of complexity (SS INT) and staining for CD45. Picture E shows the CD4+ in the blue colour (gate H) and the CD8+ lymphocytes in the magenta colour (gate I). Plot F shows the CD8+ population staining for CD38-PE and HLA-DR-APC gated from gate I.

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3.12.1.8 Analysis of flow cytometry data

Post-acquisition analysis of data was performed using Kaluza software version 1.3 (Beckman Coulter, MI, USA). Flow cytometric data was expressed as percentage of gated T lymphocytes (either CD4+ or CD8+ T lymphocytes) staining positive for each marker as shown on Figure 3.1. Gate statistics expressed in percentage were exported from Kaluza into an Excel Spreadsheet for storage and subsequent statistical analysis. Statistical analysis was performed as described in section 3.16.

3.13 Measurement of soluble CD14

Plasma soluble CD14 (sCD14) levels were measured using the HK320 Human sCD14 ELISA kit (Hycult Biotech, Uden, Netherlands) which is a ready-to-use solid phase ELISA based on the sandwich principle.

3.13.1 Dilution of standards and subjects' specimens

A standard dilution series was first prepared before the assay was performed. The dilution series was prepared by reconstituting a vial of lyophilized human sCD14 standard in a specified dilution buffer (Db) volume as stated in the certificate of analysis (CoA). Seven propylene microcentrifuge tubes were labelled as Tube 2 to Tube 8 and 225 µL of Db was pipetted into each of the tubes. A total volume of 225 µL was then transferred from the reconstituted standard vial into Tube 2 and mixed by vortexing. After mixing, 225 µL was pipetted from Tube 2 into Tube 3. The process was repeated until Tube 7. Tube 8 served as the blank and nothing else was added to the Db. A scheme of how the standard dilution series was prepared is shown in Figure 3.6. Participants EDTA plasma samples were also diluted by factor of 100 using Db before testing. Briefly a 100x dilution was made by adding 10 µL of the subject's sample to 990 µL of Db and mixing by vortexing. All dilutions were made in polypropylene tubes as recommended by the manufacturer.

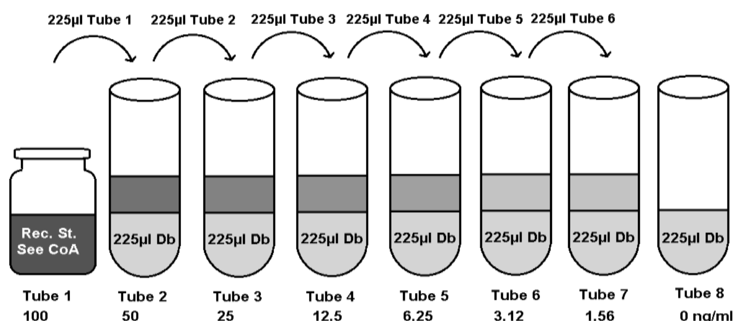

Figure 3.6: Preparation of human sCD14 standard dilution series. The concentration of the standard was halved at each further dilution point.

3.13.2 Soluble CD14 procedure

A total volume of 100 µL of diluted standards/samples was added to micotitre plates whose wells were coated with antibodies that bind to human sCD14. Each standard/ sample was assayed in duplicate. The plate was covered with a seal and tapped gently to eliminate any air bubbles. The plate was then incubated for one hour at an air-conditioned room temperature of 18°C. After the incubation, the plate was washed four times manually using a volume of 200 µL/well. A volume of 100 µL of biotinylated tracer antibody solution was then added to each well. The plate was again covered and incubated for one hour at room temperature. The wash procedure was repeated as described previously followed by the addition of 100 µL of streptavidin peroxidase to each well on the plate. This was followed by an incubation period of one hour after which the plate was again washed. A TMB volume of 100 µL was then added to each well followed by incubation for one hour. The enzymatic reaction was then stopped by the addition of 100 µL of stop solution. Optical density readings were measured using the Biochrom EZ Read LED microplate (Biochrom Limited, Cambridge, UK) reader at a single wavelength of 450 nm. An overview of the sCD14 assay is shown in Figure 3.7.

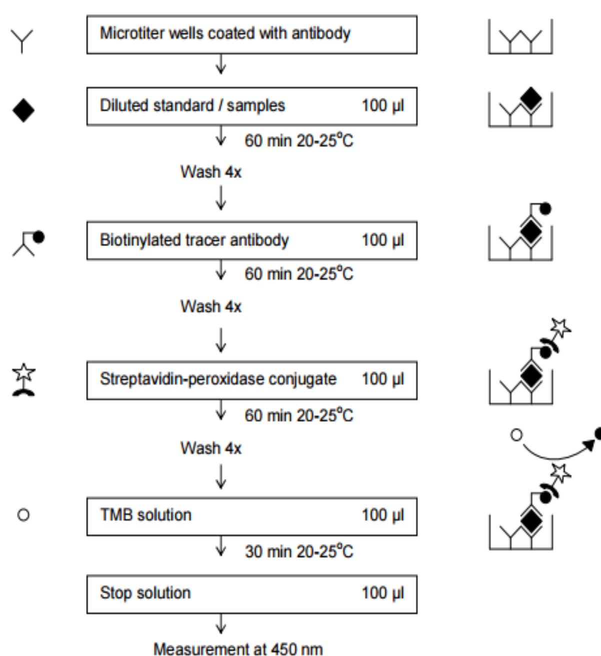

Figure 3.7: Overview of the human sCD14 procedure.

Standard curves were obtained by plotting the absorbance (linear) versus the corresponding concentrations of the human sCD14 standards (logarithmic scale) using an online ELISA analysis application available from http://elisaanalysis.com/app (Elisakit.com Pty Ltd). The

amount of sCD14 in subjects' samples were then determined from the standard curve by 5-Parameter Logistic Regression formula using the online tool. An example of the standard curve obtained for the sCD14 assay is shown in Figure 3.8.

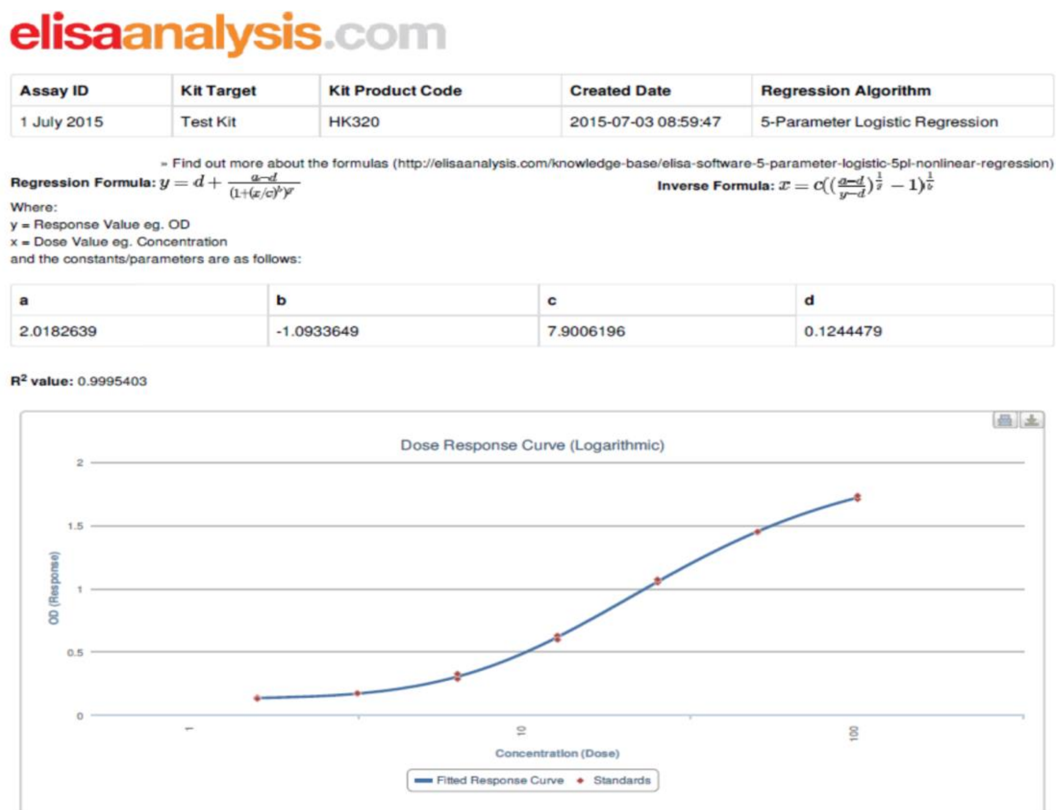

Figure 3.8 Typical standard curve for sCD14 using the Hycult Biotech assay. The figure shows the sCD14 standard concentrations on the x axis using a logarithmic scale and the corresponding optical density on the y axis in linear scale. Standards and samples were analysed in duplicate. The samples are not included on the standard curve.

3.14 Measurement of lipopolysaccharide binding protein (LBP)

LBP levels were assayed using HK315 Human LBP ELISA kit (Hycult Biotech, Uden, Netherlands). The HK315 Human LBP ELISA assay is identical to the sCD14 assay (refer to section 3.8) except for different dilution factors and diluents that are used.

The LBP standard dilution series was prepared by reconstituting a vial of lyophilized human LBP standard by pipetting the amount of wash/dilution buffer (W/Db) following the volume stated in the certificate of analysis (CoA). Seven propylene microcentrifuge tubes were labelled as Tube 2 to Tube 8 and 225 µL of (W/Db) was pipetted into each of the tubes. A total volume of 450 µL was then transferred from the reconstituted standard vial into Tube 2 and mixed by vortexing. After mixing, 450 µL was pipetted from Tube 2 into Tube 3. The

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process was repeated until Tube 7. Tube 8 served as the blank and nothing else was added to the W/Db. A scheme of how the standard dilution series was prepared is shown in Figure 3.9.

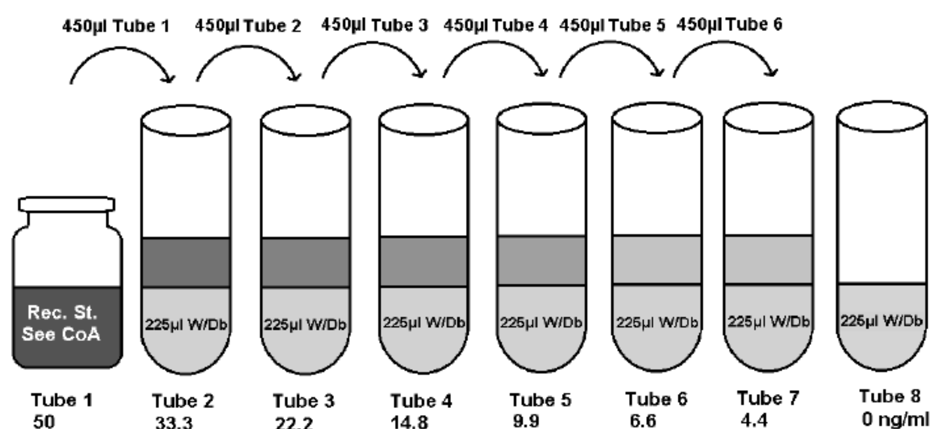

Figure 3.9: Preparation of human LBP standard dilution series. The standard concentration decreased by 1/3 at each dilution point.

Participants EDTA plasma samples were diluted to a factor of 1000x using W/Db. Briefly a 10x pre-dilution was made by adding 25 µL of EDTA plasma to 225 µL of W/Db and mixing by vortexing. A final 1000x dilution was then made by adding 10 µL of the 10x pre-diluted sample to 990 µL of W/Db and mixing by vortexing. All dilutions were made in polypropylene tubes as recommended by the manufacturer. After dilution of samples and standards, the procedure was the same as described for sCD14. Standard curves were obtained by plotting the absorbance (linear) versus the corresponding concentrations of the human LBP standards (logarithmic scale) using an online ELISA analysis application available from http://elisaanalysis.com/app (Elisakit.com Pty Ltd). The amount of LBP in subjects' samples were then determined from the standard curve by 5-Parameter Logistic Regression formula using the online tool. An example of the standard curves that were obtained for LBP is shown in Figure 3.10.

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elisaanalysis.com

Assay ID	Kit Target	Kit Product Code	Created Date	Regression Algorithm
7 July 2015	Test Kit	HK315	2015-07-07 02:56:51	5-Parameter Logistic Regression

- Find out more about the formulas (http://elisaanalysis.com/knowledge-base/elisa-software-5-parameter-logistic-5pl-nonlinear-regression)

Regression Formula: $y = d + \frac{a-d}{(1+(x/c)^b)^g}$ **Inverse Formula:** $x = c((\frac{a-d}{y-d})^{\frac{1}{g}} - 1)^{\frac{1}{b}}$

Where:
y = Response Value eg. OD
x = Dose Value eg. Concentration
and the constants/parameters are as follows:

a	b	c	d
3.5523257	-6.2285454	24.0029108	-0.1633162

R^2 value: 0.9986126

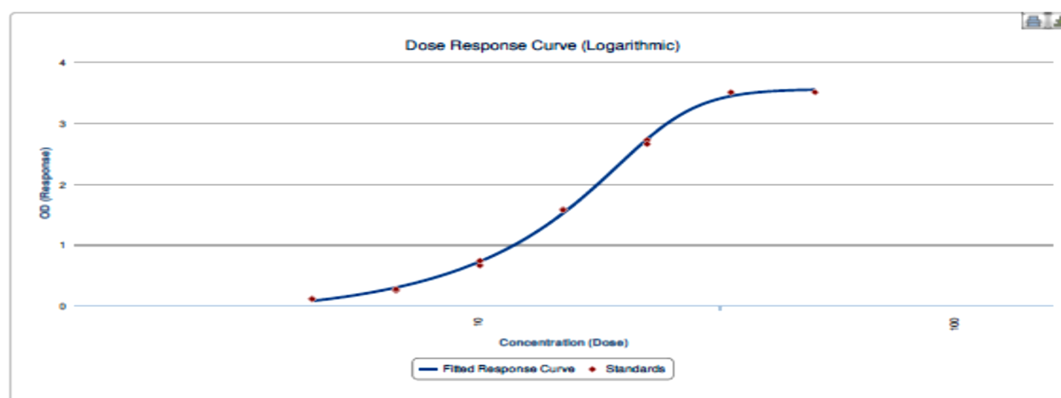

Figure 3.10 Standard curve for the Hycult Biotech LBP assay. The curve shows the LBP standard concentrations on the x axis (logarithmic scale) and the optical densities on the y axis (linear scale). Samples and controls are not included in the curve.

3.15 Cytokine measurement by Luminex technology

Luminex technology is a combination of ELISA and flow cytometry principles. The technology is based on the use of capture antibodies (directed at the analyte of interest) that are covalently coupled to fluorescently dyed microspheres (beads). Each microsphere has a unique colour code that allows distinction of individual targets within a multiplex system. The coupled antibodies react with the cytokine of interest within the sample. After a wash step to remove unbound protein, the beads are washed and biotinylated detection antibodies are added that create a sandwich complex. A detection complex of streptavidin-PE is added. The PE serves as the reporter or indicator fluorochrome during interrogation of the beads on the reader. Serum cytokines and chemokines were measured using the premixed Bio-Plex pro Human Cytokine Grp 1 panel 27-plex and the Bio-Plex Pro TGF- β Panel 3-plex assays (Bio-Rad Laboratories, CA, USA). The Bio-Plex pro Human Cytokine Grp 1 panel 27-plex kit contains the analytes; IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8. IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, basic Fibroblast Growth Factor (FGF), eotaxin, granulocyte-colony stimulating factor (G-CSF), Granulocyte Macrophage-colony stimulating factor (GM-CSF), interferon gamma (IFN-γ), IFN-γ-inducible protein 10 (IP-10), Macrophage

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Chemoattractant Protein 1 (MCP-1), Monocyte Inflammatory Protein 1 alpha (MIP-1α), Monocyte Inflammatory Protein 1 beta (MIP-1β), platelet derived growth factor-BB (PGDF-BB), regulated on activation, normal T cell expressed and secreted (RANTES), TNF-α and vascular endothelial growth factor (VEGF). The Bio-Plex Pro TGF- β Panel 3-plex kit assays for the three TGF-β isoforms TGF- β1, TGF-β2 and TGF-β3. Serum samples were thawed at room temperature and tested within 2 hours of thawing. The assays were performed according to the supplier instructions. Briefly for the 27-plex assay, 20x premixed antibody-conjugated beads of the 27 individual cytokines were diluted to 1x working concentration added 50µL were added to each well of a 96-well flat-bottom plate that was placed on a washer with a magnetic plate holder and washed twice. There is no pre-wetting of the plate necessary as would be required using a filter plate and filter washer. Serum samples were diluted 4x using Bio-Plex sample diluent and added to the plate containing the premixed beads. The plate was then sealed and covered with aluminium foil and allowed to incubate for 30 minutes at room temperature on an Orbit P4 microplate shaker (Labnet International, NJ, USA) at 850 rpm. Thereafter, the plates were washed three times using 100 µL of wash buffer on the Bio-Rad BioPlex Pro Wash Station (Bio Rad Laboratories, CA, USA). A volume of 25 µL of detection antibody was then added to each well. The plate was covered again with sealing tape and sealed with aluminium foil and incubated for 30 minutes on a shaker at 850 rpm. After incubation, the plate was washed three times using a wash buffer volume of 100 µL. A volume of 50 µL of streptavidin-PE conjugate was added to each well and incubated for 10 minutes at room temperature while seating on the shaker. Plates were washed for the last time for three cycles. One hundred and fifty microlitres of sheath fluid was then added to each well and the plate was sealed again, covered with aluminium foil and placed on the shaker for 30 seconds. The plate was read on the Bio Plex 200 platform (Bio Rad Laboratories). The Bio-Plex Pro TGF- β Panel 3-plex assay was also performed according to manufacturer's instruction and is similar to the Human Cytokine Grp 1 panel 27-plex kit except for an additional step that requires pre-treatment of sample and some of the incubation times that are also different. For the TGF-β Panel 3-plex assay, serum samples have to be first pre-treated with acid in order to render TGF-β immunoreactive. Briefly, 5 µL of 1N hydrochloric acid was added to 25 µL of participant's EDTA plasma, thoroughly mixed by vortexing and incubated at room temperature for 10 minutes. To neutralize the sample, 5 µL of a base composed of 1.2N NaOH/0.5M HEPES was then added and mixed by vortexing. To achieve a

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final sample dilution of 16x, 365 μL of Bio-Plex sample diluent was added. The assay was performed similar to the 27-plex assay except for the differences as shown in Table 3.18.

The activating acid and neutralising base for the TGF-β assay are not provided with the kit and were prepared according to the recipes recommended in the kit insert. To make 100 ml of 1N HCl, 8.33 ml of 12N HCl was slowly added to 91.67 ml of deionized water and mixed well. Preparation was done on ice because of the exothermic nature of the reaction. To make up 100 ml of 1.2N NaOH/0.5M HEPES, 12 ml of 10N NaOH was added to 75 ml of deionized water and mixed by swirling. Thereafter, 11g of HEPES powder (free acid with molecular weight 238.3g) (Sigma Aldrich, MO, USA) was added and mixed by swirling. The solution was topped with deionized water to achieve a final volume of 100 ml. Luminex data was exported onto an Excel spreadsheet. Cytokines that were below detection limit were assigned a value of zero to standardize for statistical analysis. Statistical analysis for differences between groups was performed as described in section 3.16.

Table 3.18: Similarities and differences between the Bio-Plex 27-plex assay and the Bio-Plex TGF-β 3-plex assay

Step	**27-plex**	**TGF-β 3-plex**
Pre-treat sample	No	Yes
Sample dilution	4x	16x
Beads complexed to antibody	50 μL	50 μL
Wash	2x	2x
Add samples/standards	50 μL	50 μL
Incubate	30 min, room temp, 850 rpm	2 hours, room temp, 850rpm
Wash	3x	3x
Detection antibody	25 μL	25 μL
Incubate	30 min, room temp, 850 rpm	1 hour, room temp, 850rpm
Wash	3x	3x
Streptavidin-PE conjugate	50 μL	50 μL
Incubate	10 min, room temp, 850 rpm	30 min, room temp, 850 rpm
Wash	3x	3x
Resuspend in assay buffer	125 μL	125 μL
Shake	30 sec, 850 rpm	30 sec, 850 rpm
Read plate	Bio Plex 200 platform	Bio Plex 200 platform

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3.16 Data handling and statistical analysis

All data entry and storage was performed in Epi-Info 7 (Centres for Disease Control, GA, USA) and statistical analysis was carried out in Statistica version 12 (StatSoft, Oklahoma, USA). Normality of data was tested using the D'Agostino-Pearson omnibus test. Non-parametric data was described using medians and interquartile ranges and compared using the Mann-Whitney test where there were only two groups or the Kruskal-Wallis test in cases of three or more groups. Multiple comparisons were preformed using the Dunn's post-test where the Kruskal-Wallis p value was significant.

Data with normal distribution was described using means and 95% confidence intervals (95% CI). Comparisons were performed using the unpaired t-test when data was normally distributed. Categorical data was described using proportions and 95% CI and analysed using the χ^2 test or Fisher's test depending on the number of observations. Correlational analysis was performed using the Spearman rank correlation test. Graphs were constructed using Statistica and GraphPad Prism version 5 software (GraphPad Software, CA, USA). All hypothesis testing was done at 95% confidence intervals and results were regarded as significant if $p<0.05$.

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Chapter four

4 Results

This chapter will be presented in three parts. The first part describes results from the HCC Epidemiology Study (HCC Study). The second part describes the results of the Liver Fibrosis and Immune Markers Study (Immune Study). The third part describes differences and similarities of the molecular character of HBV sequenced from the HCC Study and Immune Study.

4.1 HCC Study

4.1.1 Patient characteristics

A total of 107 HCC cases were recruited between December 2012 and August 2015 A total of 88 (83%) of the HCC cases were recruited from Tygerberg Hospital and Groote Schuur Hospital in the Western Cape Province of South Africa. There were fewer cases recruited from participating centres in Gauteng because of the unavailability of study nurses who could help the clinicians who had a heavy workload to devote time to research activities. The site of recruitment for all cases for the HCC study is shown in Table 4.1.

Table 4.1 List of recruitment sites and number of enrolled HCC cases

Recruitment site	**n (%)**
Tygerberg Hospital (Western Cape)	50 (47)
Groote Schuur Hospital (Western Cape)	38 (36)
Chris Hani Baragwanath Hospital (Gauteng)	10 (9)
Charlotte Maxeke Johannesburg Academic Hospital (Gauteng)	8 (7)
Steve Biko Academic Hospital (Gauteng)	1 (1)

The majority of the cases were male patients who made up 78% (n=83) of the cohort. Of the 107 HCC cases, 61 (57%) were born in a rural area setting. The demographic characteristics of the cases are shown in Table 4.2.

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Table 4.2 Summary of demographic characteristics of HCC cases at time of recruitment

Characteristic	**Descriptive statistic**
Number of HCC cases	107
Age at diagnosis in years, Mean (95% CI)	46 (95% CI: 43-49)
Gender	n (%)
Male	83 (78%)
Female	24 (22%)
Race	n (%)
Black	59 (55%)
Mixed	38 (35%)
Caucasian	6 (6%)
Unknown	4 (4%)
Place of birth	n (%)
Rural	61 (57%)
Urban	38 (36%)
Unknown	8 (7%)

The youngest HCC case recruited in the study was 18 years of age while the oldest case was a 90 years old male patient. A histogram plot of the results showed that the highest number of HCC cases occurred between 30-40 years of age. Figure 4.1 is a histogram of the age distribution at HCC diagnosis.

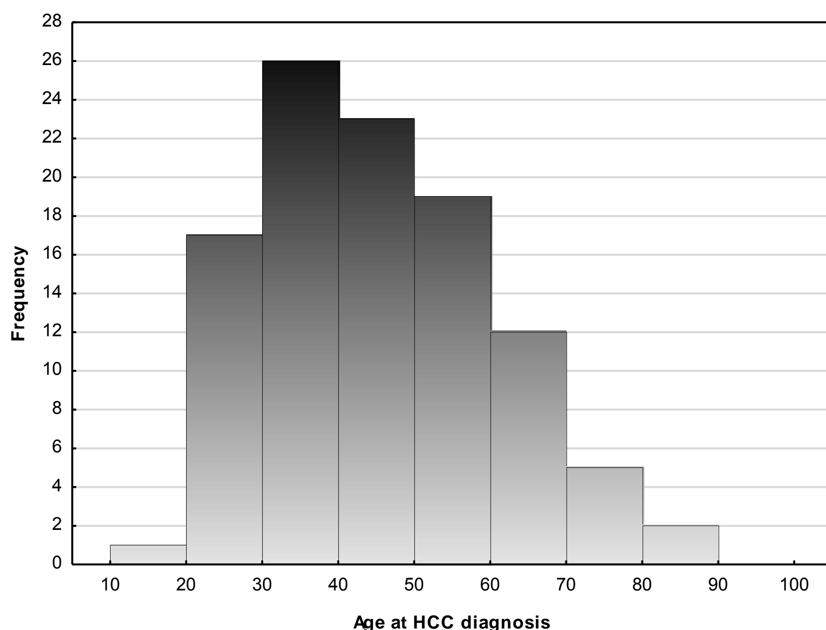

Figure 4.1 Age distribution of HCC cases. The frequency histogram shows that the modal age category and HCC presentation for the entire cohort was between 30-40 years old where 26 cases were seen.

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The median age at presentation for Caucasian cases was 60 years (IQR: 49-70) compared to 41 years (IQR: 33-51) among Black Africans and 50 years (IQR: 33-57) among those of Mixed Descent, p=0.3. There was no significant difference in the median age of Black Africans and Caucasians. The mean age of rural-born cases was 47.3 years (95% CI: 43.3-51.3) compared to 43.3 years (95% CI: 38.3-48.3) among those born in urban areas, p=0.8.

4.1.2 Diagnosis modalities for HCC in South African patients

A total of 80% of the recruited HCC cases were diagnosed using a combination of serum alpha-fetoprotein (AFP) levels plus imaging techniques. Serum AFP levels were available for 99/107 (93%) of the HCC cases with 9 cases having no results. The most common imaging modality used was contrast-enhanced computed tomography (CT) scan with 77 of 107 (72%) of the cases having received an examination by CT alone or in combination with other imaging examinations. Only three HCC cases had an USS performed together with an MRI scan without CT. Liver histological examination was performed in 29 out of 98 cases (30%) with 11 cases having incomplete data as to whether histological examination was carried out or not. The distribution of the imaging and AFP examinations is shown in Table 4.3.

Table 4.3 Imaging modalities used in diagnosing HCC among recruited cases

Modalities used for HCC diagnosis	Frequency*	%
AFP + CT alone	43	40
AFP + USS alone	23	21
AFP + USS + CT	27	25
AFP + USS + MRI	3	3
AFP + USS + CT + MRI	2	2
USS alone	2	2
USS + CT	3	3
CT alone	4	4

*Frequencies correspond to Boolean combinations of imaging modalities with denominator being 107

4.1.3 Clinical laboratory features of HCC cases at presentation

Clinical laboratory data was collected for liver function tests, full blood counts and kidney function tests. ALT values that were greater than twice the upper limit of normal were observed in 25/85 (30%) of all HCC cases. The proportion of HCC cases with serum ALT greater than 40 U/L at the time of HCC diagnosis was 58/85 (68%). A summary of available

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clinical laboratory data is shown in Table 4.4. Of 98 cases that had an AFP result, 69/98 (70%) had levels greater than 400 µg/L.

Table 4.4 Laboratory features of all recruited HCC cases on presentation

Laboratory Parameter	Reference interval	n	Median (IQR)
AFP (µg/L)	0-9.0	98	6,176 (120-47,751)
ALT (U/L)	5-40	85	51 (35-86)
AST (U/L)	5-40	62	91 (47-172)
ALP (U/L)	40-120	86	241 (172-390)
GGT (U/L)	Males: 0-60 Females: 0-35	53 9	315 (191-554) 135 (54-489)
Total bilirubin (µmol/L)	0-21	92	13 (9-26)
Conjugated bilirubin (µmol/L)	0-6.0	71	8 (4-20)
Albumin (g/L)	35-52	87	35 (30-39)
Platelets (x10^9/L)	178-400	98	302 (213-414)
MCV (fl)	79.1-98.9	80	87 (83-94)
Haemoglobin (g/dL)	Males: 13-17 Females: 12-15	68 20	12.4 (10.3-13.9) 11.4 (10.1-12.7)
Creatinine (µmol/L)	49-90	72	66 (55-80)

U/L-units per litre, fl-femtolitres

4.1.4 Clinical characteristics of HCC cases at presentation

The clinical characteristics investigated at time of recruitment of HCC cases included a known diagnosis of cirrhosis, presence of ascites, ankle oedema, abdominal mass and other features of liver disease including jaundice, hepatomegaly and splenomegaly. The most frequently observed clinical features at presentation and their frequencies are shown in Table 4.5. The most commonly observed sign among the HCC cases was the presence of an abdominal mass (reported in 67% of cases) which frequently was the cause for the patients seeking medical attention. Other findings that were observed among the HCC patients included: clubbing which was reported in eight cases, leukonychia seen in five, gynaecomastica that was found in three cases, two had hepatic arterial liver bruit and spider naevi were also seen in two HCC patients of whom one self-reported alcohol dependence. Other rare findings that were seen in no more than one case included parotid enlargement and bruising.

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Table 4.5 Prevalence of clinical features at presentation

Feature	Frequency (%)
Concomitant cirrhosis	21 (23%)*
Abdominal mass	72 (67%)
Ascites	35 (33%)
Hepatomegaly	17 (16%)
Ankle oedema	17 (16%)
Jaundice	14 (13%)
Splenomegaly	6 (6%)
Child-Pugh Score	
A	56 (61%)
B	28 (30%)
C	8 (9%)

*17 cases had data unreported.

Results of the radiological examinations showed that 58% of cases had multiple liver lesions present within the liver at the time of HCC diagnosis. A finding of hepatomegaly on radiological examination was seen among 55% of cases. Cases frequently presented with advanced disease such that only five underwent surgical resection of the tumour. Table 4.6 summarizes the observations made from the combination of radiological findings for all 107 HCC cases.

Table 4.6 Summary of findings of radiologic examinations

Imaging Findings	Frequency	Percent
Multiple liver lesions	62	58%
Enlarged liver	59	55%
Ascites	24	22%
Splenomegaly	21	20%
Single liver lesion <2 cm	13	12%
Single lesion greater than 5 cm	11	10%
Evidence of hypervascularity	7	7%
Shrunken liver	6	6%
Single lesion less/equal 5 cm	1	1%

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Data on presence or absence of metastasis was mostly incomplete and was reported for only 48/107 (45%) of the cases. Among the 48 with reported data, 27 (56%) cases had evidence of metastasis of the malignancy from/within the liver. The most common site of extrahepatic metastasis was the lungs which was reported in 12/27 (44%) patients.

4.1.5 Prevalence of lifestyle and familial risk factors for HCC

Self-reported history on alcohol dependence, smoking, consumption of traditional beer (proxy risk for iron overload) and peanut consumption (as a proxy for aflatoxin exposure) was investigated. In addition the presence of familial history of liver disease and HCC was also investigated (refer to Appendix 2). The frequency of these risk factors is shown in Table 4.7.

Table 4.7 Prevalence of familial and lifestyle risk factors among HCC patients

Risk Factor	Frequency (%)
Family history of liver disease	12/82 (15%)
Family history of HCC	5/97 (5%)
Known prior diagnosis of cirrhosis	4/90 (4%)
Self-reported alcohol dependence	22/98 (22%)
Consumption of traditional beer	24/94 (26%)
History of smoking	64/80 (80%)
Consumption of peanuts	67/85 (79%)

The mean age of HCC cases with a positive family history for liver disease was 44.0 years (95% CI: 33.2-54.7) compared to 45.6 years (95% CI: 41.6-49.5) in those without a family history of liver disease, p=0.8. Only four cases (4%) had data that indicated a prior diagnosis of cirrhosis. The remaining 17 cases that had cirrhosis had the date of cirrhosis diagnosis being the same as date of HCC diagnosis. Among the four subjects that had a confirmed prior diagnosis of cirrhosis, the mean lead time between cirrhosis diagnosis date and HCC diagnosis date was 146 days. Although only 22/98 (22%) of cases self-reported alcohol misuse (drinking patterns that have a negative impact on a person's health, relationships and work), the overall frequency of alcohol consumption was 74/107 (69%). The frequently consumed alcohol type was beer 34%, followed by spirits (19%) and wine (13%). Cases that self-reported alcohol dependence had a significantly higher mean age (52.7 years, 95% CI: 46.3-59.2) compared to those without alcohol dependence (44.0 years, 95% CI: 40.4-47.5), p=0.02

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Of the 64 subjects that had a smoking history, 11 were current smokers, 46 were past smokers who had quit less than 5 years at time of recruitment and 7 had stopped smoking for over 5 years before presentation with HCC. Males comprised 50/64 (78%) of HCC cases that had any history of smoking. Consumption of peanuts was evaluated as a proxy of aflatoxin exposure. The most commonly reported sources of peanuts were supermarkets that are thought to be low risk of aflatoxin contamination (n=25) and vegetable/farmers' markets that are thought to have a high risk of aflatoxin contamination (n=24). Fifteen subjects reported that they grew their own peanuts. HCC cases consuming peanuts from supermarkets presented at median age 41 years (IQR: 31-52), those from vegetable/farmers' markets had median age 36 years (IQR: 29.5-53.5) and those that grew their own had median age of 42 years (IQR: 33-62) at HCC presentation, p=0.59.

4.1.6 Prevalence of HBV serologic markers as risk factors among HCC cases

4.1.6.1 Prevalence of HBsAg

Of the 107 HCC cases that were recruited, HBsAg testing was performed for 106 (99.1%). The HBsAg status for one male subject was not determined because of the unavailability of serum/plasma to perform the test. Overall, seropositivity for HBsAg was seen in 68/106 (64.1%, 95% CI: 59-77) of the HCC cases. The results of HBsAg reactivity according to gender are shown in Table 4.8.

Table 4.8. Frequency of HBsAg seropositivity according to gender among HCC cases

Gender	**HBsAg positive, n (%)**
Male, n (%)*	55/82 (67%)
Female, n (%)	13/24 (54%)

Although there was a higher prevalence of HBsAg positivity among male cases (67%) compared to females (54%), the difference in proportions was not statistically significant with p=0.33.

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4.1.6.2 Prevalence of anti-HBc

Antibodies to HBV core antigen are indicative of exposure to HBV infection regardless of outcome. The prevalence of anti-HBc among HCC cases was 85/103 indicating that 83% (95% CI: 76-90) had been exposed to HBV. Four cases were not tested due to insufficient serum specimen. Among the HBsAg negative cases, 17/34 (50%, 95% CI: 33-67) were positive for anti-HBc. The prevalence of exposure to HBV among male cases was 68/81 (84%, 95% CI: 76-92) compared to 17/22 (77%, 95% CI: 54-95) among female cases, p= 0.29.

4.1.6.3 Prevalence of HBeAg

The overall prevalence of HBeAg among the HBsAg positive cases was 20/65 (31%, 95% CI: 20-42). HBeAg status was not determined in three cases because of insufficient serum. HBeAg which is considered a marker of higher HBV infectivity occurred more frequently among females with 5/11 (45%, 95% CI: 15-74) compared to male HCC cases of whom 15/54 (28%, 95% CI: 14-40) were HBeAg positive, p=0.29.

4.1.6.4 Prevalence of anti-HBe

From the 68 HBsAg positive cases, the anti-HBe status was determined in 64, of whom 45/64 (70%) were anti-HBe seropositive. There was no significant difference in carriage of anti-HBe according to gender with 8/11 (72%) of HBsAg-positive females compared to 37/53 (70%) of males, p=1.00.

4.1.6.5 Effect of HBsAg status and gender on age at presentation

Overall, there was a trend towards a younger age at HCC diagnosis in the HBsAg positive cases compared to the HBsAg negative individuals with mean ages 44.1±13.9 years and 48.5±17.1 years, respectively; p=0.15. The most frequent age range at HCC diagnosis among HBsAg positive cases was 30-35 years, 48% of HBV-related HCC cases occurred by the age of 40 years and 72% by the age of 50 years. There were no statistically significant differences in the age between the genders according to HBsAg status, p =0.44. Results of the effect of gender and HBsAg status on age at HCC diagnosis are reflected in Figure 4.2.

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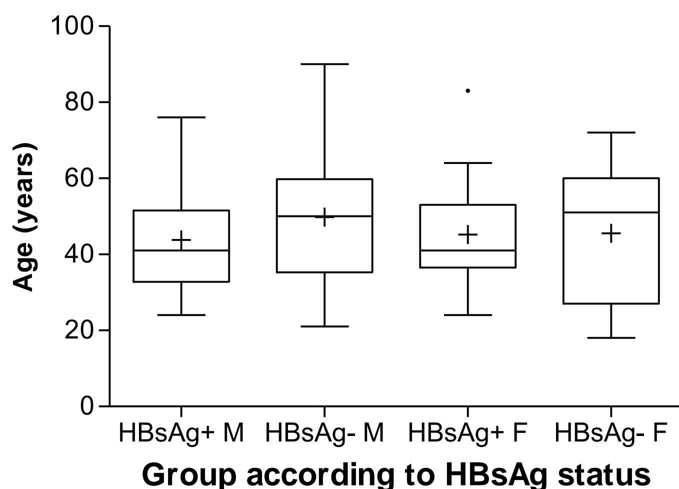

Figure 4.2 Age at HCC diagnosis against gender and HBsAg status. The middle line represents the median; the box represents the IQR; the whiskers are representative of the non-outlier range and the mean age is indicated by the + sign. The mean age of the HBsAg negative males (HBsAg- M) was 50.1±16.9 years compared to 43.8±13.4 years among the HBsAg positive males (HBsAg+ M). The mean age for HBsAg positive females (HBsAg+ F) was 45.2±15.6 years compared to 45.6±18.8 in those that were HBsAg negative (HBsAg- F), p=0.4. There was one outlier value among the HBsAg+ F group.

4.1.7 Prevalence of HIV infection among HCC cases

HIV testing was performed on 100 HCC cases. HIV status could not be determined in seven subjects due to non-availability of specimen. Overall, the prevalence of HIV infection among all the tested HCC cases was 22/100 (22%, 95% CI: 14-30). The relative risk of being female and HIV positive was twice as likely as that of being HIV positive male with 36% (95% CI: 16-56) of HCC females being HIV-infected compared to only 18% (95% CI: 2-34), p=0.083. The breakdown in prevalence of HIV among the HCC patients according to HIV status is shown in Table 4.9.

Table 4.9 Frequency of HIV infection according to gender among HCC cases

Gender	**HIV-infected**	**HIV-uninfected**
Male, n (%)	14 (18%)	64 (72%)
Female, n (%)	8 (36%)	14 (64%)

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4.1.7.1 CD4+ T cell count and therapy

Among the 22 HIV-infected HCC cases, 18 (82%) had data available on the last CD4+ T cell count at time of HCC diagnosis. The median CD4 cell count was 293 cells/µL (IQR: 200-602). HAART administration was reported for 14 cases. The periods between HAART duration and HCC diagnosis ranged from 2 weeks to more than 10 years. Of the 14 cases to have reported HAART, 9 were on a regimen of tenofovir, lamivudine and EFV while one was taking lamivudine, stavudine and nevirapine. The HAART regimen for four cases was not provided. Only 2/14 of the HIV-infected patients on therapy were HBsAg negative.

4.1.7.2 Effect of HIV and gender on age at presentation

HIV-infected cases had a mean age of 39.7 years (95% CI: 36.0-43.5). These cases were younger than the HIV-uninfected HCC cases whose mean was 46.4 years (95% CI: 42.8-50.0) and there was a trend toward a significant difference, p=0.07. The mean age of HIV-infected female HCC cases of 35.8 years (95% CI: 34.1-40.0) was 13 years (95% CI: -27 - 1.9) younger compared to HIV-uninfected females that had a mean age of 48.5 years (95% CI: 37.4-59.6), p=0.08. HIV-infected male HCC cases were only slightly younger with mean age 42.0 years (95% CI: 36.6-47.4) compared to HIV-uninfected males whose mean age was 45.9 years (95% CI: 42.2-49.8), p=0.4. These results are shown in graphic form on Figure 4.3. There was no statistically significant difference in age at presentation according to gender and HIV status, p=0.2.

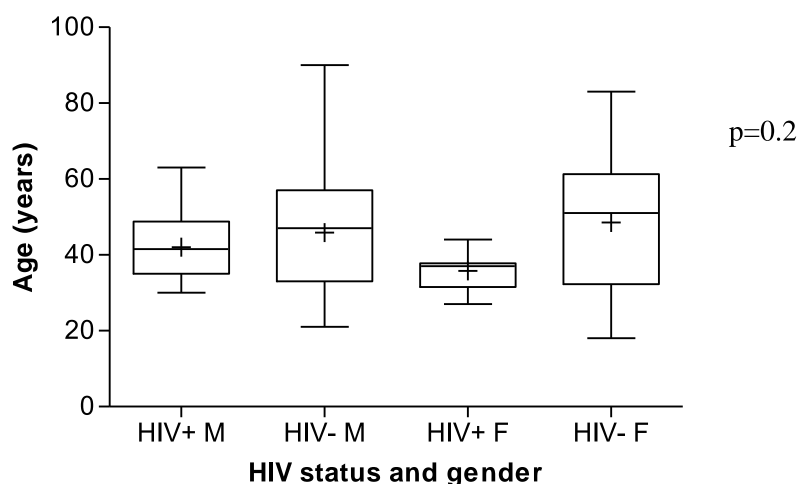

Figure 4.3 Age at HCC diagnosis against HIV status according to gender and HIV infection. The plot shows the mean age at HCC diagnosis grouped according to HIV status and gender. The middle line represents the median; the box represents the IQR; the whiskers are representative of the non-outlier range and the mean age is indicated by the + sign. HIV+ M represents HIV-infected males; HIV- M shows the HIV-uninfected males while HIV+ F represents HIV-infected females and HIV- F shows the HIV-uninfected HCC females.

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4.1.8 Co-infection with HBV and HIV infection among HCC subjects

Of the 22 HIV-infected HCC subjects, 18 (82%) were also infected with HBV as defined by seropositivity for HBsAg. Of the four HIV-infected, HBsAg negative cases, 2 (50%) were anti-HBc positive signifying exposure to HBV. Of the 18 HBV/HIV co-infected subjects, 12 (67%) were male compared to 41/47 (87%) among those that were HBsAg positive only, p=0.01. The mean age of the HBV/HIV co-infected HCC subjects was 39.8 years (95% CI: 31.4-40.0) compared to 45.4 years (95% CI: 40.9-49.8) in the HBV-mono-infected subjects, p=0.30. Table 4.10 summarises differences between HBV/HIV co-infected and HBV mono-infected HCC subjects.

Table 4.10 Comparison of demographic and virologic factors between HIV-infected and HIV-uninfected

Characteristic	HBV/HIV co-infected (n=18)	HBV mono-infected (n=47)	p value
Age in years, Mean (95% CI)	39.8 (35.6-44.0)	45.4 (40.9-49.8)	0.30
Male gender, n (%)	12 (67%)	41 (87%)	0.01
HBeAg positivity	10/17 (59%)	9/46 (20%)	0.005
AFP >400	11/18 (61%)	35/44 (80%)	0.20

Among the female HCC cases, the 6 HBV/HIV co-infected subjects had a mean age of 36.8 years (95% CI: 32.2-41.5) compared to 50.5 years (95% CI: 30.2-70.8) in the HBV mono-infected cases. The mean difference in the average age between the HBV/HIV co-infected females compared to the HBV mono-infected females was 13.7 years (95% CI: -32 to 4.4) and this had a trend toward significant difference, p=0.09. Co-infected males had a mean age of 41.3 years (95% CI: 35.1-47.4) compared to a mean age of 45.5 years (95% CI: 40.5-50.5) among the HBV mono-infected males. The difference in mean age among the males (according to HBsAg and HIV status) of 4.2 years (95% CI: -12 to 5.7) was not statistically significant, p=0.7. A graphical representation in the age at diagnosis according to HBV and HIV infection status is shown in Figure 4.4.

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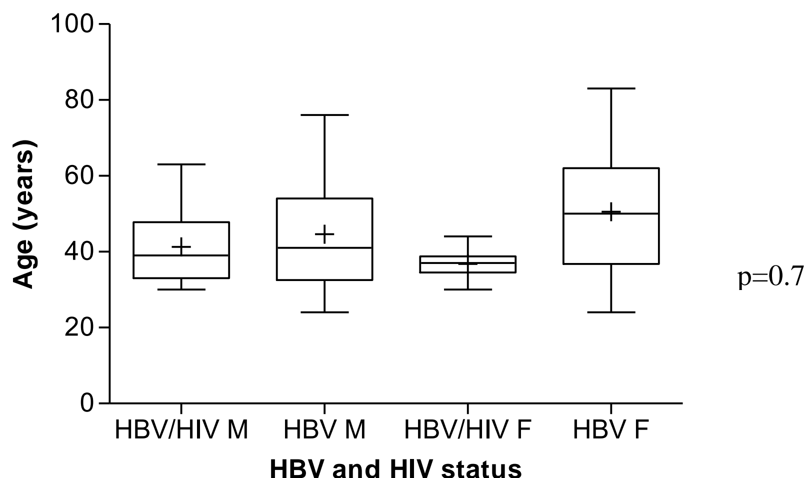

Figure 4.4 HBV and HIV infection status and gender. No significant differences in age at presentation of HCC stratified according to HBsAg, HIV and gender were detected. The middle line represents the median; the box represents the IQR; the whiskers are representative of the non-outlier range and the mean age is indicated by the + sign. HBV/HIV M represents the HBV/HIV co-infected males; HBV M represents the HBV mono-infected males; HBV/HIV F are the HBV/HIV females and HBV F shows the HBV mono-infected females.

4.1.9 Molecular characteristics of HBV in HCC patients

4.1.9.1 HBV Genotypes

HBV genotypes were determined from sequencing of the overlapping surface/polymerase gene. Sequencing was attempted for all samples with detectable HBV DNA. Genotypes were determined for 43 subjects, of whom 34 (79%) were infected with HBV genotype A, 7 (16%) had HBV genotype D and 2 (5%) carried HBV genotype E. HBV/HIV co-infected cases were infected with only genotype A1. Seven cases infected with HBV genotype D and two with genotype E were only seen in the HCC cases that were infected with HBV alone. Sub genotypes were obtained for sequences of genotypes A and D from the Max Planck Institute for Informatics web-based geno2pheno tool. Genotype A sequences were distributed as 33 subgenotype A1 (9 from co-infected cases and 24 that had HBV mono-infection) and one sub genotype A2 (from a mono-infected case). The genotype D sequences were distributed as five subgenotype D3; a single D2 and; one D4. There are no sub genotypes described in the literature for HBV genotype E. The distribution of HBV genotypes according to whether cases were infected or not infected with HIV is shown in Figure 4.5.

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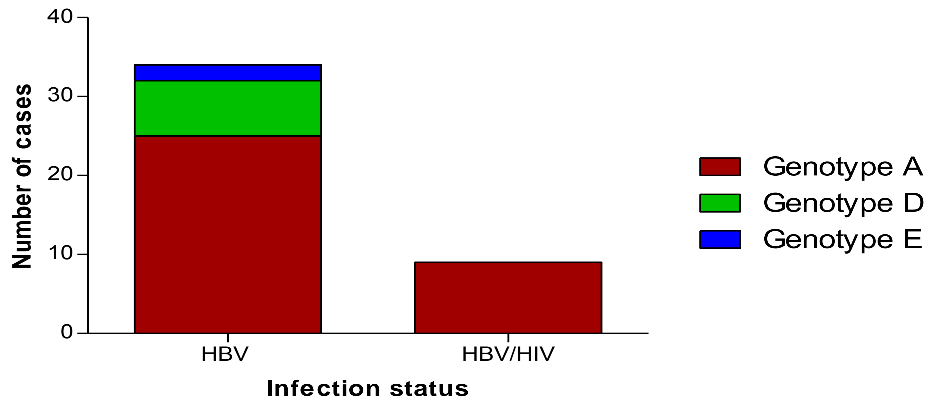

Figure 4.5 HBV genotypes according to HIV infection status among HCC cases. HCC cases with HIV co-infection were infected by only HBV genotype A while genotypes D and E were also detected among HCC cases with hepatitis B infection alone.

4.1.9.2 Phylogeny of HCC-derived sequences

The reverse-transcriptase region of HCC cases was aligned with other GenBank-deposited sequences. The sequences clustered closely to other sequences of similar genotypes. There was no monophyletic clustering of sequences according to HIV status. A phylogenetic tree of the HBV sequences from HCC cases is shown in Figure 4.6.

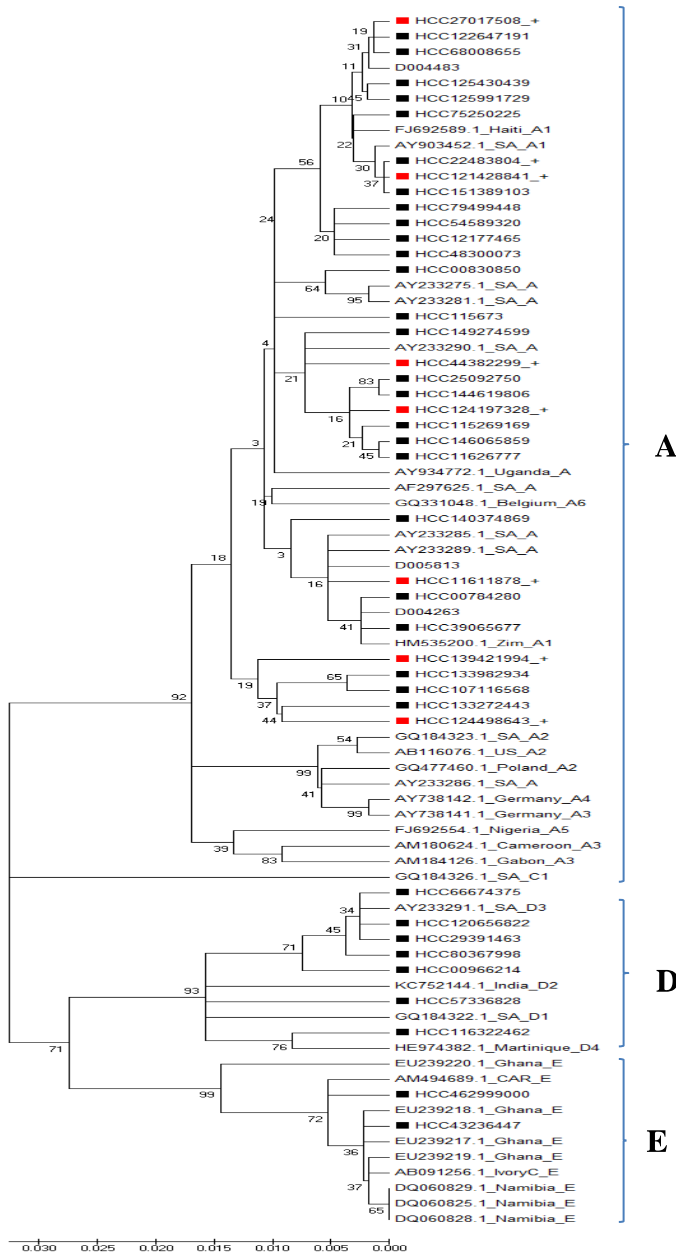

Figure 4.6 Evolutionary relationships of HBV sequences from HCC cases. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.62396497 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. The analysis involved 78 nucleotide sequences. Sequences from HIV-infected HCC cases are annotated with a red square while those from HIV-uninfected are shown with a black square. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 546 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

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4.1.9.3 Effect of HBV genotypes on age at presentation of HCC

HCC cases infected with HBV genotype A had a median age of 36 years (IQR: 31-48) compared to 53 years (IQR: 50-58) in those infected with genotype D. The difference in the median ages was statistically significant, p=0.008. The analysis did not include the two cases infected with genotype E because age at diagnosis was available for a single case only with the second case having incomplete data on the age at diagnosis.

4.1.9.4 HBV reverse transcriptase mutations

Mutations in the YMDD region of the reverse transcriptase gene of HBV are associated with development of resistance to NRTIs that are used for therapy. Drug resistance mutations were observed in only 3/43 (7%) subjects, all of whom were infected with HBV genotype A. The most common mutation observed (seen in 3/3 patients with drug-resistance mutations) which is associated with development of resistance was on codon 80 where the leucine (L) was replaced with either isoleucine (I) or valine (V). The L80I/V mutation is a compensatory mutation associated with resistance to lamivudine and telbivudine. In 2/3 cases, L80I/V occurred together with L180M and M204V/I mutations that are also associated with resistance to lamivudine, telbivudine and entecavir. A single subject had the L80I substitution as the solitary drug-resistance associated mutation.

4.1.9.5 HBV surface antigen mutations

Because of the overlapping nature of the reading frames of the RT region and the small HBsAg gene, mutations were also investigated in the latter. All except three samples had at least one mutation in the surface antigen region. The majority of surface antigen mutations occurred between amino acid residues 1-40; 111-129 and 175-205. Three cases were infected by a variant with a stop codon mutation at amino acid L216* while a single subject had a W182* mutation. Interestingly, the chromatograms revealed mixed virus populations in the four samples with stop codon mutation as there was a mixture of wildtype and mutant virus. A variety of mutations that have been described to cause detection and vaccine escape by the Max-Planck-Institute's geno2pheno web-based genotyping tool were detected. These were seen in 14/43 (33%) samples. The most common change observed was the P120T substitution that was found in six of the fourteen samples with escape mutations. Table 4.11 shows the list of escape mutations that were observed together with other co-occurring substitutions in the surface antigen region and the genotype.

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Table 4.11 List of escape mutations in the surface antigen region of HCC cases.

HBsAg mutations	Escape mutations	Genotype
F41FS, S45A, L94LS, L109ILM, G130DGNS, A166AG, A194V, I208T	109I, 130D, 130N	A1
S45P, P127PR, V177A, F220L, C221CG, V224A	127R	A1
F8FL, F20FS, S45PS, L49R, G112GR, T118AT, P120PT, G130DGNS, V184AV, A194AV, S204NS,	120T, 130D, 130N	A1
S45A, L49CR, I68IT, M133MT, V190AV, S204N,	133T	A1
S143LS	143L	E
Y100CY, T126NT, M133IM, Y161FY, S193LS,	100C, 126N, 133I	A1
S45A, T118A, P120T, P211HP	120T	A1
R24K, S45P, P120T, Y206C,	120T	A1
L42R, P120T, T127P, G159A, S207T, S210R, V224A	120T	D3
S45P, P120T, K122R	120T	A1
L21LS, S45AS, L49HL, L109IL, Y161F,	109I	A1
F20FS, L21LW, Q51QR, R79HR, T127P, T131NT, M133MT, Y134NY, F158FL, E164DE, S207RS, L213IL, L216*L	131N, 133T, 134N	D3
P120PT, T189IT,	120PT	A1
S45P, S132FS, W182*W, S204NS,	132F	A1

4.1.9.6 Precore mutations

Precore stop codon mutations were observed in 10/33 (30%) patients' plasma samples. The most common precore mutation seen was the W28* mutation that was in eight samples. Seven of eight of the HBV strains with the W28* mutation were genotype D, the eighth was genotype E. Two of the ten patients were infected by an HBV strain with the Q2* mutation. The two samples with the Q2* mutation were genotype A.

In addition, other mutations that were not stop codon mutations were seen in five patients. The most frequent of these mutations were the S13T and G29D which were seen in three patients each. The distribution of these mutations is shown in Table 4.12. All patients with the precore stop codon mutations were HIV uninfected. The patients with S13T alone and S13T and V17F were HIV-infected.

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Table 4.12 Combination of precore mutations and their occurrence in HCC patients

Combination of mutations	Number of cases
Q2* alone	2
I9V + G29D	1
S13T alone	1
S13T + V17F	1
S13T + G29D	1
W28*	7
W28* + G25E +G29D	1

4.1.9.7 BCP mutations

Sequencing of the BCP region was successful in 31 HBV-infected HCC subjects. The region covering nucleotide positions 1700-1834 of the HBV genome were examined. Much of the region was conserved except the positions 1703, 1719, 1753, 1757, 1762, 1764, 1773, 1809 and 1812 as shown in Figure 4.7.

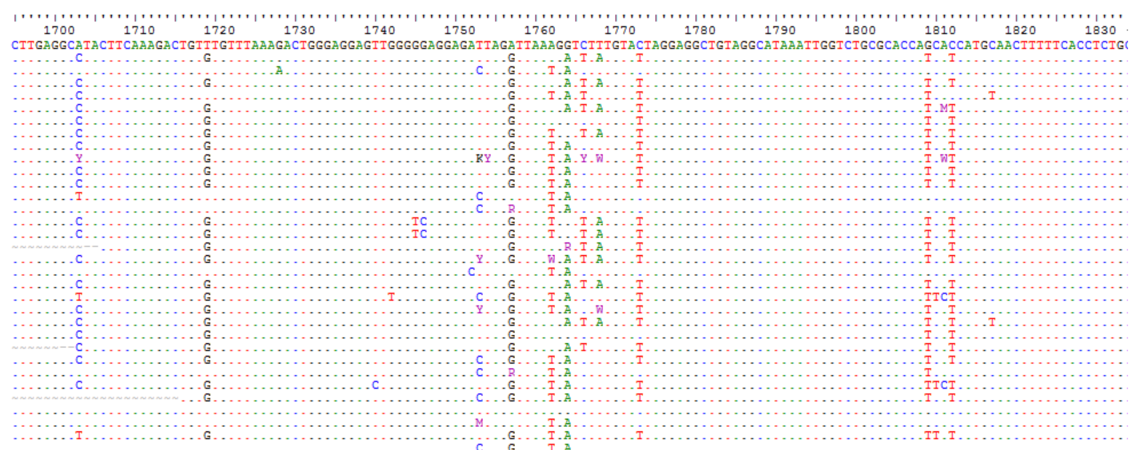

Figure 4.7 Nucleotide alignment showing the variable regions of the BCP. Dots show the conserved sites while the nucleotide letters show the nucleotide substitutions or the polymorphisms.

The A1762T, G1764A double BCP mutation was observed in 21/33 (64%) of the sequenced viruses. The 1762T mutation without 1764A was seen in three cases while the 1764A alone without the 1762T change was observed in six cases. The patterns and frequency of mutations in the BCP region together with the frequency of HIV infection are shown in Table 4.13.

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Table 4.13 Frequency of mutation patterns in the BCP region and HIV infection

BCP mutation pattern	Frequency	Number of HIV infected (%)
T1753C + A1762T + G1764A	12	2 (17%)
A1762T + G1764A	9	1 (1%)
A1762T alone	3	2 (67%)
G1764A alone	6	2 (33%)
None	3	0

With regard to the effect of the BCP mutations on the age at presentation with HCC, cases with the T1753C + A1762T + G1764A mutation pattern presented at median age of 53 years (IQR:50-60) compared to 38 years (IQR: 32-53) among those with A1762T + G1764A, 33 years (IQR: 32-34) among those with A1762T mutation alone, 36 years (IQR: 32-45) of those with the G1764A mutation alone and 37 years (IQR: 29-45) among the HBV-infected HCC cases with no BCP mutations, p=0.07.

4.1.9.8 Mutations in the X region of HBV among HCC cases

The BCP region overlaps with the HBX region and as a result mutations in the former also result in amino acid changes in the latter. The mutations T1753C, A1762T, G1764A and T1768A in the BCP result in the corresponding amino acid changes I127T, K130M, V131I and F132Y in the X region. As a result, there were 12 cases with the triple mutation I127T, K130M and V131I. Six cases had the K130M and V131I double mutation. The F132Y mutation was seen in 12/31(40%) of the cases and it occurred in combination with the K130M or the V131I but never alone.

4.1.10 Prevalence of HCV infection among HCC cases

4.1.10.1 Prevalence of anti-HCV

Anti-HCV serology was performed on 101 subjects of whom 10 were sero-reactive, resulting in a seroprevalence of 9.9% (95% CI: 4.1-15.7). Testing for anti-HCV was not performed on 6 subjects because of insufficient sample. The mean age of the anti-HCV positive subjects was 57.5 years (95% CI: 45.8-69.2) and was significantly higher than in the anti-HCV negative cases which had a mean age of 44.6 years (95% CI: 41.8-48.0), p=0.01. HCV antibodies were predominantly detected from male subjects with 9/10 (90%) of anti-HCV positive patients being male.

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4.1.10.2 Detection of HCV RNA in anti-HCV positive HCC cases

HCV RNA was tested in 8/10 of the anti-HCV positive samples. Among these, 5/8 (63%) had detectable HCV RNA with a median of 6.27 log10 copies/ml (IQR: 5.67 log10–6.42 log10). Samples from 4/5 HCC cases whose plasma had detectable HCV RNA were successfully sequenced - two cases were infected with HCV genotype 5a while one case each had HCV genotype 1a and genotype 1b.

4.1.10.3 Clinical presentation of HCV infected subjects

Among the 10 anti-HCV positive HCC cases, 5/10(50%) were also co-infected with HBV as evidenced by simultaneous detection of HBsAg. In addition HCV infected subjects presented with advanced malignancy demonstrated by imaging examinations revealing multiple lesions or a single lesion greater than 5 cm within the liver in 7/10 (70%) of the subjects. Only 2/10 of the anti-HCV positive HCC cases had a reported diagnosis of cirrhosis prior to presentation with HCC. The occurrence of HIV and a summary of the clinical findings among the 10 anti-HCV positive subjects are shown in Table 4.14.

Table 4.14 Co-occurrence of HBV and HIV and demographic characteristics of anti-HCV positive subjects

Gender	Race	Age	HIV	HBsAg	Log HCV RNA	HCV Genotype	Imaging findings
Male	Black	90	Negative	Negative	6.42	1B	Multiple liver lesions
Male	Black	47	Negative	Negative	5.67	1A	Single lesion ≤5cm
Male	Black	36	Positive	Positive	Insuff. sample	Insuff. sample	Single liver lesion <2cm
Male	Mixed	36	Negative	Positive	Insuff. sample	Insuff. sample	Multiple liver lesions
Female	Black	64	Not tested*	Positive	4.49	not amplified	Single liver lesion <2cm
Male	Black	71	Negative	Positive	6.33	5A	Multiple liver lesions
Male	Black	62	Not tested*	Negative	Not tested	Not tested	Multiple liver lesions
Male	Mixed	62	Negative	Positive	Insuff. sample	Insuff. sample	Single lesion >5cm
Male	Mixed	57	Negative	Negative	6.6	5A	Multiple liver lesions
Male	Mixed	50	Negative	Negative	Not tested	Not tested	Multiple liver lesions

*not tested due to non-availability of sample; Insuff -insufficient

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4.2 Liver Fibrosis and Immune Markers Study

4.2.1 Study population

A total of 169 participants were recruited into the study. The distribution of the subjects was: 46 HBV/HIV co-infected; 47 HBV mono-infected; 39 HIV mono-infected and; 37 HBV/HIV uninfected controls. The demographic and anthropometric characteristics of these subjects are shown in Table 4.15. The prevalence of alcohol consumption and current or past herbal medicine use is also indicated.

Table 4.15 Demographic characteristics of immune study participants according to group

	HBV/HIV (n=46)	**HBV (n=47)**	**HIV (n=39)**	**Controls (n=37)**	**p**
Age (years)*	38.5 ± 8.2	37.9 ± 12.3	37.9 ± 9.7	43.8 ± 11.4	0.04
Males (%)	22 (47%)	22 (47%)	16 (39%)	25 (41%)	0.1
Race, *n* (%)					
African	31 (67%)	18 (38%)	17 (44%)	5(14%)	
Mixed	14 (32%)	25 (53%)	22 (56%)	30 (81%)	0.0001
Caucasian	1 (1%)	3 (7%)	-	2 (5%)	
Asian	-	1 (2%)	-	-	
Body mass index	24.3 ± 5.1	28.0 ± 9.0	24.6 ± 3.5	27.0 ± 5.9	0.23
Alcohol consumption	8/46 (17%)	9/47 (19%)	11/39 (28%)	14/37 (38%)	0.12
Herbal medicine use (current/past)	1/46 (2%)	5/47 (11%)	1/39 (3%)	5/37 (14%)	0.11

*Mean ± standard deviation (SD)

4.2.2 Immunologic and virologic characteristics

All HIV infected subjects (co-infected and HIV mono-infected) were on therapy for at least 3 months, median 35 months (IQR: 13-61). All co-infected subjects had a HAART regimen that included tenofovir plus emtricitabine or lamivudine. HIV mono-infected subjects were on similar therapy except that four subjects were on zidovudine and one on stavudine in combination with lamivudine and ritonavir-boosted lopinavir. The median duration of antiretroviral therapy for the co-infected cases was 36 months (IQR: 23-63) with the participants that had the shortest duration of therapy having received ARVs for 3 months and the longest for 174 months. In comparison, HIV mono-infected subjects had a median duration of 36 months (IQR: 12-63), with range of HAART duration being 3-156 months. Co-infected cases had median CD4 T lymphocyte count of 328 cells/µl (IQR: 242-562) that

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was significantly lower than that of the HIV mono-infected participants whose median was 528 cells/µl (IQR: 367-567), p=0.04. HIV mono-infected participants had a significantly elevated median CD4/CD8 ratio of 0.7 (IQR: 0.6-1.0) compared to 0.5 (0.3-0.7) among the HBV/HIV co-infected subjects, p=0.0003. Not all of the HBV mono-infected subjects were on therapy with only 12/47 (25%) receiving anti-HBV therapy. Treatment duration in this group varied widely from just one week to more than 24 months. Of the 12 HBV mono-infected participants on therapy, 11 (92%) were receiving tenofovir. The outstanding participant had been administered lamivudine. The virological characteristics of the study subjects are shown in Table 4.16.

Table 4.16 Immunologic and virologic characteristics of study participants

	HBV/HIV n=46*	**HBV, n=47***	**HIV, n=39**	**Control, n=37**	**p**
HAART, months Median (IQR)	36 (23– 63)	n/a	36 (12- 63)	n/a	ns
CD4 count, (cells/µL) Median (IQR)	328 (242- 562)	922 (647- 1297)	528 (367- 657)	1031 (790- 1215)	<0.0001
CD4/CD8 ratio Median (IQR)	0.5 (0.3-0.7)	1.5 (1.1- 2.1)	0.7 (0.6-1.0)	1.6 (1.3- 2.1)	<0.0001
Detectable plasma HIV viral load	10/36 (27.8%)	n/a	6/33 (18.1%)	n/a	0.4
HIV viral load >1000 copies/ml	6/36 (17 %)	n/a	3/33 (9.1%)	n/a	0.48
Detectable plasma HBV DNA	22/45 (49%)	32/44 (73%)	n/a	n/a	0.03
Plasma HBV DNA >2000 IU/ml	12/45 (26%)	15/44 (32%)	n/a	n/a	0.5
HBeAg positive, (%)	13/46 (28%)	6/46 (13%)	n/a	n/a	0.12

*Not all participants were tested due to insufficient specimen. The effect of therapy in the HBV group was also evaluated and the results are described in section 2.2.2.1.

Plasma HIV RNA was detected in 10/36 (27.8%) of the co-infected subjects compared to 6/33 (18%) among those that were HIV mono-infected, p=0.4. The proportion of co-infected subjects with HIV viral load greater than 1000 copies/ml was 6/36 (17%) compared to HIV 3/33 (9%) among the HIV mono-infected, p=0.48.

There was a higher proportion of HBV mono-infected subjects [32/44 (73%)] with detectable HBV DNA compared to those that were co-infected [22/45 (49%)], p=0.03. Results showed statistically significant negative correlation between HBV DNA and duration of antiretroviral

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therapy within the HBV/HIV group with Spearman rho=-0.53, $p<0.05$. The proportion of HBV mono-infected subject with HBV DNA viral load greater than 2000 IU/ml was 15/42 (32%) compared to 12/45 (26%) among the co-infected subjects, p=0.49. HBeAg, a marker of high HBV replication and infectivity, was more frequently detected among the co-infected participants with 13/46 (28%) being HBeAg positive compared to 6/46 (13%) among HBV mono-infected participants, p=0.12.

Among the co-infected subjects, 4/29 (13.8%) had detectable HBV DNA with undetectable HIV RNA. Also among the HBV/HIV group, 3/29 (10.3%) had detectable HIV viral loads with undetectable HBV DNA whilst co-detection of HIV RNA and HBV DNA was seen in 5/29 (17.2%) of the co-infected participants.

4.2.2.1 Comparison between treated and untreated HBV subjects

Since not all HBV mono-infected subjects were on therapy, an analysis to compare the treated and untreated individuals was performed. The treatment duration ranged from one week to 36 months. Among the untreated HBV mono-infected subjects, 3/34 (9%) were positive for HBeAg compared to 3/12 (25%) among the treated HBV mono-infected participants, p=0.17. Detectable HBV DNA was observed in 24/32 (75%) of the untreated subjects compared to 8/12 (67%) of the treated subjects, p=0.7. Two untreated participants could not be tested for HBV DNA because of unavailability of sample. HBV viral loads greater than 2000 IU/ml were recorded in 11/32 (34%) of the untreated subjects compared to 4/12 (33%), p=1.00.

4.2.3 Prevalence of liver fibrosis using non-invasive markers

The prevalence of liver fibrosis was assessed using the Fibroscan as well as the APRI and FIB-4 scores. Sub-group analysis was also performed among the HBV group to detect any differences in non-invasive markers of liver fibrosis between the treated and untreated patients. The results of the HBV sub-group analysis are reported in section 4.2.3.6.

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4.2.3.1 Relationship of Fibroscan and participants' BMI values

Of the 148 Fibroscan measurements that were performed, 135 (91%) had success rates ≥60% and IQR/median <30%. These were considered as the valid Fibroscan measurements that are included in the analysis. The control group had the highest number of participants with valid readings, although it also had the lowest number of participants that were eligible for a Fibroscan examination based on BMI. There was a weak and inverse relationship between success rate of Fibroscan measurements and the BMI with a slope of -0.14 ± 0.035, p=0.0001. A representation of the relationship between success rate of Fibroscan measurements and BMI is shown in Figure 4.8.

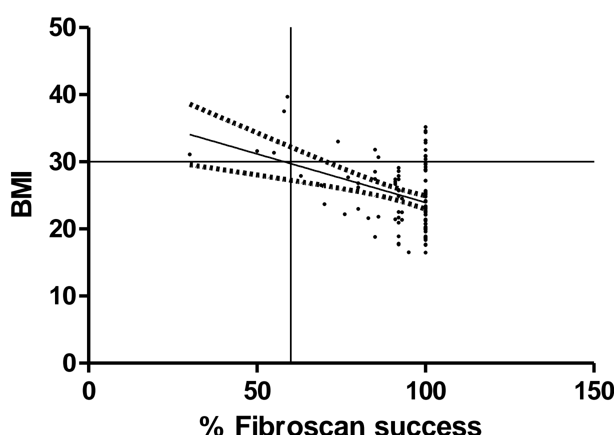

Figure 4.8 Correlation of Fibroscan success rate against the BMI. The plot shows 95% confidence interval of the slope and also the cut-off point (60%) for a success rate that was considered for a valid result on the x axis. The recommended BMI cut-off value of 30 on which the Fibroscan may be performed using the medium probe is indicated on the y axis. The figure shows that the majority of participants had a success rate of 100%, even among some with a BMI greater than 30.

4.2.3.2 Association of gender and Fibroscan scores

Differences in Fibroscan scores between males and females were calculated for the entire Immune Study cohort. The median of Fibroscan scores among males of 6.3 kPa (IQR: 5.0-8.7) was significantly higher compared to that in females whose median was 5.2 kPa (IQR: 4.7 -7.1), p=0.003. When reanalysed according to group; the co-infected and controls showed males having significantly higher median stiffness scores, p=0.03 and p=0.049, respectively. The HBV mono-infected and HIV mono-infected group showed no significant difference in Fibroscan scores according to gender with p=0.1 and p=0.41, respectively. The median and IQR of Fibroscan scores according to gender are shown in Table 4.17.

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Table 4.17 Fibroscan scores and gender showing medians and IQR in parenthesis (kPa)

	Males	Females	p
HBV/HIV	7.4 (5.6-8.7)	5.1 (4.7-7.3)	0.03
HBV	7.8 (6.0-14.2)	5.9 (4.9-8.1)	0.1
HIV	4.8 (4.3-6.1)	4.7 (3.8-5.2)	0.4
Controls	6.2 (5.4-8.1)	5.3 (4.5-6.0)	0.048

4.2.3.3 Distribution of Fibroscan scores

There was a statistically significant difference in the medians of the liver stiffness measurements using the Kruskal Wallis (K-W) test; p=0.0001. The HBV mono-infected group had the highest median of 6.9 (5.5 – 8.8), followed by the co-infected group 6.2 kPa (4.8 – 8.0); control group that had a median of 5.6 kPa (4.6 -6.8) and finally the HIV mono-infected group that had the lowest median of 4.7 kPa (3.9 – 5.9). These results are shown in Table 1-11. Following statistically significant Kruskal-Wallis tests, the Dunn's Multiple Comparison Test was used to detect the groups with the significant differences. There were statistically significant differences in rank sums between the co-infected group and the HIV mono-infected group and also between the HBV mono-infected compared to the HIV mono-infected group. The distribution of Fibroscan values is shown in Figure 4.9.

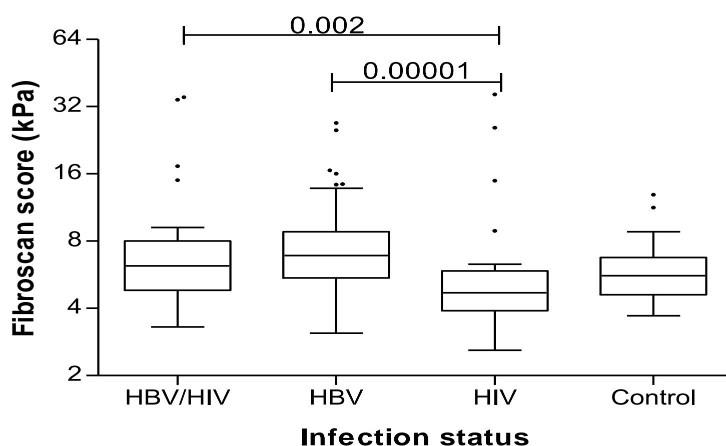

Figure 4.9 Box-and-whisker plot of Fibroscan scores grouped according to infection status. The middle line represents the median; the box represents the IQR while the whiskers correspond to the non-outlier range. The dots beyond the whiskers show outlier and extreme values. The figure shows that HBV mono-infected subjects had a statistically significant higher median of Fibroscan values compared to those with HIV alone (p=0.00001). Similarly the HBV/HIV co-infected individuals had statistically significant higher Fibroscan scores compared to those with HIV alone.

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4.2.3.4 Distribution of APRI scores

The median APRI score of the HBV mono-infected group was highest at 0.34 (0.21-0.66), followed by the co-infected and HIV mono-infected groups, both with median APRI scores of 0.32. The control group had the lowest median of the APRI score at 0.21 (0.13-0.42). The differences in APRI scores between the groups were not statistically significant, p=0.08. The distribution of the APRI scores for the four groups is shown graphically in Figure 4.10.

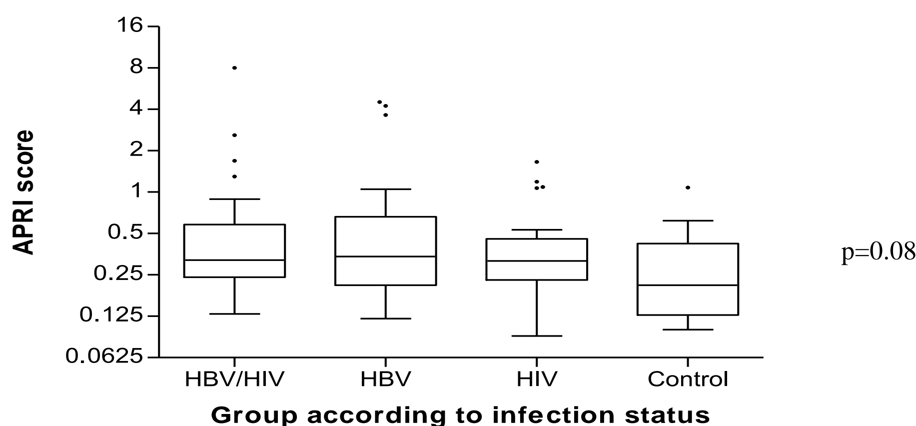

Figure 4.10 Box-and-whisker plot of APRI scores. The middle lines represent the median values while the boxes correspond to the interquartile range. The whiskers show the non-outlier range while the dots show extreme and outlier measurements. There were no statistically significant differences in the APRI score between any of the groups, p=0.8.

4.2.3.5 Distribution of FIB-4 scores

The FIB-4 score showed the co-infected group having the highest median score compared to the other groups. The median FIB-4 score for the co-infected group was 0.96 (0.58-1.30); 0.86 (0.59-1.20) in the HBV mono-infected; 0.77 (0.55-1.30) in the HIV group and 0.63 (0.41-1.00) in the control group. The difference in medians across the groups were not statistically significant, p=0.11. A scatter dot plot of FIB-4 scores is shown in Figure 4.11.

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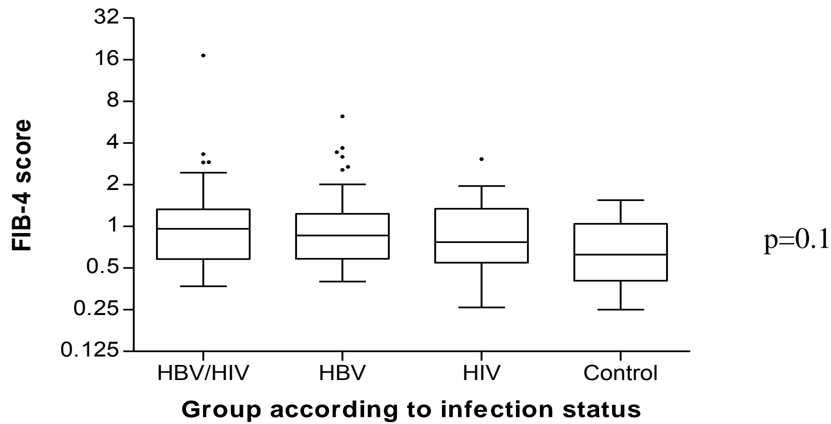

Figure 4.11 Box-and-whisker plot of FIB-4 scores according to group. The middle lines represent the median values; boxes correspond to the 25th and 75th percentiles while the whiskers show the non-outlier range. The dots beyond the whiskers represent the outliers and extreme values. There were no statistically significant differences in the APRI score between any of the groups, p=0.1.

In order to determine the distribution of fibrosis measurements derived from the three different tests, the scores obtained were classified into three strata representing F0-F1, F2-F3 and F4 stages of the Metavir system for fibrosis grading. The different strata were constructed using values that were previously published from elsewhere. The frequency of fibrosis stages according to group determined by the three different tests is shown in Table 1-12. These results are shown in Table 4.18.

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Table 4.18 Summary and distribution of fibrosis results according to group

	HBV/HIV	**HBV**	**HIV**	**Controls**	**p**
Valid Fibroscan readings	36/40 (90%)	38/41 (93%)	32/37 (86%)	29/30 (97%)	0.5
Fibroscan (kPa) <7.0 7.0 - 13 ≥13	n=36 21 (58) 11 (31) 4 (11)	n=38 19 (50) 12 (32) 7 (18)	n=32 28 (88) 1 (3) 3 (9)	n=29 23 (79) 6 (21) 0 (0)	0.002
APRI <0.5 0.5 – 1.5 >1.5	n=43 31 (72) 9 (21) 3 (7)	n=35 25 (71) 7 (20) 3 (9)	n=26 21 (81) 4 (15) 1 (4)	n=22 19 (86) 3 (14) 0 (0)	0.85
FIB-4 <1.30 1.30 – 3.25 >3.25	n=42 31 (74) 9 (21) 2 (5)	n=34 27 (79) 4 (12) 3 (9)	n=26 19 (73) 7 (27) 0	n=22 20 (91) 2 (9) 0	0.32
ALT, U/L*	34 (24-45)	25 (22-47)	31 (21-44)	24 (19-39)	0.32
AST, U/L*	31 (25-46)	27 (23-42)	32 (27-45)	21 (18-32)	0.001
Fibroscan, kPa*	6.2 (4.8 – 8.0)	6.9 (5.5 -8.8)	4.7 (3.9 -5.9)	5.6 (4.6 -6.8)	0.001
APRI*	0.32 (0.24-0.58)	0.34 (0.21-0.66)	0.32 (0.23-0.46)	0.21 (0.13-0.420	0.08
FIB-4*	0.96 (0.58-1.30)	0.86 (0.59-1.20)	0.77 (0.55-1.30)	0.63 (0.41-1.00)	0.11

*Median values and IQRs are shown in parenthesis.

4.2.3.6 Effect of therapy on non-invasive markers of liver fibrosis in the HBV group

Non-invasive markers of liver fibrosis as well as liver enzyme activities of the treated and untreated HBV patients were compared using the Mann-Whitney test. There were no statistically significant differences in any of the markers between the two subgroups. The summary of results is shown in Table 4.19.

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Table 4.19 Differences in liver fibrosis markers between treated and untreated HBV mono-infected subjects.

	Untreated HBV	Treated HBV	p
AST (U/L)	27 (24-37)	31 (28-60)	0.13
ALT (U/L)	24 (21-43)	41 (24-87)	0.11
Fibroscan (kPa)	7.6 (5.7-8.7)	7.2 (5.5-11.8)	1.00
FIB-4	0.88 (0.61-1.25)	0.76 (0.58-1.88)	0.70
APRI	0.34 (0.20-0.52)	0.40 (0.28-0.91)	0.27

Medians and interquartile ranges in parenthesis are shown

4.2.3.7 Effect of alcohol consumption and herbal medicine use on fibrosis scores

The results from this study showed no significant effect of alcohol consumption or herbal medicine use on fibrosis scores. The median (IQR) of median stiffness among those that consumed alcohol was 5.8 kPa (4.7-6.2) compared to 5.9 kPa (4.7-8.0) in non-drinkers, p=0.4. Past or current use of herbal medicine did not seem to have an effect on median stiffness scores. The median (IQR) of current herbal users was 9.2 kPa (4.5-10.4) compared to 5.9 kPa (4.7-7.8) in those that had never used herbal medicine, p=0.6.

4.2.3.8 Correlation between the Fibroscan, APRI, FIB-4 and liver enzymes

Using the Spearman rank test, there was statistically significant correlation between the Fibroscan, liver enzymes (AST and ALT) and the serum-based APRI and FIB-4 for all subjects combined. The Spearman rho value between the Fibroscan and APRI was 0.47 (95% CI 0.30 - 0.61) while that between Fibroscan and FIB-4 was 0.4 (95% CI 0.22 - 0.56). However, there was a stronger correlation between the APRI and FIB-4 with rho=0.88.

The correlations were not all maintained when analyses was performed according to infection status. In the HBV group, there was no statistically significant correlation between the FIB-4 versus Fibroscan and also between the FIB-4 against ALT. In the HBV/HIV group, Fibroscan scores had no statistically significant correlation with AST or ALT. There was also no significant correlation between FIB-4 and ALT. In the HIV group, the Fibroscan had no statistically significant correlation with ALT. There was no statistically significant correlation between Fibroscan against APRI; FIB-4; AST and ALT in the control group. The correlation matrices for all participants and according to group are shown in Table 4.20 A-E

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Table 4.20 Table A shows correlation for all study subjects with statistically significant correlation between all markers. Table B shows correlations between the different markers within the HBV group. Table C is a correlation matrix for the non-invasive markers of liver fibrosis with the HBV/HIV group. Table D and E show the HIV and control groups respectively. Statistically significant correlations at p<0.05 are shown in red font

A	All Groups				
	AST	APRI	Fibroscan	FIB-4	ALT
AST	1.00	0.78	0.27	0.53	0.81
APRI Score	0.78	1.00	0.44	0.76	0.60
Median stiffness	0.27	0.44	1.00	0.39	0.21
FIB-4 SCORE	0.53	0.76	0.39	1.00	0.32
ALT	0.81	0.60	0.21	0.32	1.00

B	HBV group				
	AST	APRI	Fibroscan	FIB-4	ALT
AST	1.00	0.81	0.43	0.49	0.79
APRI Score	0.81	1.00	0.65	0.75	0.59
Median stiffness	0.43	0.65	1.00	0.34	0.43
FIB-4 SCORE	0.49	0.75	0.34	1.00	0.25
ALT	0.79	0.59	0.43	0.25	1.00

C	HBV/HIV Group				
	AST	APRI	Fibroscan	FIB-4	ALT
AST	1.00	0.77	0.20	0.55	0.72
APRI Score	0.77	1.00	0.40	0.85	0.48
Median stiffness	0.20	0.40	1.00	0.36	0.10
FIB-4 SCORE	0.55	0.85	0.36	1.00	0.20
ALT	0.72	0.48	0.10	0.20	1.00

D	HIV Group				
	AST	APRI	Fibroscan	FIB-4	ALT
AST	1.00	0.76	0.48	0.47	0.89
APRI Score	0.76	1.00	0.47	0.79	0.73
Median stiffness	0.48	0.47	1.00	0.51	0.26
FIB-4 SCORE	0.47	0.79	0.51	1.00	0.42
ALT	0.89	0.73	0.26	0.42	1.00

E	Control Group				
	AST	APRI	Fibroscan	FIB-4	ALT
AST	1.00	0.78	0.03	0.57	0.84
APRI Score	0.78	1.00	0.04	0.57	0.67
Median stiffness	0.03	0.04	1.00	0.25	0.05
FIB-4 SCORE	0.57	0.57	0.25	1.00	0.53
ALT	0.84	0.67	0.05	0.53	1.00

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4.2.3.9 Association of Fibroscan score and viraemia

Among the HBV mono-infected subjects, those with detectable HBV DNA had significantly higher median Fibroscan scores (median 7.8 kPa: IQR 6.2-10.1) compared to those without undetectable HBV DNA (median 5.2 kPa, IQR: 4.3-7.4), p=0.03. Among the co-infected subjects, those with detectable HBV DNA had higher (median 6.4 kPa, IQR: 5.1-8.2) but non-statistically significant Fibroscan scores compared to those without DNAaemia (median 5.9 kPa, IQR: 4.8-7.9), p=0.3.

4.2.4 Microbial translocation markers

The hypothesis of the study was that microbial translocation is increased in HIV infection and drives immune activation that will lead to increased liver fibrosis in HBV infected individuals. Microbial translocation was investigated by measuring the serum concentrations of sCD14 and LBP which are released upon recognition of microbial products. As such the two biomarkers serve as proxies of microbial translocation.

4.2.4.1 Plasma sCD14 concentration

The HBV/HIV group had a median of 3.6 µg/ml (IQR: 2.4-6.2) compared to 1.8 µg/ml (IQR: 1.1-2.4) in the HBV group; 2.4 µg/ml (1.8-4.3) in the HIV group and 1.6 (1.2-2.2) in the control group. The plasma sCD14 concentration for the four study groups are also summarised in Table 4.21. Levels of sCD14 were significantly higher in the co-infected group compared to all other groups, p<0.0001. Following the statistically significant result of the Kruskal-Wallis test, post-test was done using the Dunn's Multiple Comparison test to detect the groups that had significant differences. Statistically significant differences were detected between: co-infected compared to the HBV mono-infected (p<0.0001); co-infected against the controls (p<0.00001), HBV mono-infected versus HIV mono-infected (p=0.02) and controls compared to HIV mono-infected (p=0.0007). These results are also shown in the box and whisker plot in Figure 4.13.

Table 4.21 Summary of plasma sCD14 levels in µg/ml

	HBV/HIV	HBV	HIV	Control
n	42	38	38	37
Median (IQR), (µg/ml)	3.6 (2.4-6.2)	1.8 (1.1-2.4);	2.4 (1.8-4.3)	1.6 (1.2-2.2).

There was no significant correlation between sCD14 and HBV DNA load for all participants, rho=0.14; p=0.3. There was also no significant correlation between plasma sCD14 and HIV viral load for all participants, rho= -0.02, p=0.8. Correlation between sCD14 and HIV viral load was stronger but not significant in the co-infected group (rho= -0.21, p=0.22) compared to the HIV group, (rho=-0.1, p=0.6). The sCD14 results showed a statistically significant inverse correlation with HAART duration for the HBV/HIV group but not for the HIV group. The Spearman rho calculated for sCD14 and HAART duration was -0.34, p=0.03 ($p<0.05$) in the HBV/HIV group compared to -0.23 with p=0.16 in those with HIV alone.

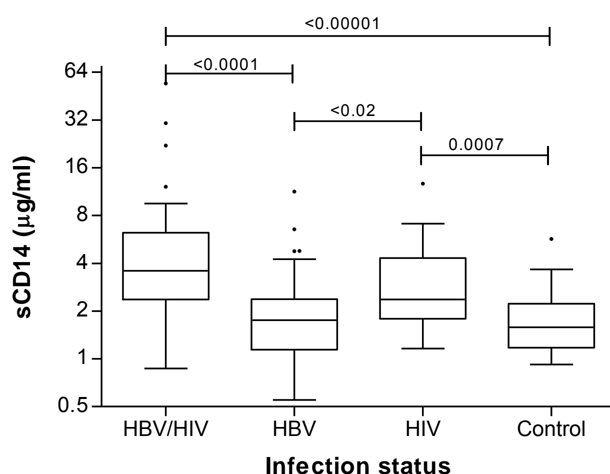

Figure 4.12 Box and whisker plot of CD14 grouped according to infection status. The middle lines show the median while the boxes represent the 25th and 75th percentiles. The whiskers show the non-outlier range, asterix represent the outliers and the solid black squares indicate the extreme values. The y axis showing the sCD14 values is drawn using a logarithmic scale. The medians and interquartile ranges in parenthesis were as follows: Co-infected 3.6 µg/ml (IQR, 2.4-6.2); HBV mono-infected 1.8 µg/ml (1.1-2.4); HIV mono-infected 2.4 µg/ml (1.8-4.3) and control 1.6 (1.2-2.2).

The effect of whether HBV treatment impacts microbial translocation was evaluated by comparing the sCD14 levels between on-treatment against untreated individuals within the HBV group. Levels of sCD14 were higher but not statistically significant in the untreated compared to the treated group with medians and interquartile ranges of 2.6 µg/ml (1.7- 3.6) and 1.6 µg/ml (1.1- 2.2) respectively; p=0.06.

4.2.4.2 Plasma levels of LBP

The results of the quantitative detection of LBP showed a trend toward increased LBP levels in the co-infected group relative to the other groups although the difference was not statistically significant, p=0.06. The medians, 25th- and 75th percentiles are shown in Table

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4.21. Differences in plasma LBP levels between on-treatment and untreated HBV mono-infected subjects were also investigated. There was no difference in the LBP levels with on-treatment subjects having median 10.0 (8.4- 12.5) compared to 13.1 (9.0- 14.7) in those that were untreated, p=0.14.

Table 4.22 Summary of plasma LBP levels (µg/ml)

	Co-infected	**HBV mono**	**HIV mono**	**Control**
n	42	38	38	37
Median (IQR)	12.4 (10.2-15.6)	10.8 (8.4-13.4)	9.9 (7.8-16.0)	9.6 (8.2-13.3)

4.2.4.3 Correlation between LBP and sCD14

Soluble CD14 can only bind to LPS in the presence of LBP. Overall, there was weak but statistically significant positive correlation between sCD14 and LBP with Spearman rho =0.24 (95% CI, 0.08-0.31) and p=0.003. The scatter plot of the LBP and sCD14 is shown in Figure 4.14. There was no correlation between the microbial translocation markers when stratified according to the four groups of subjects. The rho values and their 95% confidence interval were: co-infected 0.2 (-0.12-0.49), HBV mono-infected 0.27 (-0.06-0.55), HIV mono-infected 0.3 (-0.029-0.57) and controls -0.15 (-0.46 - 1.9).

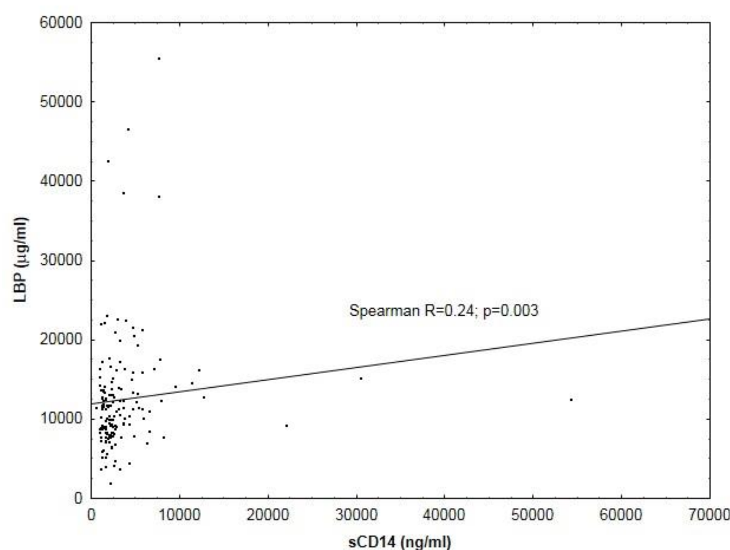

Figure 4.13 Scatter plot of sCD14 against LBP for all subjects. The scatter plot reflects weak but significant positive correlation between plasma sCD14 and LBP, with Spearman rho=0.24, p=0.003. Spearman correlation was used because of the non-parametric distribution of the sCD14 and LBP plasma concentrations.

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4.2.5 Cell-associated markers of activation

CD38 and HLA-DR are markers of immune activation and their increased expression on T lymphocytes in HIV infection is associated with a poor prognosis (Levacher et al., 1992). Differences in the percentage of CD4+ and CD8+ T lymphocytes expressing CD38 and HLA-DR were assessed using 6-colour flow cytometry. Group statistics were expressed used medians and interquartile ranges because the results did not follow a normal distribution pattern. Comparative analyses were carried out using the Kruskal-Wallis test followed by Dunn's test for multi-group comparison where the p statistic was significant.

4.2.5.1 Expression of CD38 and HLA-DR on CD8 and CD4 T lymphocytes

Statistically significant differences were observed for the expression of the following activation marker combinations on CD4+ T-lymphocytes: HLA-DR+/CD38- (single expression), HLA-DR+/CD38+ (dual expression), CD38+/HLA-DR-, HLA-DR+ (total) and CD38+ (total). Statistically significant differences in expression on CD8+ T-lymphocytes were observed for CD38+/HLA-DR+, CD38+/HLA-DR- and total HLA-DR. The p values, median, first and third quartiles of percentage expression of the different activation marker combinations on the respective T-lymphocytes are shown in Table 4.23. Representative plots of CD38 and HLA-DR expression by CD8+ T lymphocytes are shown in Figure 4.15.

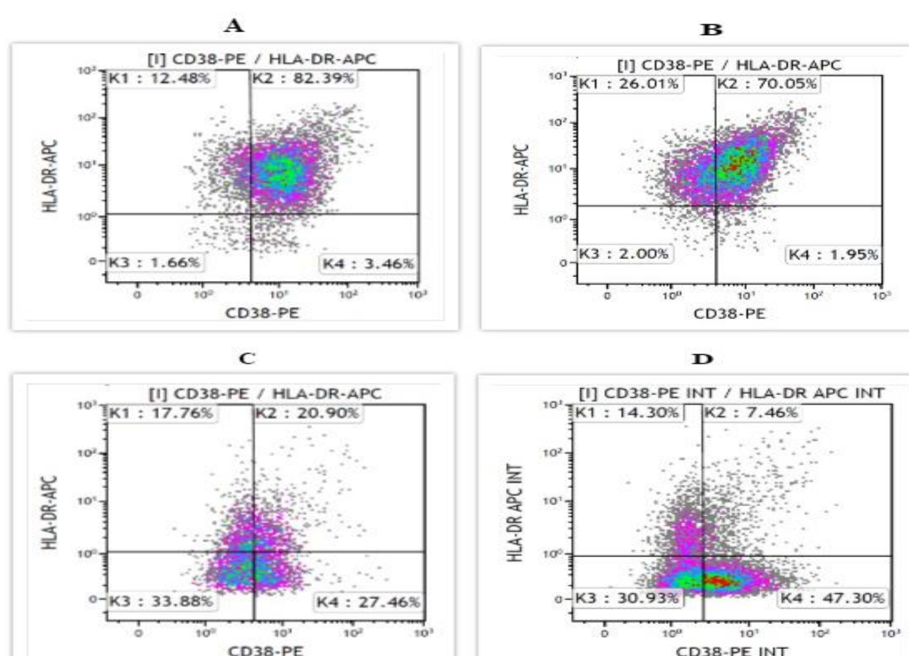

Figure 4.14 Representative density plots of cell-associated activation markers. Percentage co-expression of CD38 and HLA-DR by CD8+ T lymphocytes is shown for an HBV/HIV co-infected participant in A; HIV mono-infected subject in B, HBV-mono-infected subject in C and a control in D. Figure A shows that 82.4% of CD8+ lymphocytes co-expressed CD38 and HLA-DR while there was 70.1% in B; 20.9% in C and 7.5% in D.

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Table 4.23 Expression of activation markers (CD38 and HLA-DR) on CD4+ and CD8+ T lymphocytes.

	Co-infected	HBV Mono	HIV Mono	Control	p
%CD4/CD38-/HLA-DR+	24 (16-33)	13 (9.8-22)	16 (13-22)	12 (8.1-18)	<0.0001
%CD4/CD38+/HLA-DR+	9.9 (6.2-17)	4.7 (3.6-6.3)	7 (4.5-12)	3.5 (2.6-6)	<0.0001
%CD4/HLA-DR+	37 (28-50)	19 (14-27)	25 (21-31)	17 (12-22)	<0.0001
%CD4/CD38+	49 (37-65)	42 (34-52)	56 (44-68)	44 932-51)	0.0002
%CD4/CD38+/HLA-DR-	32 (18-48)	36 (30-46)	41 (35-57)	38 (28-46)	0.0362
%CD8/CD38-/HLA-DR+	39 (19-55)	38 (22-46)	37 (25-50)	33 (25-40)	0.794
%CD8/CD38+/HLA-DR+	30 (17-53)	17 (14-22)	23 (16-33)	16 (7.7-26)	<0.0001
%CD8/CD38+/HLA-DR$^-$	11 (5.5-17)	22 (18-27)	16 (9.8-26)	16 (14-26)	0.0005
%CD8/CD38+	46 (33-68)	40 (35-53)	45 (34-64)	38 (31-50)	0.11
%CD8/HLA-DR+	79 (66-87)	57 (43-68)	65 (51-78)	55 (43-63)	<0.0001

Median and IQR (in parenthesis) are shown. Values are percentages of either CD4+ or CD8+ T cells expressing marker of interest.

Co-infected subjects had significantly higher co-expression of HLA-DR and CD38 on CD4+ T lymphocytes compared to HBV mono-infected and controls. There was no statistically significant difference between the co-infected and HIV mono-infected participants. HIV mono-infected participants had significantly higher co-expression of CD38 and HLA-DR on CD4+ cells compared to HBV mono-infected and controls. There was no statistically significant difference between the HBV mono-infected and control groups. A graphical representation of differences in the co-expression of CD38 and HLA-DR on CD4 T cells is shown on Figure 4.16.

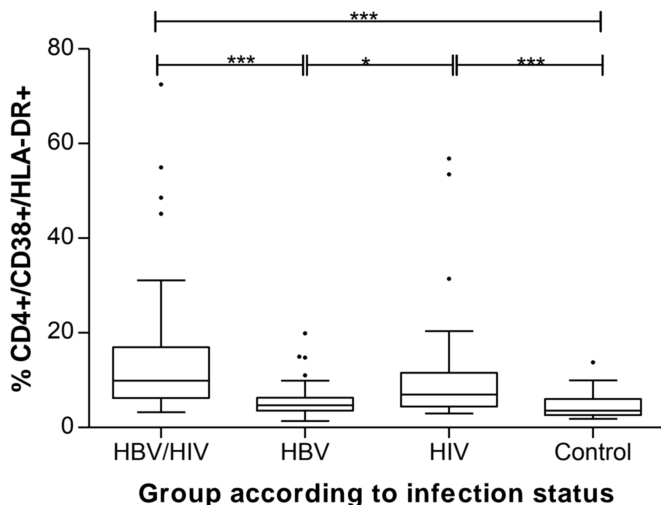

Figure 4.15 Co-expression of CD38 and HLA-DR on CD4+ T lymphocytes. The middle lines show the medians, boxes represent interquartile range while the whiskers show the non-outlier range. The dots are the outliers and extreme values. The asterix signs represent the strength of significant differences as calculated by Dunn's post-test. Only the significant differences are indicated. HBV/HIV vs HBV, p=0.00001; HBV vs HIV, p=0.01; HIV vs control, p=0.00001, HBV/HIV vs control, p<0.000001.

The co-expression of CD38 and HLA-DR on CD8+ lymphocytes in co-infected subjects was significantly higher than in the HBV mono-infected and control groups. There were no other significant differences between the groups. Conversely, the expression pattern CD38+/HLA-DR- on CD8 lymphocytes was higher on the HBV mono-infected group compared to those with co-infection. Controls also had higher expression of CD38 on CD8 lymphocytes in the absence of HLA-DR compared to the co-infected group. Figure 4.17 is a box plot of the percentage of CD8 T lymphocytes co-expressing CD38+/HLA-DR+.

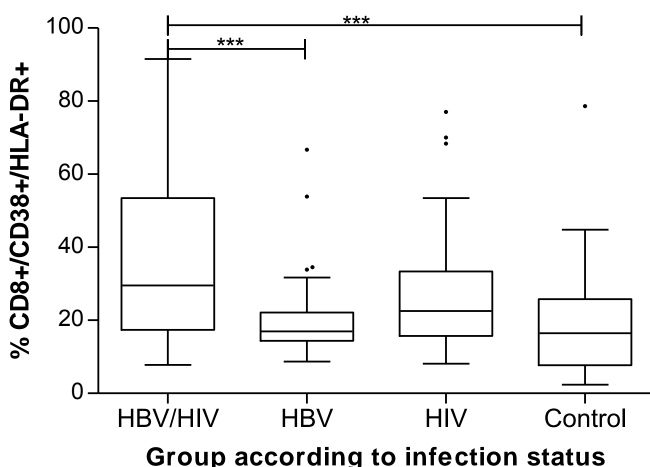

Figure 4.16 Expression of CD38/HLA-DR on CD8+ lymphocytes according to group. The middle lines show the medians, boxes represent interquartile range while the whiskers show the non-outlier range. The dots are the outliers and extreme values. The asterix signs represent the strength of significant differences as calculated by Dunn's post-test. Only the significant differences are indicated. HBV/HIV vs HBV, p=0.001; HBV/HIV vs controls, p=0.0004.

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The independent expression of CD38 and HLA-DR by CD8+ T lymphocytes was also evaluated as shown in Table 4.22. There was no significant difference in the total percentage of CD8+ T cells expressing CD38 alone or in combination with HLA-DR across the four groups as shown in Table 4.22 and Figure 4.18A. Significant differences were observed in the expression of total HLA-DR on CD8+ T lymphocytes (refer to Table 4.22. These results are also shown in Figure 4.18B.

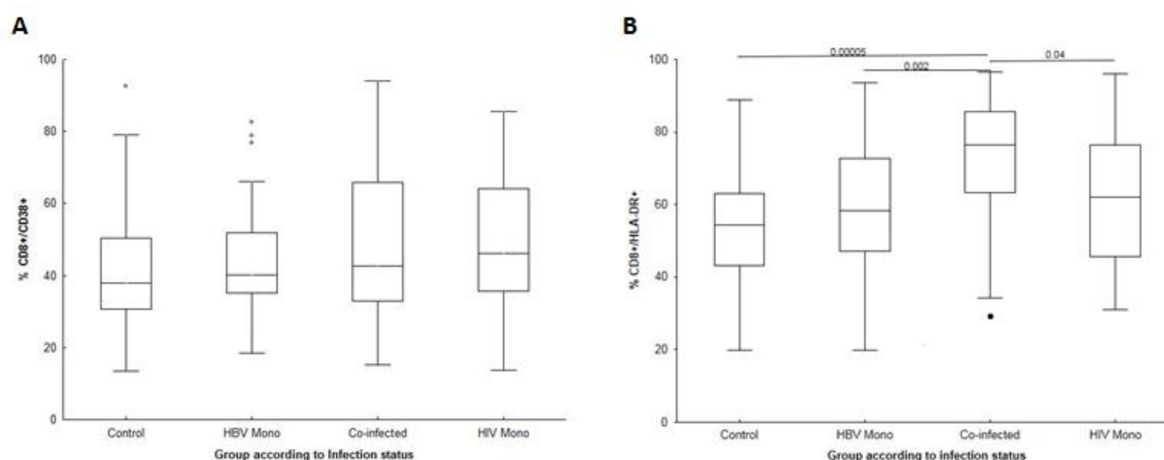

Figure 4.17 Total percentage of CD8+ T cells expressing CD38 and HLA-DR. No differences were observed for total % of CD8+ T cells expressing CD38 shown in figure A, p=0.1. There were statistically significant differences in the % of CD8+ T cells expressing HLA-DR between HBV/HIV compared to controls (p=0.00005); HBV/HIV vs HIV (p=0.002) and HBV/HIV compared to HIV (p=0.04) as shown in Figure B. The middle lines show the medians, boxes represent interquartile range while the whiskers show the non-outlier range. The dots are the outliers. The p values as calculated by the Dunn's post-test are indicated on top of the arrows. Only the significant differences are indicated.

Correlational analysis within the HBV/HIV group of the co-expression of CD38 and HLA-DR by CD8+ T cells against CD4+ cells count and CD4/CD8 ratio showed a statistically significant negative correlation with Spearman rho values of -0.48 and -0.38 respectively ($p<0.05$). No similar statistically significant correlations were observed in the other groups. Co-expression of CD38 and HLA-DR by CD8+ T cells within the HBV/HIV group was also significantly correlated with HBV viral load ($R=0.37$, $p<0.05$) and HIV viral load ($R=0.40$, $p<0.05$). There was no correlation between viral loads and co-expression of CD38 and HLA-DR on CD8+ T cells in the HBV- or the HIV- group.

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4.2.6 Cell associated markers of immune exhaustion

4.2.6.1 PD-1 and CTLA-4 expression on CD4+ and CD8+ T lymphocytes

The percentage of CD4+ and CD8+ T lymphocytes with surface expression of CTLA-4 was consistently low (<2% of both CD4+ and CD8+ T cells). As a result of low surface expression of CTLA-4, its co-expression with PD-1 was also low. Representative density plots of CTLA-4 and PD-1 expression are shown in Figure 4.19.

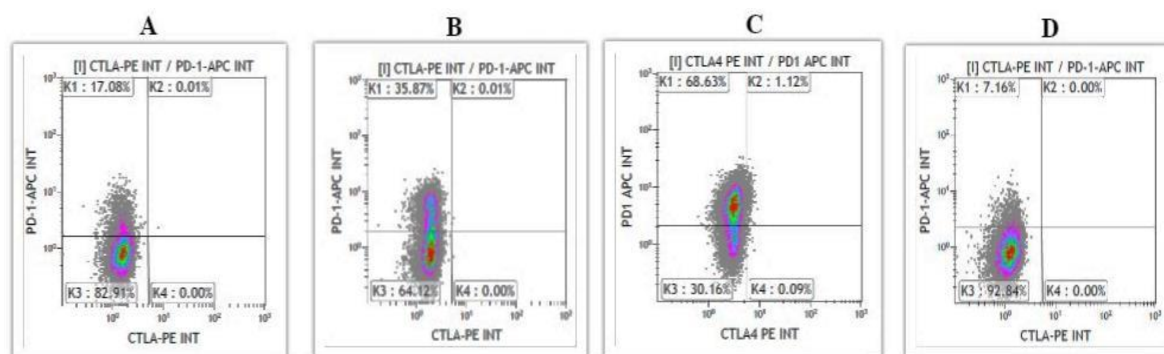

Figure 4.18 Density plot of PD-1 and CTLA-4 expression by CD8+ T cells. Plot A shows 17.1% PD-1 expression by CD8 T lymphocytes in an HBV subject, B shows 35.9% in an HIV patient, C reveals high expression (68.3%) expression in an HBV/HIV subject while D shows the expression of PD-1 in a control individual. All graphs consistently show <2% expression of CTLA-4 on the CD8+ T lymphocytes.

The expression of PD-1 on both CD4+ and CD8+ T lymphocytes was highest amongst co-infected subjects compared to all other groups, $p<0.0001$ in each instance. The median, first and third quartiles of total percentage expression of CTLA-4 on CD4+ and CD8+ lymphocytes is shown in Table 4.24.

Table 4.24 % Expression of CTLA-4 and PD-1 by CD4+ and CD8+ T lymphocytes

	HBV/HIV	HBV	HIV	Control	p
%CD4+/CTLA4+	1.79 (0.94-3.56)	1.64 (0.65-2.30)	1.81 (1.24-3.33)	1.80 (0.85-2.48)	0.71
%CD8+/CTLA4+	1.49 (0.99-2.94)	1.65 (0.91-2.42)	1.57 (1.57-2.44)	1.67 (0.69-3.23)	0.99
%CD4+/PD-1+	24.8 (18.2-33.7)	17.9 (13.2-22.8)	20.9 (15.0-27.2)	12.8 (7.8-21.9)	<0.0001
%CD8+/PD-1+	21.9 (15.3-33.0)	13.2 (11.2-20.2)	21.7 (14.9-26.3)	15.3 (8.5-21.3)	<0.0001

Post-test analysis showed that the expression of PD-1 on CD8+ cells was significantly higher on co-infected subjects compared to HBV mono-infected and controls. HIV mono-infected subjects also had significantly higher CD8+/PD-1+ expression compared to HBV mono-infected and controls but not to the same magnitude as the co-infected participants. The

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percentage expression of PD-1 on CD4+ lymphocytes was significantly higher in the co-infected group compared to HBV mono-infected and controls but not HIV mono-infected. The HIV mono-infected group had higher expression of PD-1 on CD4+ lymphocytes compared to the control group. The results for post-test analysis of percentage PD-1expression on CD8+ lymphocytes are shown graphically on Figure 4.20.

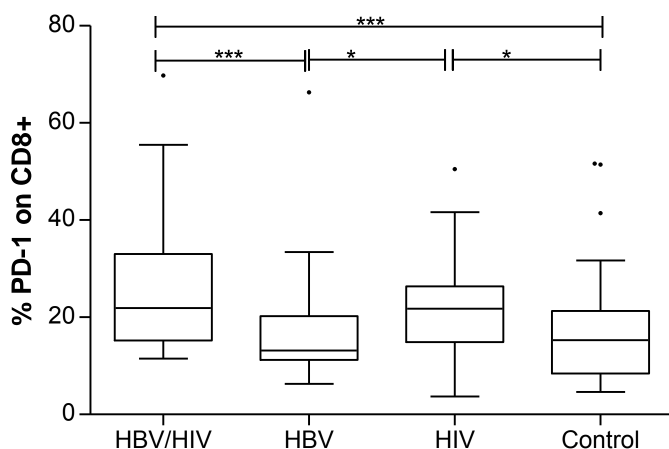

Figure 4.19 Percentage expression of PD-1 on CD8+ lymphocytes. The middle lines show the medians, boxes represent interquartile range while the whiskers show the non-outlier range. The dots are the outliers and extreme values. The * signs represent the strength of significant differences as calculated by Dunn's post-test. Only the significant differences are indicated. HBV/HIV vs HBV, p=0.0008; HBV/HIV vs control, p=0.0008; HIV vs HBV, p=0.03: HIV vs controls, p=0.02.

4.2.7 Correlation of cell-associated immune markers, microbial translocation makers and non-invasive liver fibrosis markers

The HBV group showed significant positive correlation between CD38 and HLA-DR co-expression and PD-1 expression by CD8+ T lymphocytes with Spearman rho=0.34, $p<0.05$. In addition, there was also significant correlation between CD8/CD38+/HLA-DR+ and CD8/CD38+ (total) with rho=0.45 and between CD8/CD38+/HLA-DR+ and CD8/HLA-DR+ (total) with R=0.51 ($p<0.05$ in both instances). In the HBV/HIV group, only CD8/HLA-DR+ (total) had a significant positive correlation with PD-1 expression by CD8+ T lymphocytes, R=0.31, $p<0.05$. There was a statistically significant inverse correlation between CD38 and PD-1 expression by CD8+ cells in the HIV group with rho=-0.38, $p<0.05$. No statistically significant correlations between PD-1 expression and either CD38 or HLA-DR on CD8 cells were observed in the control group. There was significant positive correlation observed between the percentage of CD8+ T cells co-expressing CD38 and HLA-DR as well as HLA-

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DR alone and LBP in the HIV group. The other groups did not show similar correlation between cell-associated biomarkers and microbial translocation proxies.

Correlation was also performed for all participants without stratification of groups. The results showed that sCD14 positively correlated with the % of CD8+ T cells co-expressing the activation markers CD38 and HLA-DR and also %CD8+/PD-1+ expression ($p<0.05$ in each instance). In addition, sCD14 showed significant negative correlation to CD4+ T cell count (rho=-0.35) and CD4/CD8 ratio (rho=-0.47). Similar, although weaker, significant inverse correlation were observed between LBP and CD4+ T cell count (rho=-0.18) and also LBP against CD4/CD8 ratio (-0.21). Correlation matrices for the cell-based immune markers, microbial translocation products and non-invasive markers of liver fibrosis for all ungrouped participants are shown in Figure 4.21. The correlation markers according to group are shown in Appendices 9-12.

	CD4 count	HBV DNA load	HIV viral load	CD4/CD8 ratio	CD8 count	Median stiffness	APRI Score	FIB-4 SCORE	ALT	Length on HAART	Soluble CD14	LBP
CD4 count	1.00	0.11	-0.29	0.69	0.18	-0.06	-0.22	-0.22	-0.11	0.12	-0.35	-0.18
HBV DNA load	0.11	1.00	0.53	0.05	0.09	0.28	0.29	0.19	0.31	-0.53	-0.07	0.06
HIV viral load	-0.29	0.53	1.00	-0.41	0.24	0.29	0.15	0.14	0.03	-0.18	-0.02	0.04
CD4/CD8 ratio	0.69	0.05	-0.41	1.00	-0.52	0.04	-0.10	-0.07	-0.12	0.23	-0.47	-0.21
CD8 count	0.18	0.09	0.24	-0.52	1.00	-0.10	-0.13	-0.15	-0.01	-0.15	0.18	0.09
Median stiffness	-0.06	0.28	0.29	0.04	-0.10	1.00	0.44	0.39	0.21	0.04	-0.09	-0.05
APRI Score	-0.22	0.29	0.15	-0.10	-0.13	0.44	1.00	0.76	0.60	0.12	0.06	-0.12
FIB-4 SCORE	-0.22	0.19	0.14	-0.07	-0.15	0.39	0.76	1.00	0.32	0.16	0.00	-0.08
ALT	-0.11	0.31	0.03	-0.12	-0.01	0.21	0.60	0.32	1.00	0.13	0.19	0.04
Length on HAART	0.12	-0.53	-0.18	0.23	-0.15	0.04	0.12	0.16	0.13	1.00	-0.28	-0.12
Soluble CD14	-0.35	-0.07	-0.02	-0.47	0.18	-0.09	0.06	0.00	0.19	-0.28	1.00	0.24
LBP	-0.18	0.06	0.04	-0.21	0.09	-0.05	-0.12	-0.08	0.04	-0.12	0.24	1.00
CD4/CD38+/HLADR+	-0.58	0.10	0.34	-0.57	0.10	0.08	0.08	0.14	0.14	-0.03	0.29	0.13
CD4/CD38-/HLADR+	-0.38	-0.01	0.34	-0.39	0.11	0.02	0.00	0.10	0.08	0.05	0.15	0.14
CD4/CD38+/HLADR-	0.19	-0.22	-0.35	0.14	-0.02	-0.11	-0.08	-0.13	-0.09	-0.08	-0.08	-0.11
CD4/CD38	-0.11	-0.13	-0.10	-0.14	0.02	-0.09	-0.03	-0.06	-0.04	-0.03	0.03	-0.03
CD8/CD38+/HLADR+	-0.39	0.08	0.36	-0.38	0.08	0.15	0.06	0.08	0.13	-0.11	0.19	0.08
CD4/HLADR	-0.51	0.06	0.43	-0.51	0.11	0.05	0.04	0.14	0.11	0.05	0.20	0.16
CD8/CD38	-0.22	0.01	0.17	-0.12	-0.06	0.17	0.04	-0.05	0.03	-0.15	0.10	0.01
CD8/HLADR	-0.44	0.03	0.38	-0.49	0.17	0.02	0.02	0.15	0.16	0.06	0.19	0.27
CD4/PD1+/CTLA4+	-0.38	0.06	0.16	-0.47	0.19	-0.05	-0.08	-0.18	-0.01	-0.09	0.22	0.17
CD4/PD1/CTLA4-	-0.59	-0.03	0.31	-0.52	0.02	0.07	0.10	0.06	0.19	-0.06	0.26	0.19
CD4/CTLA4+	-0.17	0.01	0.08	-0.31	0.22	-0.05	-0.12	-0.28	-0.04	-0.08	0.15	0.11
CD4/PD1+	-0.59	-0.04	0.31	-0.53	0.03	0.06	0.09	0.04	0.18	-0.06	0.27	0.19
CD8/PD1+/CTLA4+	-0.14	-0.16	0.14	-0.19	0.07	-0.07	-0.07	-0.06	0.06	0.07	0.16	-0.17
CD8/PD1+	-0.35	-0.33	0.14	-0.33	0.05	-0.16	-0.18	-0.14	-0.01	0.05	0.21	0.10

	CD4/CD38+/HLADR+	CD4/CD38-/HLADR+	CD4/CD38+/HLADR-	CD4/CD38	CD8/CD38+/HLADR+	CD4/HLADR	CD8/CD38	CD8/HLADR	CD4/PD1+/CTLA4+	CD4/PD1/CTLA4-	CD4/CTLA4+	CD4/PD1+	CD8/PD1+/CTLA4+	CD8/PD1+
CD4 count	-0.58	-0.38	0.19	-0.11	-0.39	-0.51	-0.22	-0.44	-0.38	-0.59	-0.17	-0.59	-0.14	-0.35
HBV DNA load	0.10	-0.01	-0.22	-0.13	0.08	0.06	0.01	0.03	0.06	-0.03	0.01	-0.04	-0.16	-0.33
HIV viral load	0.34	0.34	-0.35	-0.10	0.36	0.43	0.17	0.38	0.16	0.31	0.08	0.31	0.14	0.14
CD4/CD8 ratio	-0.57	-0.39	0.14	-0.14	-0.38	-0.51	-0.12	-0.49	-0.47	-0.52	-0.31	-0.53	-0.19	-0.33
CD8 count	0.10	0.11	-0.02	0.02	0.08	0.11	-0.06	0.17	0.19	0.02	0.22	0.03	0.07	0.05
Median stiffness	0.08	0.02	-0.11	-0.09	0.15	0.05	0.17	0.02	-0.05	0.07	-0.05	0.06	-0.07	-0.16
APRI Score	0.08	0.00	-0.08	-0.03	0.06	0.04	0.04	0.02	-0.08	0.10	-0.12	0.09	-0.07	-0.18
FIB-4 SCORE	0.14	0.10	-0.13	-0.06	0.08	0.14	-0.05	0.15	-0.18	0.06	-0.28	0.04	-0.06	-0.14
ALT	0.14	0.08	-0.09	-0.04	0.13	0.11	0.03	0.16	-0.01	0.19	-0.04	0.18	0.06	-0.01
Length on HAART	-0.03	0.05	-0.08	-0.03	-0.11	0.05	-0.15	0.06	-0.09	-0.06	-0.08	-0.06	0.07	0.05
Soluble CD14	0.29	0.15	-0.08	0.03	0.19	0.20	0.10	0.19	0.22	0.26	0.15	0.27	0.16	0.21
LBP	0.13	0.14	-0.11	-0.03	0.08	0.16	0.01	0.27	0.17	0.19	0.11	0.19	-0.17	0.10
CD4/CD38+/HLADR+	1.00	0.58	-0.23	0.25	0.76	0.82	0.39	0.68	0.30	0.50	0.12	0.50	0.08	0.18
CD4/CD38-/HLADR+	0.58	1.00	-0.58	-0.37	0.31	0.91	-0.15	0.68	0.27	0.44	0.09	0.44	0.17	0.27
CD4/CD38+/HLADR-	-0.23	-0.58	1.00	0.76	-0.12	-0.54	0.30	-0.44	-0.25	-0.29	-0.13	-0.30	-0.14	-0.12
CD4/CD38	0.25	-0.37	0.76	1.00	0.28	-0.12	0.58	-0.08	-0.06	-0.08	-0.04	-0.08	-0.16	-0.07
CD8/CD38+/HLADR+	0.76	0.31	-0.12	0.28	1.00	0.56	0.65	0.60	0.21	0.32	0.10	0.32	0.05	0.13
CD4/HLADR	0.82	0.91	-0.54	-0.12	0.56	1.00	0.08	0.79	0.30	0.50	0.10	0.49	0.12	0.24
CD8/CD38	0.39	-0.15	0.30	0.58	0.65	0.08	1.00	-0.02	0.07	0.15	0.05	0.15	-0.04	0.00
CD8/HLADR	0.68	0.68	-0.44	-0.08	0.60	0.79	-0.02	1.00	0.28	0.39	0.12	0.39	0.02	0.24
CD4/PD1+/CTLA4+	0.30	0.27	-0.25	-0.06	0.21	0.30	0.07	0.28	1.00	0.56	0.87	0.61	0.32	0.35
CD4/PD1/CTLA4-	0.50	0.44	-0.29	-0.08	0.32	0.50	0.15	0.39	0.56	1.00	0.30	1.00	0.20	0.61
CD4/CTLA4+	0.12	0.09	-0.13	-0.04	0.10	0.10	0.05	0.12	0.87	0.30	1.00	0.34	0.29	0.19
CD4/PD1+	0.50	0.44	-0.30	-0.08	0.32	0.49	0.15	0.39	0.61	1.00	0.34	1.00	0.22	0.61
CD8/PD1+/CTLA4+	0.08	0.17	-0.14	-0.16	0.05	0.12	-0.04	0.02	0.32	0.20	0.29	0.22	1.00	0.48
CD8/PD1+	0.18	0.27	-0.12	-0.07	0.13	0.24	0.00	0.24	0.35	0.61	0.19	0.61	0.48	1.00

Figure 4.20 Correlation matrix of cell-associated immune markers, microbial translocation biomarkers and liver fibrosis markers for all participants ungrouped. Analysis was performed using Spearman rank correlation test. Significant correlation at $p<0.05$ are indicated in red font. Median stiffness, but not APRI or FIB-4, was positively correlated with %CD8+ T cells expressing CD38. Median stiffness also positively correlated with both HBV DNA and HIV viral load.

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4.2.8 Detection of soluble immune markers

As the study investigated the association between expression of immune markers and non-invasive markers of liver fibrosis, the presence and concentration of soluble/plasma immune markers (cytokines and chemokines) was measured using Luminex technology.

4.2.8.1 Detection rates of cytokines

The majority of cytokines were detected in all samples except for some whose detection rates were variable between groups. The reason for non-detection of certain cytokines was due to plasma levels that were below the limit of detection for that particular assay. Detection rates were particularly low for IL-5 and IL-15 on which none of the groups had more than 50% detection. Rates of detection of IL-5 were especially low among the HIV mono-infected where only 7% of the plasma samples had detectable cytokine. The detection of IL-15 was poor across the groups with only the HBV mono-infected group having 20% of samples with concentrations above the lower limit of detection. The other groups all had less than 10% of subjects with detectable IL-15, with the control group not having any participant with detectable IL-15. The non-detection of these soluble cytokines may hold clinico-pathological significance. IL-15 was excluded from further statistical analysis because of the low number of samples with detectable amounts (all, except the HBV group, had less than 10% of samples with detectable plasma IL-15). All samples with a concentration of cytokine below the lower limit of quantitation were assigned a value of zero in order to standardize the data for statistical analysis. The list of cytokines with detection rates less than 90% is shown in Table 4.25.

Table 4.25 List of plasma cytokines with detection rates of less than 90% of samples in any group

	HBV/HIV, n=45	**HBV, n=44**	**HIV, n=39**	**Controls, n=19**
IL-2	15 (33%)	34 (77%)	16 (41%)	8 (42%)
IL-5	22 (49%)	21 (48%)	3 (7%)	8 (42%)
IL-6	35 (78%)	42 (95%)	31 (79%)	16 (84%)
IL-7	31 (69%)	34 (77%)	20 (51%)	16 (84%)
IL-15	2 (4%)	9 (20%)	3 (8%)	0 (0%)
GM-CSF	15 (33%)	31 (70%)	17 (44%)	10 (53%)
MCP-1	27 (60%)	33 (75%)	20 (51%)	12 (63%)
MIP-1a	36 (80%)	42 (95%)	36 (92%)	18 (95%)

The table shows cytokines that were detected in less than 90% of all samples within any group. Low levels across all groups were particularly seen for IL-15.

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4.2.8.2 Plasma cytokine concentrations across groups

The HBV group was heterogeneous because not all the subjects were on therapy. In order to determine the effect of anti-HBV therapy on the expression of cytokines, data of treated and untreated HBV mono-infected subjects was analysed using the Mann-Whitney test. There were no statistically significant differences in expression of the majority of cytokines between the treated and untreated individuals and as a result the HBV mono-infected participants were subsequently analysed as a single homogenous group. The only cytokine that showed statistically significant differences between the on-treatment and non-treated HBV mono-infected subjects was platelet derived growth factor (PDGF) which was higher in the treated with median 3843 pg/ml (IQR: 3275-4777) compared to the untreated with mean 2699 pg/ml (IQR: 1807-3570), p=0.04. The results of the comparison between the on-treatment and untreated HBV mono-infected patients are shown on Table 4.26.

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Table 4.26 Comparison between on-treatment and treatment-naïve HBV mono-infected subjects.

Variable	Units	On treatment HBV	Non-treated HBV	p
IL-1b	pg/ml	2.1 (1.6- 3.0)	2.3 (1.5- 5.2)	0.71
IL-1ra	pg/ml	112.5 (95.3- 407.9)	106.3 (80.5- 184.3)	0.65
IL-2	pg/ml	8.7 (3.9- 20.0)	5.9 (0- 15.2)	0.57
IL-4	pg/ml	2.9 (2.1- 3.7)	2.8 (2.2- 5.4)	0.95
IL-5*	pg/ml	0 (0- 14.1)	7.0 (0- 19.2)	0.45
IL-6	pg/ml	12.0 (8.0- 26.2)	11.0 (6.9- 32.4)	0.67
IL-7	pg/ml	6.9 (2.5- 12.9)	10.4 (3.7- 22.9)	0.42
IL-8	pg/ml	53.0 (24.7- 119.4)	51.9 (28.3- 192.8)	0.78
IL-9	pg/ml	74.4 (51.6- 127.1)	70.2 (62.1- 112.9)	0.85
IL-10	pg/ml	15.9 (11.9- 30.6)	15.0 (12.3- 26.2)	0.90
IL-12	pg/ml	43.6 (25.5- 73.2)	70.5 (39.1- 98.7)	0.11
IL-13	pg/ml	13.4 (7.5- 29.1)	17.5 (11.4- 48.0)	0.38
IL-15*	pg/ml	0 (0- 0)	0 (0.0- 0.0)	0.58
IL-17	pg/ml	87.1 (54.0- 133.2)	81.7 (74.2- 133.2)	0.45
EOTAXIN	pg/ml	129.3 (68.0- 164.8)	140.1 (89.4- 213.8)	0.50
Basic FGF	pg/ml	65.7 (45.4- 91.5)	71.1 (48.1- 93.0)	0.80
G-CSF	pg/ml	59.1 (44.2- 97)	63.8 (34.0- 132.2)	0.97
GM-CSF	pg/ml	35.2 (7.2- 89.6)	4.0 (0.0- 85.5)	0.51
IFN-γ	pg/ml	130.2 (88.7- 168.0)	84.1 (75.7- 253.4)	0.27
IP-10	ng/ml	0.89 (0.70- 1.2)	1.2 (0.9- 1.5)	0.46
MCP-1	pg/ml	21.2 (0- 47.0)	33.9 (18.6- 73.3)	0.15
MIP-1a	pg/ml	7.2 (4.8- 11.5)	8.2 (3.9- 10.0)	0.97
PDGF	ng/ml	2.97 (1.81- 3.57)	3.8 (3.3- 4.8)	0.04
MIP-1b	pg/ml	129.4 (93.7- 204.7)	154.7 (138.0- 182.2)	0.29
RANTES	ng/ml	19.0 (13.6- 22.5)	19.0 (14.4- 23.7)	0.68
TNF-α	pg/ml	21.8 (18.5- 47.2)	40.6 (22.9- 57.4)	0.36
VEGF	pg/ml	111.2 (50.1- 187.7)	141.8 (117.3- 239.2)	0.08
TGF-β1	ng/ml	44.4 (38.3- 56.8)	46.4 (41.6- 54.2)	0.71
TGF-β2	ng/ml	2.2 (1.8- 2.4)	1.9 (1.8- 2.5)	0.99
TGF-β3	ng/ml	1.9 (1.3- 2.2)	1.5 (1.5- 2.1)	0.93
CD8/CD38+/HLADR+	%	19.37 (15.1- 26.32)	18.0 (13.8- 31.7)	1.00
CD8/PD1+	%	13.62 (11.52- 17.98)	13.1 (11.2- 25.8)	0.92

*IL-5 and IL-15 had very low detection rates across all groups of participants.

Statistical differences in serum concentration were observed for 21/28 (75%) of the analysed cytokines. The majority of cytokines had highest concentrations in the HBV mono-infected group compared to the other groups which also had the highest CD4+ T cell counts. The summary statistics different plasma cytokine concentrations according to infection status are shown in Table 4.27 using the medians and inter-quartile ranges. Because of the low concentration of IL-5 and IL-15, the medians and 25th percentiles had values of zero across

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all groups. The difference in plasma levels are best indicated using means and one standard deviation for each group. For IL-5, the mean ± standard deviation in ng/ml for each group was - HBV 18.0±48.2; HBV/HIV 28.7±166.8; HIV 1.1±3.9 and controls – 2.4±3.9. For IL-15, the means and standard deviations for the each group were: HBV- 31.8 pg/ml ±101.8; HBV/HIV -8.0 pg/ml ±52.8 and HIV- 1.2 pg/ml ± 4.5. There was no specimen with detectable IL-15 within the control group.

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Table 4.27 Median plasma cytokine concentrations.

	Units	LDL	HBV/HIV, n=45	HBV, n=44	HIV, n=39	Control, n=19	p
IL-1β	pg/ml	0.465	1.3 (0.9- 2.8)	2 (1.5- 3.1)	1.2 (0.9- 1.9)	1.7 (1.2- 2.8)	*0.001*
IL-1ra	pg/ml	5.33	59 (40.4- 100.8)	110.8 (85.1- 376.7)	62.2 (40.4- 85.8)	69.8 (53.7- 106.3)	*<0.00001*
IL-2	pg/ml	1.52	0 (0- 5.4)	8 (0.5- 18.4)	0 (0- 7.3)	0 (0- 4.7)	*0.0002*
IL-4	pg/ml	0.24	2.2 (1.6- 3)	2.8 (2.1- 4.1)	2.2 (1.6- 3.2)	2.3 (2- 2.9)	*0.03*
IL-5*	pg/ml	1.00	0 (0- 7)	0 (0- 15.5)	0 (0- 0)	0 (0- 4)	*0.0005*
IL-6	pg/ml	1.68	10 (0.8- 18.3)	11.3 (7.2- 29.3)	4.3 (0.5- 10.3)	6.3 (1.5- 12.5)	*0.002*
IL-7	pg/ml	0.84	9 (3.7- 14.6)	7.4 (2.5- 14.8)	1.2 (0- 7.9)	5.9 (1.2- 9)	*0.002*
IL-8	pg/ml	2.44	26.8 (19.5- 53.3)	51.9 (22.8- 121.8)	21.7 (13.9- 43.6)	120.1 (35.2- 437.2)	*<0.00001*
IL-9	pg/ml	1.19	54.7 (44.5- 73.8)	73.1 (55.6- 118.5)	59.5 (45.5- 83.7)	78.2 (57.6- 97.1)	*0.01*
IL-10	pg/ml	2.21	10.2 (5.8- 18.8)	15.9 (11.3- 29.2)	9.5 (5.6- 14.5)	9.7 (7.7- 15.9)	*0.001*
IL-12	pg/ml	2.25	50.8 (31.9- 63.6)	47.4 (27.1- 87.9)	35.2 (19.4- 59.9)	44.5 (31.7- 65.4)	0.3
IL-13	pg/ml	0.40	12 (6.2- 24.8)	13.8 (8- 29.6)	9.5 (5.2- 13.7)	6.5 (4.8- 10.4)	*0.004*
IL-15*	pg/ml	5.73	0 (0- 0)	0 (0- 0)	0 (0- 0)	0 (0- 0)	*0.02*
IL-17	pg/ml	5.81	44.5 (27.7- 84.3)	81.7 (61.6- 133.2)	67.7 (54.1- 111.9)	78.5 (64.1- 110.4)	*0.004*
EOTAXIN	pg/ml	1.75	107.9 (81.2- 172.8)	129.3 (72.1- 171.3)	115.8 (73.9- 160.1)	142.5 (93.1- 261.1)	0.4
Basic-FGF	pg/ml	3.91	43.1 (21.8- 66.9)	65.7 (46.2- 92.3)	44.8 (24.2- 61.3)	50.4 (34.2- 65.7)	*0.0006*
G-CSF	pg/ml	3.06	51.1 (32.1- 66.1)	59.7 (44.1- 98.6)	41.9 (30.3- 58.1)	37.6 (32.1- 66.1)	*0.006*
GM-CSF	pg/ml	2.94	0 (0- 22.2)	25.5 (0- 87.6)	0 (0- 35.7)	0.6 (0- 15.3)	*0.006*
IFN-γ	pg/ml	42.93	88.7 (57.5- 145.3)	121.2 (80.1- 182.0)	66.7 (48- 125.7)	97.2 (51.1- 116.7)	*0.004*
IP-10	ng/ml	4.94	1.5 (1.0- 2.8)	0.9 (0.7- 1.5)	1.1 (0.8- 1.5)	1 (0.8- 1.2)	*0.0001*
MCP-1	pg/ml	1.24	4.7 (0- 27.2)	21.3 (0- 54.4)	0.4 (0- 20.5)	17.3 (0- 78.8)	*0.03*
MIP-1a	pg/ml	0.15	4.4 (1.3- 7.7)	7.3 (4.6- 11.4)	4.7 (2.5- 6.3)	8.2 (5.3- 10.3)	*0.001*
PDGF	ng/ml	1.00	2.4 (1.7- 3.3)	3.2 (1.8- 3.9)	2.9 (1.9- 3.8)	3.3 (2.3- 4.5)	0.09
MIP-1b	pg/ml	0.46	140.9 (95.5- 218.8(	147.3 (102.7- 199.1)	128.8 (103.8- 227.4)	217 (155.5- 264.7)	0.06
RANTES	ng/ml	3.72	19.9 (16.7- 26.7)	19.0 (13.8- 23.1)	19.1 (16.7- 22.6)	22.1 (17.8- 24.7)	0.23
TNF-a	pg/ml	3.77	19.5 (15.1- 27.4)	23.5 (18.5- 54.5)	19.5 (15- 24)	18.5 (15.1- 26.2)	*0.01*
VEGF	pg/ml	2.30	88.9 (52.2- 147.7)	117.7 (60- 211.5)	77.8 (45.2- 138)	89.6 (51.5- 160.5)	0.22
TGF-β1	ng/ml		41.7 (34.1- 53.6)	46.4 (38.3- 56.8)	49.7 (42.1- 55.7)	48.2 (42.5- 55)	0.10
TGF-β2	ng/ml		1.8 (1.7- 1.9)	2.1 (1.8- 2.4)	2.3 (1.8- 2.5)	2 (1.7- 2.4)	*<0.00001*
TGF-β3	ng/ml		1.3(1.2- 1.5)	1.9 (1.4- 2.2)	2.2 (1.5- 2.3)	1.8 (1.4- 2.2)	*<0.00001*

*Plasma levels of IL-5 and IL-15 were below detection limit in most samples across the groups hence the statistics (refer to Table 4.25).

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4.2.8.3 Multi-group comparison of cytokines with significantly different concentrations

Multiple comparison analyses were performed on cytokines with significant p values on the Kruskal-Wallis test to determine the groups that had statistically significant cytokine concentrations. The majority of cytokines with significant differences in concentration had the highest quantities in the HBV mono-infected group and the lowest concentration among the HIV mono-infected. IL-Ira was significantly higher in HBV compared to HBV/HIV ($p<0.0001$); HIV ($p<0.0001$) and controls ($p=0.03$). Exceptions of cytokines without highest concentration in HBV were observed for IL-8 where the controls had the highest plasma concentration compared to all other groups and also for IP-10 which had the highest concentration in the co-infected subjects compared to HIV mono-infected, HBV mono-infected and controls. Multi-group comparison statistics for soluble markers with statistically significant p values as calculated using the Kruskal Wallis test are shown in Figure 4.22.

Cytokine expression within the control group might suggest that the control subjects were not altogether healthy as was expected or that the HBV and/or HIV infected patient groups had lower levels than the "normal" controls. Analysis of differences in cytokine expression was rerun excluding the control group. The results still showed that the 9/27 (33%) of cytokines were significantly more highly expressed in the HBV group compared to both the HBV/HIV and the HIV group. The 9 cytokines were IL-1b, IL-1ra, IL-2, IL-4, IL-10, basic FGF, GM-CSF, MIP-1α and TNF-α. The HIV group showed significantly lower IL-5 and IL-7 expression compared to both HBV and HBV/HIV groups whilst the HBV/HIV had significantly lower TGF-β2 and -β3 isoforms compared to HBV and HIV. Results of multiple comparison analyses excluding the control group are shown on Figure 4.23

IL-1b	K-W p =.0014			
	Control	HBV	HBV/HIV	HIV
Control		1.0000	1.0000	0.4929
HBV	1.0000		0.0296	0.0011
HBV/HIV	1.0000	0.0296		1.0000
HIV	0.4929	0.0011	1.0000	

IL-7	K-W p =.0023			
	Control	HBV	HBV/HIV	HIV
Control		1.0000	0.6468	1.0000
HBV	1.0000		1.0000	0.0165
HBV/HIV	0.6468	1.0000		0.0034
HIV	1.0000	0.0165	0.0034	

FGF-BASIC	K-W p =.0006			
	Control	HBV	HBV/HIV	HIV
Control		0.2682	1.0000	1.0000
HBV	0.2682		0.0019	0.0020
HBV/HIV	1.0000	0.0019		1.0000
HIV	1.0000	0.0020	1.0000	

MIP-1a	K-W p =.0010			
	Control	HBV	HBV/HIV	HIV
Control		1.0000	0.0370	0.0180
HBV	1.0000		0.0452	0.0195
HBV/HIV	0.0370	0.0452		1.0000
HIV	0.0180	0.0195	1.0000	

IL-1ra	KW p =.0000			
	Control	HBV	HBV/HIV	HIV
Control		0.0346	1.0000	1.0000
HBV	0.0346		0.0000	0.0000
HBV/HIV	1.0000	0.0000		1.0000
HIV	1.0000	0.0000	1.0000	

IL-8	K-W p =.0000			
	Control	HBV	HBV/HIV	HIV
Control		0.4056	0.0024	0.0000
HBV	0.4056		0.1657	0.0039
HBV/HIV	0.0024	0.1657		1.0000
HIV	0.0000	0.0039	1.0000	

G-CSF	K-W p =.0058			
	Control	HBV	HBV/HIV	HIV
Control		0.0884	1.0000	1.0000
HBV	0.0884		0.1008	0.0077
HBV/HIV	1.0000	0.1008		1.0000
HIV	1.0000	0.0077	1.0000	

TNF-a	K-W p =.0102			
	Control	HBV	HBV/HIV	HIV
Control		0.1636	1.0000	1.0000
HBV	0.1636		0.0760	0.0152
HBV/HIV	1.0000	0.0760		1.0000
HIV	1.0000	0.0152	1.0000	

IL-2	K-W p =.0002			
	Control	HBV	HBV/HIV	HIV
Control		0.0337	1.0000	1.0000
HBV	0.0337		0.0024	0.0075
HBV/HIV	1.0000	0.0024		1.0000
HIV	1.0000	0.0075	1.0000	

IL-9	K-W p =.0100			
	Control	HBV	HBV/HIV	HIV
Control		1.0000	0.0625	0.4981
HBV	1.0000		0.0261	0.4634
HBV/HIV	0.0625	0.0261		1.0000
HIV	0.4981	0.4634	1.0000	

GM-CSF	K-W p =.0055			
	Control	HBV	HBV/HIV	HIV
Control		0.3562	1.0000	1.0000
HBV	0.3562		0.0131	0.0695
HBV/HIV	1.0000	0.0131		1.0000
HIV	1.0000	0.0695	1.0000	

TGF-b2	K-W p =.0000			
	Control	HBV	HBV/HIV	HIV
Control		1.0000	0.0758	1.0000
HBV	1.0000		0.0023	1.0000
HBV/HIV	0.0758	0.0023		0.0000
HIV	1.0000	1.0000	0.0000	

IL-4	K-W p =.0272			
	Control	HBV	HBV/HIV	HIV
Control		0.8234	1.0000	1.0000
HBV	0.8234		0.0651	0.0467
HBV/HIV	1.0000	0.0651		1.0000
HIV	1.0000	0.0467	1.0000	

IL-10	K-W p =.0010			
	Control	HBV	HBV/HIV	HIV
Control		0.1791	1.0000	1.0000
HBV	0.1791		0.0232	0.0008
HBV/HIV	1.0000	0.0232		1.0000
HIV	1.0000	0.0008	1.0000	

IFN-g	K-W p =.0042			
	Control	HBV	HBV/HIV	HIV
Control		0.1093	1.0000	1.0000
HBV	0.1093		0.1880	0.0036
HBV/HIV	1.0000	0.1880		1.0000
HIV	1.0000	0.0036	1.0000	

TGF-b3	K-W p =.0000			
	Control	HBV	HBV/HIV	HIV
Control		1.0000	0.0440	0.6129
HBV	1.0000		0.0034	0.4291
HBV/HIV	0.0440	0.0034		0.0000
HIV	0.6129	0.4291	0.0000	

IL-5	K-W p =.0005			
	Control	HBV	HBV/HIV	HIV
Control		1.0000	1.0000	0.4802
HBV	1.0000		1.0000	0.0037
HBV/HIV	1.0000	1.0000		0.0211
HIV	0.4802	0.0037	0.0211	

IL-13	K-W p =.0039			
	Control	HBV	HBV/HIV	HIV
Control		0.0138	0.0532	1.0000
HBV	0.0138		1.0000	0.0720
HBV/HIV	0.0532	1.0000		0.2909
HIV	1.0000	0.0720	0.2909	

IP-10	K-W p =.0001			
	Control	HBV	HBV/HIV	HIV
Control		1.0000	0.0054	1.0000
HBV	1.0000		0.0002	1.0000
HBV/HIV	0.0054	0.0002		0.0178
HIV	1.0000	1.0000	0.0178	

IL-6	K-W p =.0020			
	Control	HBV	HBV/HIV	HIV
Control		0.0663	1.0000	1.0000
HBV	0.0663		0.3105	0.0017
HBV/HIV	1.0000	0.3105		0.4688
HIV	1.0000	0.0017	0.4688	

IL-17	K-W p =.0035			
	Control	HBV	HBV/HIV	HIV
Control		1.0000	0.1122	1.0000
HBV	1.0000		0.0026	0.6320
HBV/HIV	0.1122	0.0026		0.4500
HIV	1.0000	0.6320	0.4500	

MCP-1	K-W p =.0278			
	Control	HBV	HBV/HIV	HIV
Control		1.0000	1.0000	0.5695
HBV	1.0000		0.2827	0.0388
HBV/HIV	1.0000	0.2827		1.0000
HIV	0.5695	0.0388	1.0000	

Figure 4.21 Multiple group comparison statistics for cytokines with significantly different plasma concentration including control group. Statistically significant results are shown in red font. The p statistic calculated using the Kruskal-Wallis (K-W) test is also indicated on the top row of each analyte.IL-1ra shows that the HBV group had statistically significant higher levels compared to all other groups while IP-10 shows that the HBV/HIV subjects had higher expression relative to others subject groups.

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IL-1b	K-W test p =0.0004		
	HBV/HIV	HBV	HIV
HBV/HIV		0.0139	0.8915
HBV	0.0139		0.0005
HIV	0.8915	0.0005	

IL-1ra	K-W p =0.0000		
	HBV/HIV	HBV	HIV
HBV/HIV		0.0000	1.0000
HBV	0.0000		0.0000
HIV	1.0000	0.0000	

IL-2	K-W test p=0.0002		
	HBV/HIV	HBV	HIV
HBV/HIV		0.0017	1.0000
HBV	0.0017		0.0045
HIV	1.0000	0.0045	

IL-4	K-W p =0.0127		
	HBV/HIV	HBV	HIV
HBV/HIV		0.0403	1.0000
HBV	0.0403		0.0266
HIV	1.0000	0.0266	

IP-10	K-W p =0.0001		
	HBV/HIV	HBV	HIV
HBV/HIV		0.0001	0.0095
HBV	0.0001		0.9712
HIV	0.0095	0.9712	

IL-5	K-W p =0.0002		
	HBV/HIV	HBV	HIV
HBV/HIV		1.0000	0.0125
HBV	1.0000		0.0023
HIV	0.0125	0.0023	

IL-7	K-W p =0.0014		
	HBV/HIV	HBV	HIV
HBV/HIV		1.0000	0.0026
HBV	1.0000		0.0105
HIV	0.0026	0.0105	

IL-10	K-W p =0.0005		
	HBV/HIV	HBV	HIV
HBV/HIV		0.0143	0.9588
HBV	0.0143		0.0006
HIV	0.9588	0.0006	

FGF-BASIC	K-W p =0.0002		
	HBV/HIV	HBV	HIV
HBV/HIV		0.0011	1.0000
HBV	0.0011		0.0013
HIV	1.0000	0.0013	

GM-CSF	K-W p =0.0029		
	HBV/HIV	HBV	HIV
HBV/HIV		0.0089	1.0000
HBV	0.0089		0.0418
HIV	1.0000	0.0418	

MIP-1a	K-W p =0.0058		
	HBV/HIV	HBV	HIV
HBV/HIV		0.0213	1.0000
HBV	0.0213		0.0137
HIV	1.0000	0.0137	

TNF-a	K-W p =0.0067		
	HBV/HIV	HBV	HIV
HBV/HIV		0.0420	1.0000
HBV	0.0420		0.0100
HIV	1.0000	0.0100	

TGF-b2	K-W p =0.0000		
	HBV/HIV	HBV	HIV
HBV/HIV		0.0011	0.0000
HBV	0.0011		0.9687
HIV	0.0000	0.9687	

TGF-b3	K-W p =0.0000		
	HBV/HIV	HBV	HIV
HBV/HIV		0.0021	0.0000
HBV	0.0021		0.2393
HIV	0.0000	0.2393	

Figure 4.22 Multi-group comparison of soluble markers excluding controls. The figure shows statistically significant differences shown in red font. The Kruskal Wallis p statistic values are also indicated for each marker. The expression of IL-10 was statistically significant higher in the HBV group compared to the HBV/HIV and the HIV groups.

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4.2.9 Multiple correlation analysis

Correlational analysis was performed using the Spearman Rank test on biomarkers that showed statistically significant differences between groups as determined by the Kruskal-Wallis test. Correlation was also performed for all study participants as a single group as well according to their infection status using all continuous variables measured in the study. There was significant positive correlation between IP-10 and sCD14 (rho=0.32) and IP-10 against LBP (rho=0.19). IL-2 and IL-1ra both had significant negative correlation with sCD14 with correlation coefficients of -0.18 in both instances. Median stiffness had significant negative correlation with the three TGF-β isoforms and IL-5. Not all correlations that were observed in the ungrouped analysis were replicated in the grouped analysis.

No statistically significant correlation between median stiffness and any of the soluble or cell-associated immune markers was observed within the HBV group. Median stiffness in this group had significant positive correlation with co-expression of CD38 and HLA-DR expression on CD8 cells (rho=0.43) and negative correlation with TGF-β2 (rho=-0.44). Within the HIV group, median stiffness had statistically significant negative correlation with IL-2 (rho=-0.34); IL-4 (-0.46) and GM-CSF (-0.38). There were correlations between cytokines that were observed across all patient groups. There was positive correlation between IL-1b with- IL-1ra; IL-2; IL-4; IL-6; IL-10; MIP-α; GM-CSF and TNF-α. These correlation matrices are shown graphically in the collage of tables shown in Figure 4.24.

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A. All Groups

	CD4/CD8	HBV DNA load	Fibroscan	sCD14	IL-1b	IL-1ra	IL-2	IL-4	IL-5	IL-7	IL-10	Basic-FGF	GM-CSF	IP-10	MIP-1a	TNF-a	TGF-b2	TGF-b3	CD8/CD38+/HLADR+	CD8/PD1+	HIV viral load
CD4/CD8	1.00	0.05	0.04	-0.47	0.12	0.19	0.15	0.12	-0.01	-0.09	0.02	0.15	0.14	-0.37	0.21	0.02	0.23	0.15	-0.38	-0.33	-0.41
HBV DNA load	0.05	1.00	0.28	-0.07	0.22	0.27	0.27	0.21	0.13	0.13	0.26	0.26	0.22	0.17	0.31	0.24	0.14	0.24	0.08	-0.33	0.53
Fibroscan	0.04	0.28	1.00	-0.09	0.01	0.04	-0.05	-0.10	0.17	0.15	0.03	-0.05	0.01	0.13	0.13	-0.10	-0.31	-0.31	0.15	-0.16	0.29
sCD14	-0.47	-0.07	-0.09	1.00	-0.07	-0.18	-0.18	-0.16	-0.02	0.14	-0.05	-0.14	-0.22	0.32	-0.15	-0.13	-0.27	-0.13	0.19	0.21	-0.02
IL-1b	0.12	0.22	0.01	-0.07	1.00	0.74	0.63	0.67	0.40	0.47	0.60	0.53	0.46	0.17	0.61	0.62	0.13	0.09	-0.08	-0.23	0.12
IL-1ra	0.19	0.27	0.04	-0.18	0.74	1.00	0.69	0.66	0.47	0.37	0.69	0.57	0.54	0.12	0.50	0.62	0.17	0.06	-0.05	-0.27	0.13
IL-2	0.15	0.27	-0.05	-0.18	0.63	0.69	1.00	0.67	0.22	0.25	0.58	0.68	0.75	-0.05	0.48	0.61	0.37	0.25	-0.09	-0.18	0.13
IL-4	0.12	0.21	-0.10	-0.16	0.67	0.66	0.67	1.00	0.39	0.38	0.55	0.67	0.68	-0.02	0.51	0.59	0.42	0.27	-0.15	-0.18	0.06
IL-5	-0.01	0.13	0.17	-0.02	0.40	0.47	0.22	0.39	1.00	0.58	0.38	0.34	0.13	0.20	0.16	0.40	-0.20	-0.28	0.08	-0.04	0.24
IL-7	-0.09	0.13	0.15	0.14	0.47	0.37	0.25	0.38	0.58	1.00	0.42	0.27	0.24	0.25	0.25	0.36	-0.28	-0.33	0.13	0.02	0.20
IL-10	0.02	0.26	0.03	-0.05	0.60	0.69	0.58	0.55	0.38	0.42	1.00	0.55	0.51	0.10	0.46	0.61	0.17	0.14	0.01	-0.15	0.07
Basic-FGF	0.15	0.26	-0.05	-0.14	0.53	0.57	0.68	0.67	0.34	0.27	0.55	1.00	0.61	-0.04	0.59	0.58	0.35	0.28	-0.17	-0.21	0.06
GM-CSF	0.14	0.22	0.01	-0.22	0.46	0.54	0.75	0.68	0.13	0.24	0.51	0.61	1.00	-0.21	0.44	0.50	0.44	0.29	-0.15	-0.29	0.04
IP-10	-0.37	0.17	0.13	0.32	0.17	0.12	-0.05	-0.02	0.20	0.25	0.10	-0.04	-0.21	1.00	0.11	0.06	-0.36	-0.32	0.33	0.16	0.36
MIP-1a	0.21	0.31	0.13	-0.15	0.61	0.50	0.48	0.51	0.16	0.25	0.46	0.59	0.44	0.11	1.00	0.41	0.10	0.09	-0.09	-0.44	0.15
TNF-a	0.02	0.24	-0.10	-0.13	0.62	0.62	0.61	0.59	0.40	0.36	0.61	0.58	0.50	0.06	0.41	1.00	0.24	0.25	-0.03	-0.11	0.13
TGF-b2	0.23	0.14	-0.31	-0.27	0.13	0.17	0.37	0.42	-0.20	-0.28	0.17	0.35	0.44	-0.36	0.10	0.24	1.00	0.86	-0.31	0.01	-0.21
TGF-b3	0.15	0.24	-0.31	-0.13	0.09	0.06	0.25	0.27	-0.28	-0.33	0.14	0.28	0.29	-0.32	0.09	0.25	0.86	1.00	-0.25	0.03	-0.22
CD8/CD38+/HLADR+	-0.38	0.08	0.15	0.19	-0.08	-0.05	-0.09	-0.15	0.08	0.13	0.01	-0.17	-0.15	0.33	-0.09	-0.03	-0.31	-0.25	1.00	0.13	0.36
CD8/PD1+	-0.33	-0.33	-0.16	0.21	-0.23	-0.27	-0.18	-0.18	-0.04	0.02	-0.15	-0.21	-0.29	0.16	-0.44	-0.11	0.01	0.03	0.13	1.00	0.14
HIV viral load	-0.41	0.53	0.29	-0.02	0.12	0.13	0.13	0.06	0.24	0.20	0.07	0.06	0.04	0.36	0.15	0.13	-0.21	-0.22	0.36	0.14	1.00

B. HBV

Variable	CD4/CD8	HBV DNA load	Fibroscan	sCD14	IL-1b	IL-1ra	IL-2	IL-4	IL-5	IL-7	IL-10	Basic-FGF	GM-CSF	IP-10	MIP-1a	TNF-a	TGF-b2	TGF-b3	CD8/CD38+/HLADR+	CD8/PD1+
CD4/CD8	1.00	-0.32	-0.08	-0.30	-0.07	-0.10	-0.07	-0.10	-0.13	-0.05	-0.13	-0.13	-0.06	-0.35	-0.38	-0.18	0.07	0.05	-0.14	0.25
HBV DNA load	-0.32	1.00	0.24	0.12	0.08	0.06	0.05	0.12	0.10	0.01	0.29	0.06	0.05	0.25	0.20	0.09	0.13	0.17	0.00	-0.27
Fibroscan	-0.08	0.24	1.00	0.00	-0.04	-0.13	-0.12	0.01	-0.04	-0.11	-0.02	-0.27	0.00	0.16	-0.01	-0.21	0.02	0.02	-0.01	0.24
sCD14	-0.30	0.12	0.00	1.00	-0.13	-0.08	-0.21	-0.13	0.03	0.25	0.15	-0.03	-0.20	0.30	0.07	-0.01	-0.28	-0.03	-0.12	0.07
IL-1b	-0.07	0.08	-0.04	-0.13	1.00	0.61	0.73	0.55	0.27	0.43	0.59	0.29	0.52	0.25	0.46	0.65	0.08	0.10	-0.15	-0.21
IL-1ra	-0.10	0.06	-0.13	-0.08	0.61	1.00	0.61	0.40	0.42	0.29	0.59	0.33	0.50	0.23	0.43	0.56	0.08	0.02	-0.08	-0.33
IL-2	-0.07	0.05	-0.12	-0.21	0.73	0.61	1.00	0.50	0.17	0.31	0.66	0.54	0.71	0.10	0.41	0.63	0.22	0.27	-0.12	-0.20
IL-4	-0.10	0.12	0.01	-0.13	0.55	0.40	0.50	1.00	0.46	0.46	0.36	0.61	0.64	0.03	0.47	0.56	0.30	0.15	-0.26	-0.30
IL-5	-0.13	0.10	-0.04	0.03	0.27	0.42	0.17	0.46	1.00	0.63	0.18	0.44	0.19	0.03	0.17	0.36	-0.11	-0.27	-0.17	-0.08
IL-7	-0.05	0.01	-0.11	0.25	0.43	0.29	0.31	0.46	0.63	1.00	0.38	0.46	0.36	0.07	0.29	0.37	-0.13	-0.17	-0.19	-0.15
IL-10	-0.13	0.29	-0.02	0.15	0.59	0.59	0.66	0.36	0.18	0.38	1.00	0.41	0.58	0.20	0.52	0.59	0.19	0.31	-0.15	-0.29
Basic-FGF	-0.13	0.06	-0.27	-0.03	0.29	0.33	0.54	0.61	0.44	0.46	0.41	1.00	0.62	-0.08	0.57	0.64	0.20	0.11	-0.28	-0.18
GM-CSF	-0.06	0.05	0.00	-0.20	0.52	0.50	0.71	0.64	0.19	0.36	0.58	0.62	1.00	-0.05	0.47	0.58	0.42	0.29	-0.33	-0.33
IP-10	-0.35	0.25	0.16	0.30	0.25	0.23	0.10	0.03	0.03	0.07	0.20	-0.08	-0.05	1.00	0.23	0.13	-0.29	-0.22	0.13	-0.09
MIP-1a	-0.38	0.20	-0.01	0.07	0.46	0.43	0.41	0.47	0.17	0.29	0.52	0.57	0.47	0.23	1.00	0.63	0.06	0.05	-0.10	-0.38
TNF-a	-0.18	0.09	-0.21	-0.01	0.65	0.56	0.63	0.56	0.36	0.37	0.59	0.64	0.58	0.13	0.63	1.00	0.19	0.23	-0.13	-0.27
TGF-b2	0.07	0.13	0.02	-0.28	0.08	0.08	0.22	0.30	-0.11	-0.13	0.19	0.20	0.42	-0.29	0.06	0.19	1.00	0.85	-0.18	-0.10
TGF-b3	0.05	0.17	0.02	-0.03	0.10	0.02	0.27	0.15	-0.27	-0.17	0.31	0.11	0.29	-0.22	0.05	0.23	0.85	1.00	-0.11	0.02
CD8/CD38+/HLADR+	-0.14	0.00	-0.01	-0.12	-0.15	-0.08	-0.12	-0.26	-0.17	-0.19	-0.15	-0.28	-0.33	0.13	-0.10	-0.13	-0.18	-0.11	1.00	0.23
CD8/PD1+	0.25	-0.27	0.24	0.07	-0.21	-0.33	-0.20	-0.30	-0.08	-0.15	-0.29	-0.18	-0.33	-0.09	-0.38	-0.27	-0.10	0.02	0.23	1.00

C. HBV/HIV

Variable	CD4/CD8	HBV DNA load	Fibroscan	sCD14	IL-1b	IL-1ra	IL-2	IL-4	IL-5	IL-7	IL-10	Basic-FGF	GM-CSF	IP-10	MIP-1a	TNF-a	TGF-b2	TGF-b3	CD8/CD38 +/HLADR+	CD8/PD1+	HIV viral load
CD4/CD8	1.00	-0.43	-0.27	-0.20	0.02	0.02	-0.02	0.00	0.01	0.02	-0.10	-0.01	-0.13	-0.12	0.13	-0.13	0.02	-0.11	-0.38	-0.15	-0.53
HBV DNA load	-0.43	1.00	0.21	0.16	0.13	0.17	0.31	0.09	0.10	0.30	0.11	0.19	0.23	0.52	0.23	0.23	-0.13	-0.01	0.37	-0.10	0.53
Fibroscan	-0.27	0.21	1.00	-0.28	-0.20	-0.11	-0.01	-0.22	0.08	-0.03	-0.24	-0.09	0.03	0.21	-0.03	-0.17	-0.44	-0.28	0.43	-0.25	0.23
sCD14	-0.20	0.16	-0.28	1.00	0.11	0.02	0.08	0.05	-0.17	0.04	0.00	0.07	0.01	0.06	-0.06	0.06	0.11	0.41	0.09	0.03	-0.21
IL-1b	0.02	0.13	-0.20	0.11	1.00	0.74	0.67	0.73	0.45	0.56	0.61	0.56	0.54	0.14	0.66	0.73	0.31	0.30	0.08	-0.07	0.10
IL-1ra	0.02	0.17	-0.11	0.02	0.74	1.00	0.74	0.81	0.58	0.55	0.83	0.65	0.61	0.25	0.48	0.68	0.29	0.11	0.17	-0.12	0.19
IL-2	-0.02	0.31	-0.01	0.08	0.67	0.74	1.00	0.71	0.29	0.41	0.57	0.66	0.82	0.04	0.54	0.61	0.32	0.15	0.00	-0.26	0.23
IL-4	0.00	0.09	-0.22	0.05	0.73	0.81	0.71	1.00	0.50	0.62	0.75	0.74	0.71	0.01	0.56	0.70	0.48	0.28	-0.03	-0.03	0.05
IL-5	0.01	0.10	0.08	-0.17	0.45	0.58	0.29	0.50	1.00	0.66	0.53	0.36	0.23	0.28	0.13	0.51	-0.03	-0.10	0.34	0.26	0.27
IL-7	0.02	0.30	-0.03	0.04	0.56	0.55	0.41	0.62	0.66	1.00	0.51	0.36	0.36	0.25	0.34	0.58	0.03	0.02	0.28	0.28	0.06
IL-10	-0.10	0.11	-0.24	0.00	0.61	0.83	0.57	0.75	0.53	0.51	1.00	0.55	0.54	0.15	0.37	0.72	0.33	0.20	0.22	0.03	0.10
Basic-FGF	-0.01	0.19	-0.09	0.07	0.56	0.65	0.66	0.74	0.36	0.36	0.55	1.00	0.66	0.05	0.62	0.56	0.34	0.30	-0.09	-0.18	0.23
GM-CSF	-0.13	0.23	0.03	0.01	0.54	0.61	0.82	0.71	0.23	0.36	0.54	0.66	1.00	-0.06	0.47	0.47	0.42	0.24	-0.03	-0.28	0.16
IP-10	-0.12	0.52	0.21	0.06	0.14	0.25	0.04	0.01	0.28	0.25	0.15	0.05	-0.06	1.00	0.10	0.15	-0.16	-0.10	0.50	0.17	0.48
MIP-1a	0.13	0.23	-0.03	-0.06	0.66	0.48	0.54	0.56	0.13	0.34	0.37	0.62	0.47	0.10	1.00	0.40	0.16	0.18	0.06	-0.33	0.18
TNF-a	-0.13	0.23	-0.17	0.06	0.73	0.68	0.61	0.70	0.51	0.58	0.72	0.56	0.47	0.15	0.40	1.00	0.32	0.27	0.13	0.10	0.20
TGF-b2	0.02	-0.13	-0.44	0.11	0.31	0.29	0.32	0.48	-0.03	0.03	0.33	0.34	0.42	-0.16	0.16	0.32	1.00	0.73	-0.29	0.01	-0.18
TGF-b3	-0.11	-0.01	-0.28	0.41	0.30	0.11	0.15	0.28	-0.10	0.02	0.20	0.30	0.24	-0.10	0.18	0.27	0.73	1.00	-0.11	0.01	-0.24
CD8/CD38+/HLADR+	-0.38	0.37	0.43	0.09	0.08	0.17	0.00	-0.03	0.34	0.28	0.22	-0.09	-0.03	0.50	0.06	0.13	-0.29	-0.11	1.00	0.17	0.40
CD8/PD1+	-0.15	-0.10	-0.25	0.03	-0.07	-0.12	-0.26	-0.03	0.26	0.28	0.03	-0.18	-0.28	0.17	-0.33	0.10	0.01	0.01	0.17	1.00	0.20
HIV viral load	-0.53	0.53	0.23	-0.21	0.10	0.19	0.23	0.05	0.27	0.06	0.10	0.23	0.16	0.48	0.18	0.20	-0.18	-0.24	0.40	0.20	1.00

D. HIV

	CD4/CD8 ratio	Fibroscan	sCD14	IL-1b	IL-1ra	IL-2	IL-4	IL-5	IL-7	IL-10	Basic-FGF	GM-CSF	IP-10	MIP-1a	TNF-a	TGF-b2	TGF-b3	CD8/CD38 +/HLADR+	CD8/PD1+	HIV viral load
CD4/CD8	1.00	-0.10	-0.17	-0.13	-0.17	0.07	0.12	0.07	-0.30	-0.17	0.11	0.02	-0.14	0.14	-0.06	0.15	0.01	-0.27	-0.26	-0.15
Fibroscan	-0.10	1.00	-0.07	-0.26	-0.29	-0.34	-0.46	-0.14	0.13	-0.12	-0.25	-0.38	0.14	-0.05	-0.20	-0.31	-0.29	-0.06	0.08	0.07
sCD14	-0.17	-0.07	1.00	0.23	0.10	-0.12	-0.15	0.29	0.36	0.12	-0.12	-0.34	0.37	0.06	-0.10	-0.51	-0.47	0.31	-0.13	0.10
IL-1b	-0.13	-0.26	0.23	1.00	0.69	0.56	0.59	0.33	0.39	0.58	0.52	0.36	0.43	0.51	0.56	0.19	0.18	0.19	-0.14	0.12
IL-1ra	-0.17	-0.29	0.10	0.69	1.00	0.68	0.70	0.28	0.19	0.63	0.52	0.52	0.36	0.52	0.55	0.29	0.25	0.03	-0.16	0.09
IL-2	0.07	-0.34	-0.12	0.56	0.68	1.00	0.77	0.02	0.05	0.51	0.72	0.61	0.24	0.59	0.53	0.55	0.39	-0.13	-0.25	0.12
IL-4	0.12	-0.46	-0.15	0.59	0.70	0.77	1.00	0.11	-0.01	0.41	0.63	0.68	0.14	0.60	0.59	0.65	0.57	-0.02	-0.25	0.16
IL-5	0.07	-0.14	0.29	0.33	0.28	0.02	0.11	1.00	0.23	0.35	0.12	-0.14	0.41	0.05	0.31	-0.17	-0.19	0.26	0.11	-0.08
IL-7	-0.30	0.13	0.36	0.39	0.19	0.05	-0.01	0.23	1.00	0.34	-0.10	0.12	0.39	0.09	0.06	-0.41	-0.46	0.37	-0.12	0.31
IL-10	-0.17	-0.12	0.12	0.58	0.63	0.51	0.41	0.35	0.34	1.00	0.50	0.39	0.35	0.42	0.49	0.20	0.09	0.09	-0.13	0.03
Basic-FGF	0.11	-0.25	-0.12	0.52	0.52	0.72	0.63	0.12	-0.10	0.50	1.00	0.45	0.25	0.58	0.45	0.61	0.48	-0.24	-0.11	-0.19
GM-CSF	0.02	-0.38	-0.34	0.36	0.52	0.61	0.68	-0.14	0.12	0.39	0.45	1.00	-0.11	0.43	0.45	0.56	0.39	-0.11	-0.38	0.00
IP-10	-0.14	0.14	0.37	0.43	0.36	0.24	0.14	0.41	0.39	0.35	0.25	-0.11	1.00	0.42	0.03	-0.22	-0.28	0.10	0.01	0.02
MIP-1a	0.14	-0.05	0.06	0.51	0.52	0.59	0.60	0.05	0.09	0.42	0.58	0.43	0.42	1.00	0.28	0.28	0.22	0.00	-0.35	0.10
TNF-a	-0.06	-0.20	-0.10	0.56	0.55	0.53	0.59	0.31	0.06	0.49	0.45	0.45	0.03	0.28	1.00	0.36	0.41	0.05	-0.16	0.09
TGF-b2	0.15	-0.31	-0.51	0.19	0.29	0.55	0.65	-0.17	-0.41	0.20	0.61	0.56	-0.22	0.28	0.36	1.00	0.83	-0.29	0.02	-0.06
TGF-b3	0.01	-0.29	-0.47	0.18	0.25	0.39	0.57	-0.19	-0.46	0.09	0.48	0.39	-0.28	0.22	0.41	0.83	1.00	-0.18	0.07	-0.03
CD8/CD38+/HLADR+	-0.27	-0.06	0.31	0.19	0.03	-0.13	-0.02	0.26	0.37	0.09	-0.24	-0.11	0.10	0.00	0.05	-0.29	-0.18	1.00	-0.23	0.19
CD8/PD1+	-0.26	0.08	-0.13	-0.14	-0.16	-0.25	-0.25	0.11	-0.12	-0.13	-0.11	-0.38	0.01	-0.35	-0.16	0.02	0.07	-0.23	1.00	0.07
HIV viral load	-0.15	0.07	0.10	0.12	0.09	0.12	0.16	-0.08	0.31	0.03	-0.19	0.00	0.02	0.10	0.09	-0.06	-0.03	0.19	0.07	1.00

Figure 4.23 Multiple correlation matrices of markers with statistical significance across groups. Table A shows the correlation for all participants combined. Table B shows correlations within the HBV group, C shows correlation within the HBV/HIV group whilst D has reveals correlations within the HIV group. All significant correlations at $p<0.05$ are marked with red font.

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4.2.10 Molecular characterisation of HBV among Immune Study subjects

4.2.10.1 HBV genotyping

Genotyping of HBV using the overlapping polymerase/surface antigen was successfully performed on 42 samples, 13 (31%) of whom were co-infected and 29 (69%) that were HBV mono-infected.

4.2.10.1.1 Distribution of genotypes according to HBV and HIV infection status

Among the thirteen co-infected participants with detectable HBV DNA that was successfully sequenced, eight (62%) were infected with HBV genotype A, three (23%) with D and two had HBV genotype E (15%). In comparison, the distribution of genotypes among the HBV mono-infected group was- 16/29 (55%) A, 11/29 (38%) D and 2/29 (7%) E. The distribution of the genotypes is shown in Figure 4.25 according to HIV status.

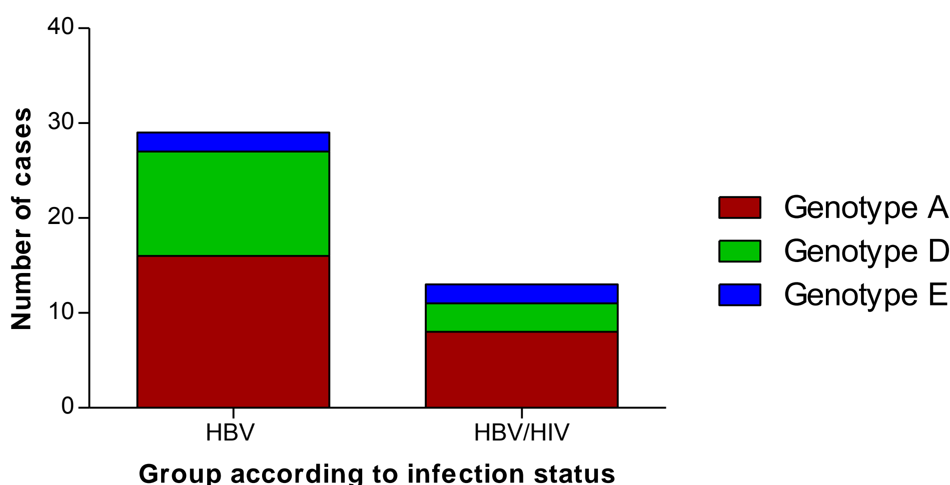

Figure 4.24 Frequency of HBV genotypes according to HBV and HIV infection status. The red columns represent HBV genotype A, green is for genotype D and the blue corresponds to genotype E. Genotyping was frequently more successful in the HBV mono-infected group compared to the co-infected group.

The overall distribution of sub-genotypes among the 24 genotype A samples was 23 subgenotype A1 and a single A2. The distribution of the 14 genotype D sequences according to subgenotype was D3 (9), D4 (4) and D2 (1). Genotype A1 sequences derived from the Immune Study subjects closely clustered with other African-derived sequences deposited onto GenBank, particularly those from South Africa. The subgenotype D4 sequences clustered together with a D4 isolate from Martinique. A phylogenetic tree of study sequences and other representative sequences is shown in Figure 4.26.

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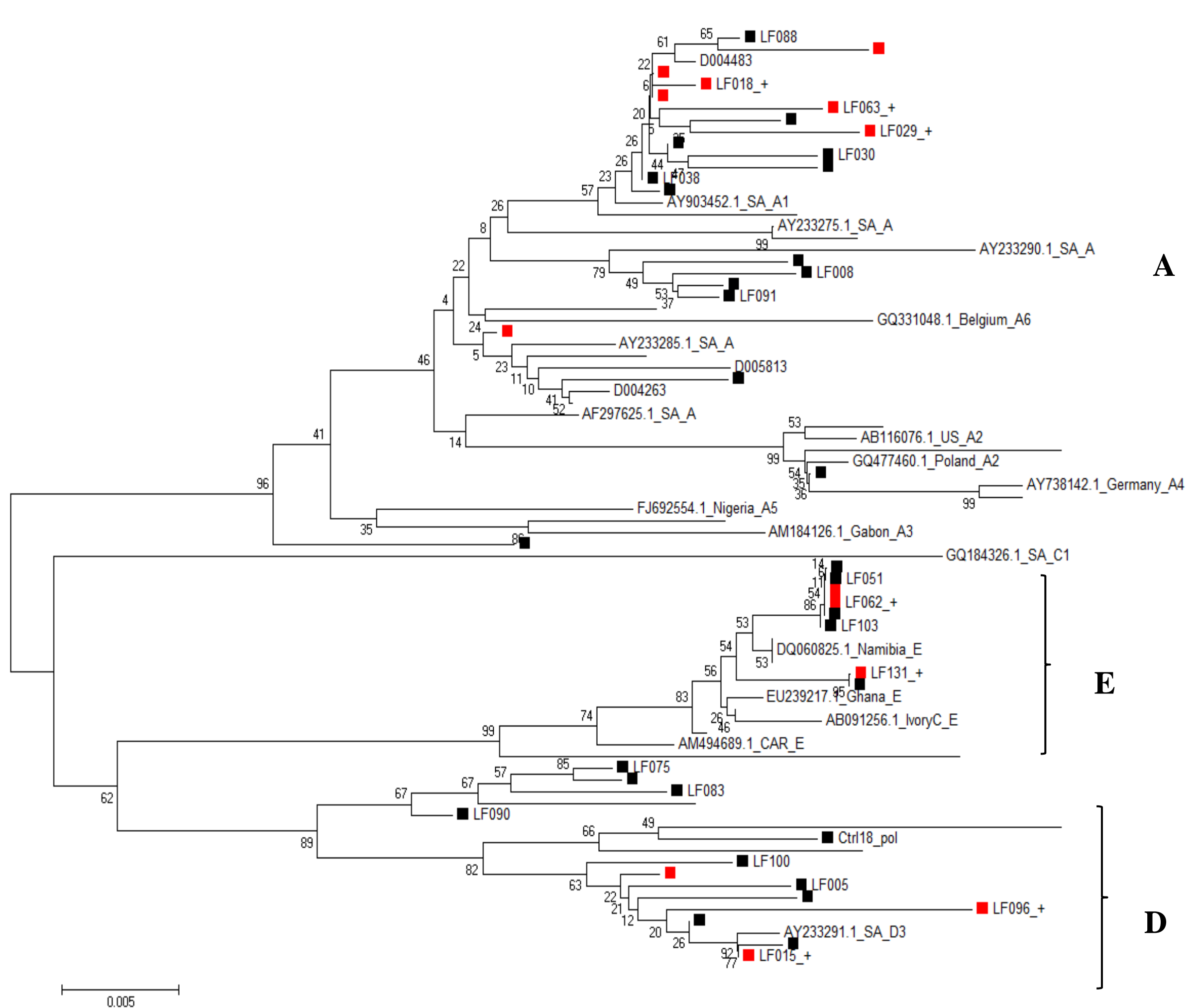

Figure 4.25 Evolutionary relationships of Immune Study HBV sequences. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.53668300 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. The analysis involved 79 nucleotide sequences. Sequences of co-infected subjects are annotated with a red square and those of HBV mono-infected with a black square. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 546 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

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4.2.10.1.2 Association between liver stiffness and HBV genotypes

The median of the Fibroscan score for the different genotypes were- 7.9kPa (5.8-12.1) for genotype A; 6.3kPa (5.8-7.6) for genotype D and 7.0kPa (5.3-7.9) for genotype E. Even though there seemed to be a trend towards more advanced fibrosis in genotype A, there was no statistically significant difference in the distribution of fibrosis scores according to the genotype of HBV, p=0.43; Fisher's exact test for proportions. There was no association between HBV genotypes and fibrosis scores (using the Fibroscan) when results were stratified according to whether participants had HBV mono-infection (p=0.47) or co-infection (p=0.73). The distribution of fibrosis frequency according to genotype is shown in Table 4.28.

Table 4.28 Proportions of fibrosis scores according to the HBV genotype

	<7.0 kPa	**7-13 kPa**	**>13 kPa**
Genotype A, n=21	7 (33%)	9 (43%)	5 (24%)
Genotype D, n=11	7 (64%)	3 (27%)	1 (9%)
Genotype E, n=4	2 (50%)	2 (50%)	0

4.2.10.2 Analysis of mutations

Analysis of mutation patterns was performed for selected regions of the polymerase region (to detect drug-resistance associated mutations), surface antigen (to detect vaccine escape mutants), precore region to detect mutations associated with the stop codon that abrogates synthesis of HBeAg and on the X region to detect the double core promoter mutation that is associated with increased risk of HCC development.

4.2.10.2.1 Polymerase gene mutations

Mutations were more commonly observed in the HBV mono-infected group of participants compared to the HBV/HIV co-infected group. No classic mutations associated with resistance to nucleot(s)ide reverse transcriptase were observed in either group. An I169L mutation was observed in one co-infected participant. The effect of the I169L mutation on resistance is unknown but occurred on a rated position that is associated is associated with resistance to entecavir.

Another mutation that was also observed was A181S in an untreated HBV mono-infected participant. Amino acid 181 of the polymerase gene is rated and the occurrence of valine (V)

or threonine (T) at this position predicts resistance to lamivudine, adefovir and telbivudine. The distribution of mutations across the polymerase region is shown in a column chart on Figure 4.27.

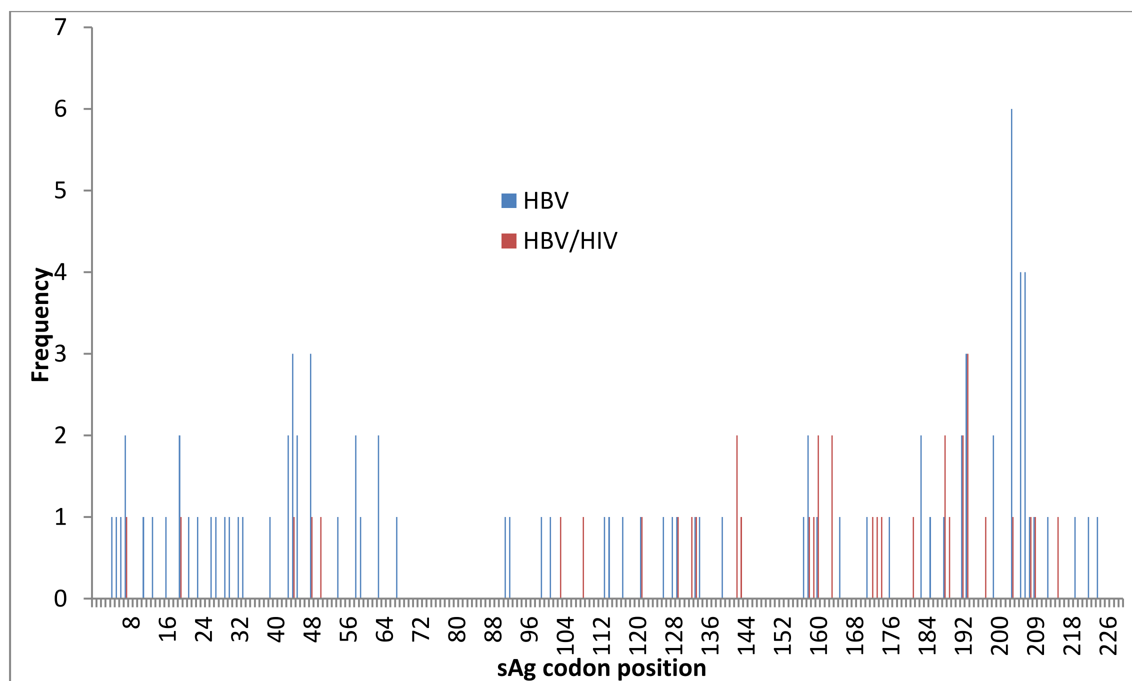

Figure 4.26 Mutations in the HBV polymerase mutations region according to HIV status. The figure shows more frequent occurrence of mutations in the polymerase region of HBV mono-infected participants compared to the co-infected subjects. The horizontal axis corresponds to the amino acid positions of the polymerase region. Mutations detected from samples of HBV mono-infected subjects are shown in blue bars while those from co-infected subjects are shown in red.

4.2.10.2.2 Surface antigen mutations

Mutations in the surface antigen region are associated with the emergence of vaccine escape mutants and also variants that may be missed by diagnostic tests. Using the list of mutations that have been associated with vaccine and detection failure as described on the geno2pheno web-based tool, the number of participants infected with mutant viruses was similar in the co-infected and HBV-mono-infected groups. Three sequences in each group had mutations that are associated with vaccine or detection escape. The escape mutations that were observed are shown in Table 4.29. Other accompanying surface antigen mutations and whether subject was co-infected are indicated.

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Table 4.29 List of escape mutations among Immune Study participants

Sample ID	Co-infected	HBsAg mutations	Escape mutations
LF003	yes	K122KR, T143M, K160N, Y161S, S207N	122K
LF029	yes	M133I, E164G, L173P, A194V, S207N	133I
LF045	no	Y100C, K122R, Y161F, A194V, S207N	100C
LF052	no	T127P, Q129HQ	129H
CTRL18	no	T45NT, Q101HQ, T118A	101H
LF096	yes	L109LM, T127P, S143L, D144E, T189I, V190A, V194A	143L, 144E

Similar to observations of the polymerase region, sequences from HBV mono-infected subjects had more mutations across the surface antigen region compared to the co-infected group although the significance of some of these mutations is unknown. One co-infected subject had a variant with a stop codon at W182*. The sample revealed a mixed virus population as there was both wild type and mutated surface antigen. Another co-infected participant had a sequence with truncated surface antigen at codon L216*.

4.2.10.2.3 HBV Precore region mutations

The W28* codon mutation was observed in 5/31 (16%) samples that were successfully sequenced in the precore region. All the five subjects were infected with genotype D. All except one of the participants with the precore stop codon mutation were HBV mono-infected. In two of the five cases, the W28* mutation occurred with the G29D mutation representing a mixed virus population.

4.2.10.2.4 HBV BCP and X mutations

The triple mutation T1753C, A1762T and G1764A was observed in 3/25 (12%) subjects. Two of the three subjects were co-infected. The double A1762T and G1764A mutation was seen in 4/25 (13%), all of whom were HBV mono-infected. The G1764A only mutation pattern was seen in 2/25 (8%) subjects. There was no subject with the A1762T mutation alone.

As a result of mutations in the BCP region, the I127T, K130M and V131I triple mutation of the X protein was seen in 3/35 (12%). The double change K130M and V131I on its own was detected in four cases. The V131I was seen on two subjects in whom it occurred together with the F132Y in keeping with the T1768A mutation in the overlapping BCP region. An image of the amino acid alignment of the X protein is shown in Figure 4.28. The presence of BCP mutants did not seem to have a statistically significant effect on the Fibroscan scores although the mean of median stiffness in those with BCP mutants was 11.3 kPa (±8.8) compared to 8.1 kPa (±4.5) in those without, Mann Whitney test p=0.2.

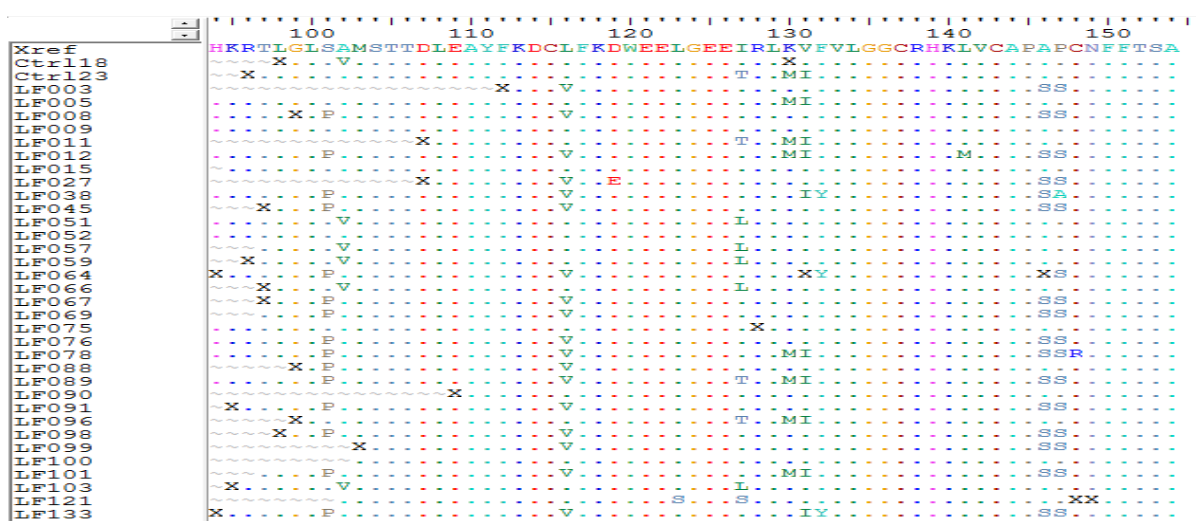

Figure 4.27 Alignment of the HBV X protein region. Conserved sites are toggled off and appear as dots while the variable sites reveal the amino acid arising from the nucleotide change. Variability was particularly observed around codons 101, 116 and 127-131.

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4.3 Differences in HBV mutation patterns between HCC cases and HBV-infected subjects with no known diagnosis of HCC

Sequence variation of HBV may be responsible for disease outcomes in chronic HBV infection. Comparison of HBV variability between the HCC Epidemiology Study cases and the HBV-infected Liver Fibrosis and Immune Markers Study participants was performed in order to determine if the distribution of genotypes and occurrence of mutations differs within the BCP, precore and surface antigen regions.

4.3.1 Differences in the distribution of HBV genotypes

HBV genotype A is the most prevalent genotype within South Africa. There was an over-representation of HBV genotype A among the HCC cases compared to the non-HCC subjects from the Liver Fibrosis study with prevalence of 34/43 (79%) and 24/42 (58%) respectively. HBV genotypes D and E were less prevalent among the HCC cases compared to those without a diagnosis of HCC, p=0.07. The prevalence of HBV genotypes grouped according to known diagnosis of HCC is shown using a stacked graph in Figure 4.29.

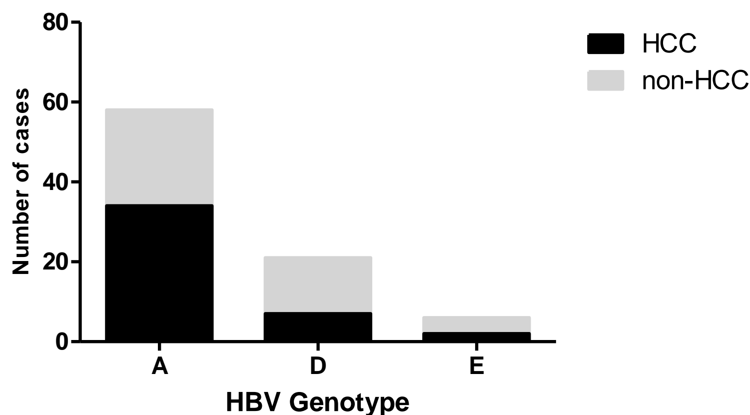

Figure 4.28 Distribution of HBV genotypes according to diagnosis of HCC. There was a high occurrence of HBV genotype A among the HCC cases compared to any other genotypes. The occurrence of genotype A was also highest among the non-HCC cases reflecting its existing background prevalence within the general population infected with hepatitis B. No other genotypes except for A, D and E were detected amongst the patients in the two studies.

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4.3.2 BCP region

The results revealed that there was a higher prevalence of the double 1762T/1764A mutation in the HBV genome of the HCC cases compared to the Immune Study subjects that did not have a diagnosis of liver malignancy. There were 21/33 (64%) of HCC cases with the double BCP mutation compared to only 7/25 (28%) among the Immune Study participants that were successfully sequenced, p=0.009. The relative risk of HCC cases having the double BCP mutation pattern compared to chronically infected subjects without a known diagnosis of HCC was 2.3 (95% CI: 1.1-4.5).

4.3.3 Precore region

The sequencing results also showed that there was a more frequent occurrence of stop codon mutations in the HBV precore region of HCC cases relative to subjects with chronic HBV but with no diagnosis of HCC. There were 10/33 (30%) of HCC cases with precore stop codon mutations compared to only 5/31 (16%) among the Immune Study subjects, p=0.24. The relative risk of HCC cases having the precore stop codon was 1.9 (95% CI: 0.7-4.9) compared to the Immune Study subjects. In addition, Immune Study subjects only had the W28* mutation pattern compared to HCC cases that had W28* (80%) and also a unique Q2* (20%) precore stop codon mutations.

4.3.4 Surface antigen region

While the P120T mutation was observed in 6/14 (43%) of HCC cases with at least one escape mutation, it was not seen among any of the Liver Fibrosis and Immune Markers Study samples. The occurrence of the L216* mutation was more frequent among HCC cases with three patients having the variant compared to only one among those without a diagnosis of HCC. The occurrence of the W182* stop codon was rare in both studies.

4.3.5 Phylogeny of HBV sequences from the HCC and Immune Study

Phylogenetic analysis using the polymerase/surface region of sequences from the two studies was performed to detect any distinct clustering patterns. The results showed no monophyletic clustering of HBV sequences according to whether the subject had a diagnosis of HCC or not. Figure 4.30 is a Neighbour-Joining phylogenetic tree showing the clustering of HBV sequences derived from HCC - (prefixed by HCC) and Immune- Study subjects.

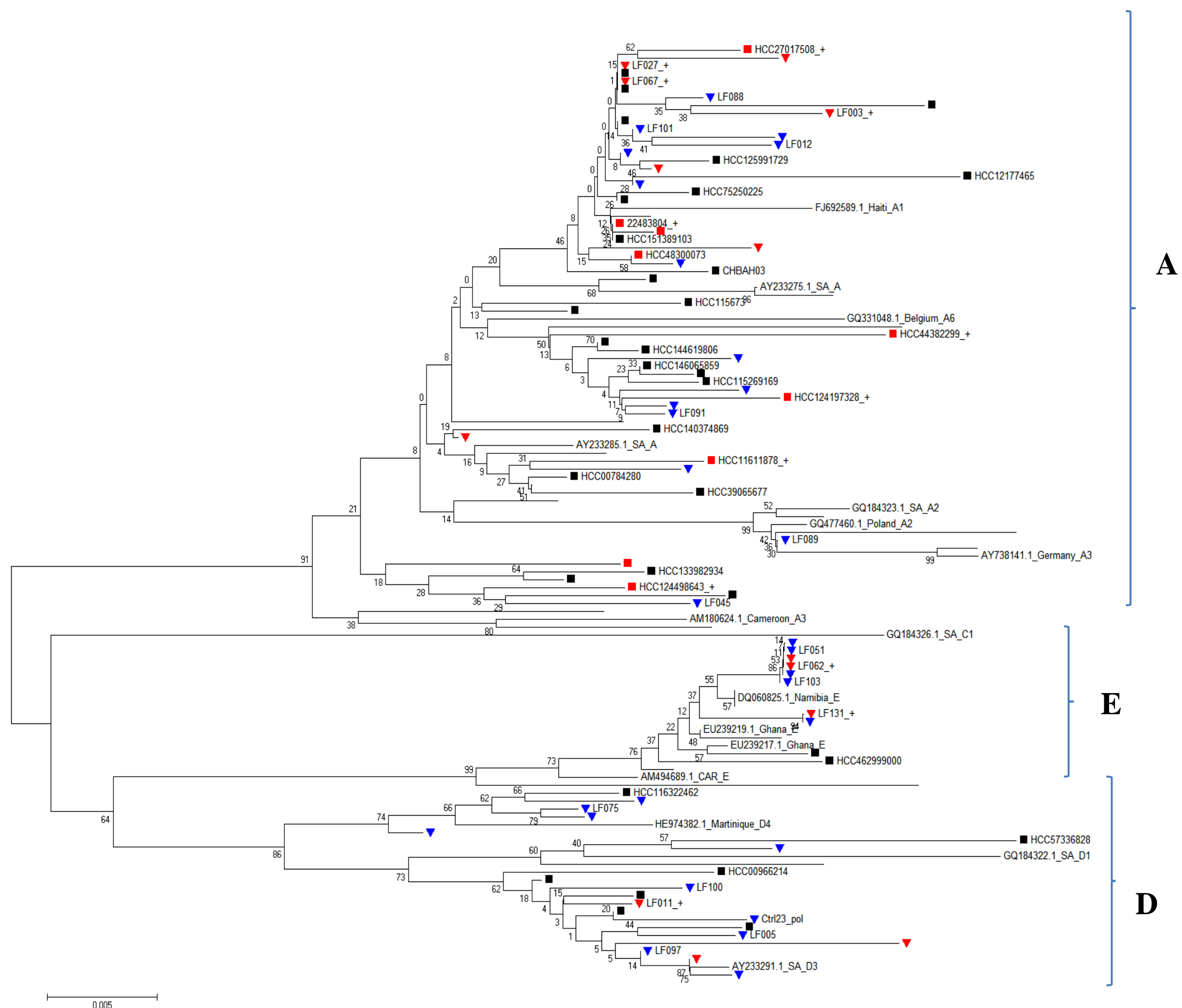

Figure 4.29 Evolutionary relationships of HCC- and Immune Study-derived HBV sequences. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.74823423 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. The analysis involved 118 nucleotide sequences. HIV co-infected HCC cases are annotated with a red square while HIV-negative HCC cases with a black square. Non-HCC co-infected sequences are shown with a red triangle while the HBV mono-infected non-HCC cases are shown with a blue triangle. All ambiguous positions were removed for each sequence pair. There were a total of 546 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). The tree shows no monophyletic clustering according to whether patients had HCC or not. There was also no apparent clustering according to HIV status.

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Chapter five

5. Discussion

This discussion chapter is presented in three parts. The first part (section 5.1) discusses the results arising of the HCC Study while the second part (section 5.2) covers the results of the Immune Study. Section 5.3 is a discussion of the strategies to mitigate against HBV-driven liver diseases.

5.1. HCC Study

The HCC Study, a multicentre, prospective longitudinal study aimed at describing the occurrence of HIV and/or HBV infection in patients presenting with HCC at selected teaching hospitals in South Africa. The study sought to determine the prevalence of hepatitis B in HIV-infected and HIV-uninfected patients with HCC and also to characterise HBV, in terms of genotypes and mutations, in cases of HBV-related HCC.

5.1.1. Age at presentation of HCC

According to GLOBOCAN 2012 data, liver cancer is the leading cause of cancer mortality among males and the third leading cause of cancer death among females in Africa (Ferlay et al., 2015). A total of 107 subjects diagnosed with HCC were prospectively recruited between December 2012 and October 2015. The mean age at presentation with malignancy was 46 years (95% CI: 43-49) with 75% of cases presenting with malignancy by 56 years of age. HCC cases were most frequently diagnosed between the ages of 30-40 years. The age at presentation tended to be younger in HBV-associated HCC patients in comparison to those that did not have HBV infection, although the difference was not statistically significant. However, patients with HCV-associated HCC were statistically significantly older compared to those that had HBV-associated malignancy. This is consistent with literature which describes that HCV-associated HCC tends to peak in the sixth decade of life compared to HBV-associated where the peak is in the fourth decade (Kew et al., 1997, Yang et al., 2015). The reasons for the differences in age at presentation are probably related to the age at which infection with the respective virus is acquired and also the different pathogenic mechanisms of the two infections. Hepatitis B is usually acquired early in childhood mainly through vertical or horizontal transmission while hepatitis C is acquired later in adulthood through horizontal transmission that includes among others injecting drug-use and sexual activity. HCV-related HCC usually presents on a background of cirrhosis and severe fibrosis while

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HBV-associated may arise with no underlying cirrhosis (Zemel et al., 2011). Also, HBV is directly carcinogenic being able to directly integrate into the host genome while HCV does not and this may also impact on the rate of hepatocarcinogenesis.

5.1.2. Effect of place of birth

The place of birth (rural vs urban) did not have an effect on the mean age at presentation of HCC in this study, contrary to previous data. The urban born cases had a mean age of 43.3 years (95% CI: 38.3-48.3) and that was four years younger than that of patients that had been born and lived in a rural setting for at least 10 years (mean 47.3 years, 95% CI: 43.3-51.3), p=0.8. Kew et al. have previously described that people born in rural areas who later move to urban areas present with HCC at a younger mean age relative to those who are born and grow up in urban areas (Kew, 1981, Kew et al., 1986). A possible reason for rural-born HCC cases presenting younger than their urban-born counterparts is the likely greater exposure to co-carcinogens such as aflatoxin and dietary iron overload (Kew et al., 1986). The results from the present study may suggest that the risk factors that were previously associated with rural birth have been diminishing over the years but this needs to be verified with further studies. One of the most prevalent risk factors that is known to have a synergistic effect on hepatocarcinogenesis in HBV-driven HCC in rural-born patients is exposure to aflatoxin (Qian et al., 1994, Wang et al., 1996, Kew, 2003). The effect of declining aflatoxin exposure on mortality due to HCC has been demonstrated in Asian countries where the number of people dying from liver cancer has decreased with reduced detection of aflatoxin adducts in urine from 219 µg/ml in 1991/1992 to 0.017 µg/ml in 2004 (Chen et al., 2013). Aflatoxin exposure is likewise decreasing in some African countries as better agricultural and food storage practices are adopted (Grace et al., 2015). Indeed, African countries now have a partnership, the Partnership for Aflatoxin Control in Africa (PACA), whose responsibilities include ensuring effective aflatoxin control so that people, animals and crops are protected from the harmful effects of the toxins. More data using archived and prospectively collected samples are needed to evaluate the level of aflatoxin exposure among HCC patients in sub-Saharan Africa.

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5.1.3. Effect of race

Black Africans account the highest proportion (55%) of the HCC cases compared 35% of Mixed Ancestry, 5% of Caucasians and 4% whose racial origin was not provided. The 2011 General Census data of the general population of the Western Cape estimates that 48.8% of the province's population is of Mixed Race descent while Black Africans make up 32.9% (StatsSA, 2012). The difference in proportion of race between the general population and the HCC cases may be reflective of Black Africans being at higher risk of HCC due to greater exposure to factors predisposing to HCC such dietary iron overload and HBV infection (Mandishona et al., 1998, Kew, 2008). Alternatively, the discrepancy may be indicative of selection bias in that Black Africans are more likely to seek healthcare from public/state-owned rather than private healthcare institutions and also migration from provinces with poorer healthcare facilities compared to other races (StatsSA, 2012). An accurate National Cancer Registry would be beneficial to ascertain the effect of race on HCC epidemiology in South Africa. There was a non-significant trend towards older age at presentation with HCC among Caucasians compared to Black Africans and those of Mixed Descent. This finding is consistent with literature describing older mean age at HCC presentation in low-risk populations that have a low prevalence of risk factors such as hepatitis B and aflatoxin exposure (Kew et al., 1986, Kew, 2010, Kew, 2013). It has been suggested that Black Africans tend to present with HCC at younger age compared to Caucasians but this current study did not observe a significant difference between the two races, p=0.1. The reason for no significant difference may be related to the small number of Caucasian cases (n=6) that were recruited compared to Black Africans (n=59).

5.1.4. HBsAg prevalence

The prevalence of HBsAg in the cohort was 64.1% and is higher than previously described data from South Africa but consistent with the estimated contribution of hepatitis B to the development of HCC (Kew et al., 1997, Parkin, 2006). Due to the study design, it was not possible to determine whether carriage of HBsAg was chronic (more than 6 months) but it is likely that infections were chronic due to the known routes of transmission of hepatitis B in South Africa. More male HCC were HBsAg positive than women that were HBsAg positive, p=0.3. This is a well-recognized phenomenon possibly explained by the contribution of hormonal differences between genders in the incidence of HBsAg (Beasley, 1988). The HBsAg positive cases were younger (mean 44.1±13.9 years) compared to those that were

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HBsAg negative (48.5±17.1 years), although this was not statistically significant. The difference between the HBsAg-positive and -negative males was larger (about 6 years) than it was between HBsAg-positive and -negative females with a similar mean. It has been described previously among South African HCC patients that HBsAg positive patients on average present at least 10 years younger than those that are HBsAg negative (Kew et al., 1997). This study did not see such a large difference, perhaps because of a smaller sample size or because of other unidentified effects. The prevalence of HBeAg was much higher among females (45%) than males (28%). The reason for this result may be due to the higher proportion of HBsAg positive females that were HIV-infected (36%) compared to males (18%). HIV infection has been demonstrated to delay the seroconversion of HBeAg to anti-HBe resulting in higher carriage of HBeAg among co-infected individuals (Oshitani et al., 1996, Thio, 2009, Andersson et al., 2012, Andersson et al., 2013). A particular cause for concern is the age at which HBV-related cases presented. Almost 50% of the cases occurred by the age of 40 years and 75% by 50 years of age, that is in age groups as most economically productive. These findings support the recommendations that African-born persons at risk for HCC should be placed under surveillance from the age of 20 years (Bruix et al., 2011).

5.1.5. Prevalence of HIV among HCC cases

There was an HIV prevalence rate of 22% among HCC cases overall, which is higher than any previously reported from Africa which ranged between 3-20% (Stein et al., 2008, Kew et al., 2010, Ocama et al., 2011, Tanon et al., 2012a). The prevalence of HIV was higher among the female HCC cases with 8/22 (36%) being HIV positive compared to 14/78 (18%) of males, p=0.08. The high prevalence may be a reflection of the underlying higher prevalence of HIV among females compared to males in much of sub-Saharan Africa where the UNAIDS statistics show that more than 50% of the 25.8 million individuals living with HIV are females (UNAIDS, 2015). Results of the 2012 South African National HIV Prevalence, Incidence and Behaviour Survey Report showed that 14.4% of females were HIV-infected compared to 9.9% of males (Zuma et al., 2016). The prevalence of HIV among HCC cases will probably increase over time as more individuals get access to HAART that would allow them to live long enough for the cancer to develop (Silverberg et al., 2015). The administration of HAART is thought to be one of the reasons for earlier studies reporting a lower HIV prevalence among HCC cases in the pre-HAART era (Thio et al., 2002, Giordano

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et al., 2004). In the pre-HAART era HIV-infected patients died from AIDS before HCC could develop. Increased longevity of HIV-infected persons in the context of HAART has resulted in an increase in age-related conditions like cardiovascular disease and non-AIDS defining malignancies such as prostate cancer (Palella et al., 2006, Deeks and Phillips, 2009).

5.1.6. Effect of HIV on age at presentation

HIV infection seems to have an effect on age at presentation of HCC especially among HBV-infected females. HIV/HBV co-infected females presented with malignancy about 10 years younger with mean 36.8 years (95% CI: 32.2-41.5) compared to HBV mono-infected women whose median age was 50.5 years (95% CI: 30.2-70.8), p=0.09. The data is consistent with the few studies from Africa that have investigated the effect of HIV on the epidemiology of HCC. A study from Western Africa reported that HIV-infected liver cancer patients were seen at an age that was 10 years younger compared to those that were HIV-uninfected and that the difference was significant (Tanon et al., 2012a). However the study from West Africa included only 60 cases overall of whom only seven were HIV-positive.

In contrast, the effect of HIV on age of presentation with HCC among males is not as clear amongst males as HBV/HIV co-infected males were only slightly younger than the HBV mono-infected males with mean age of 41.3 years (95% CI: 35.1-47.4) and 45.5 years (95% CI: 40.5-50.5), respectively, p=0.7. The fact that HIV-infected cases presented at a younger age could be argued that this is because of lead-time bias as patients with HIV are likely to have more frequent presentation at healthcare institutions. However, this is not supported by data from this cohort in which HIV-infected cases presented with a similar stage of advanced malignancy similar to those without HIV infection. This is likely to reflect the lack of strategic screening protocol for HCC in South Africa.

5.1.7. Effect of gender

There were three and a half times more males than females in this HCC cohort. This is in keeping with literature where male gender has been described as a risk factor of developing HCC (Kew, 2000, El-Serag, 2012). A Taiwanese study reported a male to female ratio of 4.5:1 among a cohort of HCC patients (Lee et al., 1999). The reason for males being more prone to developing HCC than females has been ascribed to higher levels of androgens in men (Wu et al., 2010a). HBV transgenic mouse models where genes coding for the androgen receptor within the liver were knocked-out showed that the lack of androgen receptors

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reduces HBV replication and is protective against development of HCC when the mice were exposed to carcinogenic nitrosamines (Wu et al., 2010a). Males historically have a higher prevalence of hepatitis B that would also explain the greater proportion of males than females that were diagnosed with HCC over the recruitment period (El-Serag, 2012). Other possible factors previously invoked to explain the male preponderance of HCC include an increased exposure to hepatocarcinogenic aflatoxins due to higher food intake and iron overload arising from consumption of traditional alcoholic beverages brewed in iron pots (Kew, 2013). However, no objective data in this cohort exists to support the assertion that males have higher aflatoxin exposure compared to females because of greater food intake. Comparing gender differences in the concentration of urinary metabolites of aflatoxin or other plasma biomarkers such as serum aflatoxin B1 (AFB_1) -albumin adducts may confirm or disprove if males have greater exposure to the fungal toxin and hence greater risk.

5.1.8. Lifestyle risk factors

5.1.8.1. Alcohol consumption

There was a low prevalence of self-reported alcohol dependence of only 22% (95% CI: 14-30) among the HCC cases from this study. Surprisingly, cases reporting alcohol dependence had a significantly higher mean age (52.7 years, 95% CI: 46.3-59.2) compared to those without alcohol dependence (44.0 years, 95% CI: 40.4-57.5) despite the fact that some of the cases also had HBV infection and one would expect a synergistic interaction that might result in earlier development of malignancy in cases exposed to both risk factors. This may be a result of under-reporting of alcohol misuse which is a well-recognised phenomenon. South Africa is rated as the country with highest alcohol consumption (>12.5 litres/capita/year) amongst all African countries (Ferreira-Borges et al., 2016) and alcohol misuse is a known risk factor for HCC development. Alcohol dependence was seen in 35% of cases that did not have any evidence of exposure to HBV or HCV in this study.

5.1.8.2. Aflatoxin exposure

This HCC study did not directly measure aflatoxin exposure but instead investigated peanut consumption as a proxy of aflatoxin exposure especially among cases that consumed home-grown peanuts which are more likely to be improperly stored. There was a prevalence of 22% for consumption of home-grown peanuts. The prevalence of aflatoxin contamination of food has previously been described to range from about 2.0% to 25% in the Eastern Cape province

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of South Africa (Van Rensburg et al., 1985). The questionnaire to collect aflatoxin exposure data was probably not optimal; it should have captured maize meal consumption which also carries the risk of mycotoxin contamination. Maize meal is a staple of many Black Africans who prepare it as pap. Despite the limitation of asking about peanut consumption only, previous data from South Africa show that peanuts carry the highest risk of contamination with more than 70% (Van Rensburg et al., 1975).

5.1.8.3. Iron overload

The consumption of large quantities of traditionally brewed beer that would be associated with iron overload was 26% of the entire cohort. However, none of the HCC cases in this study had a clinical history of hepatic siderosis. With increased modernisation, it can be expected that the role of dietary iron overload, associated with traditional beer consumption, in the development of HCC will diminish. Dietary iron overload is a risk factor that is common in rural Africa where people consume traditional beer that is brewed in iron pots or drums resulting in iron leaching into the brew (Walker and Arvidsson, 1953). Apart from dietary iron overload, hereditary hemochromatosis may also lead to the development of HCC (Kew, 2009).

5.1.9. Familial history of HCC

A low proportion of only 15% of the HCC cases reported a family history of liver disease with an even lower 5% reporting a family history of HCC. Having a family history of liver disease did not impact on the mean age at HCC presentation. A family history of liver disease is a recognised risk factor for the development of HCC, explained by familial clusters of HBV infection as well as shared exposure to environmental carcinogens coupled with a genetic disposition to cancer development (Yu et al., 2000). A study from Italy reported that a family history of HCC and the presence of HBV infection increased the risk of developing HCC 72 times relative to those without a family history of liver disease and with no HBV infection (Turati et al., 2012). Family history of liver disease has also been independently associated with increased risk of developing HCC and this probably points to the contribution of host genetics apart from the presence of other risk factors (Yu et al., 2000). However, the accuracy of family history data may be compromised by recall bias especially in patients with malignancy as they link unconnected events to the development of HCC. There is also the possibility of the data being inaccurate due to break-up of families and loss of the traditional family structure.

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5.1.10. Clinical presentation

Of 107 HCC cases, 72 (67%) presented with an abdominal mass which is consistent with the previous studies of HCC among African patients (Kew, 1981, Umoh et al., 2011). An abdominal mass in HCC is a sign of late presentation and is indicative of a tumour that has grown big. This in turn may be an indicator of the lack of screening for HCC in at-risk patients. Ascites which reflects decreased synthetic capacity of the liver was seen in 33% of cases. There was a low prevalence of concomitant liver cirrhosis among the HCC cases with only 23% of patients at the time of HCC presentation. This rate is lower than previously reported cirrhosis rates in South Africa and indeed in much of sub-Saharan Africa. Cirrhosis prevalence in HBV-associated HCC among African patients has previously been reported to be in the range between 44-63% (Paterson et al., 1985, Kew, 1989, Yoshida et al., 1994).

The low prevalence of cirrhosis in this cohort may in part due to under-reporting, lack of contact with gastroenterologists prior to diagnosis of malignancy and also due to the low utilization of histologic examinations for the diagnosis of HCC. The lack of histologic diagnosis of HCC is itself an indicator of cancers that are diagnosed when they are far progressed such that clinical diagnosis is often sufficient without needing to subject patients to a procedure that has a risk of post-procedure bleeding complication, especially in those that have liver-disease related coagulopathies (Terjung et al., 2003). The under-diagnosis and under-reporting of cirrhosis is not unique to sub-Saharan Africa as studies from the USA have also shown that there is 24-30% rate of not recognizing prior cirrhosis among HCC patients (Singal et al., 2012, Walker et al., 2016). The inability to detect underlying cirrhosis might be reflective of poor HCC surveillance because most algorithms are based on the monitoring of cirrhotic patients in order to have early diagnosis of HCC yet most cases with undiagnosed cirrhosis will present with more advanced cancer (Walker et al., 2016). Moreover, in places where HCC is common, more than 50% of the patients will present with asymptomatic cirrhosis (Ahmet et al., 2013). Therefore any screening modality that is targeted at cirrhotic patients would also exclude more than 50% of patients at high-risk of HCC.

The results showed that a significant proportion (70%) of the HCC cases presented with elevated concentrations of serum AFP greater than 400 μg/L. Serum AFP concentration >400 μg/L is considered diagnostic for HCC with specificity of 99.4% (Stefaniuk et al., 2010). However, the European Association for the Study of the Liver (EASL) and the American

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Association for the Study of Liver Diseases (AASLD) no longer recommend the measurement of serum AFP for the diagnosis and/or prognosis of HCC because it is deemed to be not sensitive or specific enough as it can be elevated in HBV infection without the presence of malignancy and may also remain within normal levels in some patients that have well-differentiated small HCC lesions (Bruix et al., 2011, EASL-EORTC, 2012). However, the position of EASL and AASLD regarding use of serum AFP in HCC surveillance and diagnosis has been challenged with others suggesting that AFP is still important in diagnosing HCC especially when the serum concentration is extremely elevated (El-Serag and Davila, 2011, Marrero and El-Serag, 2011).

ALT is a liver specific enzyme, the serum concentration of which may increase following liver injury (Giannini et al., 2005). Only 30% of HCC cases had overt evidence of liver injury as evidenced by serum ALT levels >80 U/L. The utility of ALT in HCC screening and prediction is inconclusive with some research groups concluding that use of ALT is inadequate as a predictor of HCC risk (Hann et al., 2012). However, a Taiwanese study described ALT as being “best able to predict HCC risk even among subjects with unknown or HBV- or HCV-negative infection status” (Wen et al., 2012). An algorithm was developed for predicting HCC in cirrhotic HCV-infected patients which combine ALT together with AFP, platelets and the patient’s age (El-Serag et al., 2014). In addition, the risk estimation for hepatocellular carcinoma in chronic hepatitis B (REACH-B) model was developed to predict the risk of developing HCC among Asian patients with CHB and it incorporates ALT together with gender, age, serum ALT concentration, HBeAg status, and serum HBV DNA load (Yang et al., 2011). It therefore appears that the ALT is useful when combined with other markers to predict HCC.

Early diagnosis of HCC is associated with better survival (5-year survival rates near 70% with resection or liver transplantation) whilst median survival is usually <1 year for those that present with advanced malignancy (Patel et al., 2015, Singal and El-Serag, 2015). More than 60% of all cases in this study presented with advanced malignancy as shown by the stage of presentation. These results suggest that there is room for improvement: Currently, there is limited recognition of at-risk patients, poor surveillance for HCC of patients with known HBV infection and/or inadequate guidelines for screening in sub-Saharan Africa (Singal and El-Serag, 2015). Data from elsewhere suggests that early diagnosis of HCC in HBV-infected patients has better outcomes than in hepatitis C because of better liver reserve and reduced hepatic inflammation (Njei et al., 2015). However, the challenge of late diagnosis of HCC is

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not limited to Africa only as 46.2% of HCC cases in the USA are diagnosed at an early and localized stage and most of the patients will not receive curative therapy (Njei et al., 2015, Singal and El-Serag, 2015). The situation becomes bleak if one considers the fact that the diagnosis rate of patients with hepatitis B in America is less than 40% and that an even smaller percentage (<10%) of those that are eligible for hepatitis B therapy are on treatment (Cohen et al., 2011). Clearly, awareness of HBV and ultimately HCC needs to be raised amongst the healthcare providers as well as the general public, especially those that are at risk of infection.

5.1.11. Molecular characteristics of HBV

5.1.11.1. Effect of HBV genotypes on HCC presentation

HBV genotype A has been described as having four times the hepatocarcinogenic potential of the non-A genotypes within the South African setting (Kew et al., 2005, Kew, 2008). Genotype A made up 79% of the sequenced isolates in the HCC study. Other genotypes observed were genotype D in 16% and genotype E in 5%. Genotype E was detected among two HCC cases of West African origin where it predominates. With increased migration within Africa, the distribution of genotypes may shift. New genotypes may be introduced from other regions reflecting the interaction between individuals from different geographical regions. Some European and North American countries have already seen introduction of foreign genotypes among immigrant populations (Palumbo et al., 2008, Pourkarim et al., 2014, Martinez et al., 2015). In addition, there is a possibility of the formation of recombinants as a result of re-infection, especially in highly endemic settings (Pourkarim et al., 2014). The study results showed an effect of HBV genotypes on age of onset of HCC with the median age of those infected with genotype A being lower than that of genotype D cases by about 17 years. The finding that cases infected with genotype A were significantly younger than those infected with non-A genotypes is consistent with the results by Kew et al. who described a mean age difference of almost 7 years (Kew et al., 2005). It is not clear as to why there exists such a difference between genotype A and D in terms of age at presentation of HCC but perhaps the absence of HBeAg and the basal core promoter mutations in genotype D may provide an explanation. HBeAg, which is a marker of high HBV replication, is a known risk factor for HCC development (Yang et al., 2002c). Prospective studies have shown that patients who are positive for HBeAg have an increased risk of developing HCC compared to those who are HBsAg but HBeAg negative (Yang et al., 2002b, You et al.,

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2004). However, the data is not clear as to whether there is also rapid progression to HCC due to HBeAg positivity.

5.1.11.2. Precore mutations

The G1896A mutation which translates to a W28* change in the precore region frequently occurs in genotype D abrogating the synthesis of HBeAg that acts as a tolerogen to the immune system and is thought to hold a lower hepatocarcinogenic potential as the mutation is not detected in all HCC tissues (Manzin et al., 1992). The W28* mutation was observed in all the genotype D sequences and in a single genotype E in this study. This data is consistent with occurrence of W28* mutation among genotype D sequences and not among genotype A (Kramvis et al., 1998). However, the results also showed that some patients were infected with mixed virus populations as their plasma still had HBeAg despite the presence of the precore stop codon mutation. Two sequences showed a rarely described Q2* stop codon mutation whose significance in hepatocarcinogenesis is not well understood (Laras et al., 1998). The Q2* stop codon is characteristic of HBV genotype G (Stuyver et al., 2000, Kato et al., 2002). One of the cases with the Q2* mutation had serum ALT concentration >40 IU/ml. Whether the detection of this Q2* mutation may actually suggest infection with two viruses of different genotypes would need to be confirmed by clonal sequencing which has not been done in this study.

5.1.11.3. BCP mutations

The basal core promoter (BCP) region of HBV guides the transcription of precore and core RNA which code for HBeAg and the HBV core antigen, respectively (Buckwold et al., 1997). The double 1762T/1762A BCP mutation causes a reduction of HBeAg synthesis through suppressing transcription of precore mRNA (Alexopoulou, 2009). There was a high occurrence of the double 1762T/1764A BCP mutation which was detected in 64% of the cases. The 1762T/1764A double BCP mutation frequently occurred with the 1753C mutation in 12 cases. This mutation was more frequently detected in the HIV-uninfected than in HIV-infected cases. The double 1762T/1764A BCP mutation has been associated with an increased risk of developing HCC (Zhang and Cao, 2011). Although the mutation is detected in HBV-infected patients without HCC its prevalence is greater in those with HCC suggesting that it may play a role in hepatocarcinogenesis (Zhang and Cao, 2011). Previous studies in South Africa have detected the mutation in 11-35% of CHB patients without HCC (Baptista et al., 1999, Selabe et al., 2007, Andersson et al., 2013, Makondo et al., 2013). A

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study from Taiwan previously reported that the likelihood of the BCP mutations parallels the development of liver disease with its prevalence increasing from 3% among inactive carriers up to 65% in those with HCC (Kao et al., 2003). Interestingly, the occurrence of the triple mutation BCP mutation pattern T1753C, A1762T and A1764G was associated with an older median age at presentation. HCC cases with the T1753C, A1762T and G1764A mutation pattern presented at median age 53 years (IQR: 50-60) compared to 37 years (IQR: 29-45) among those without any BCP mutation. A study among Korean patients with HCC has previously described that older HCC patients were more likely to have the A1762T, G1764A double mutations than younger HCC patients (Lee et al., 2011). This probably suggests that a longer time with infection may be needed for the mutation patterns to develop. Indeed, it has been described that the frequency of BCP mutations increases as the infections progressed from asymptomatic HBV carriage to chronic disease and then to HCC (Zheng et al., 2011, Yang et al., 2016). The change across these phases of HBV infection is a function of time and this probably explains the higher median age of HCC cases with the triple mutation pattern compared to those without the triple mutation pattern.

The HBV precore region partially overlaps with the X region and mutations in the former also result in changes in the latter. The T1753C, A1762T and A1764G mutations in the BCP result in amino acid changes K127T, K130M and V131I in the X region (Zhang and Ding, 2015). HBx protein is a known non-specific transactivator of cellular and viral genes that has been described to play a role in hepatocarcinogenesis. Some of the cellular genes that are affected by HBx are involved in signalling processes, cell cycle checkpoints, apoptosis and protein degradation (Murakami, 2001, Tang et al., 2006). The K127T, K130M, V131I and F132Y mutations were detected among the HCC cases. The occurrence of the K127T and F132Y mutants is consistent with literature describing that these changes usually occur with progression of HBV infection from CHB to cirrhosis and HCC (Guo et al., 2008, Liu et al., 2009, Yan et al., 2015). A combination of the K127T, K130M, V131I and F132Y mutations has been shown to reduce expression of p53 tumour suppressor gene in hepatoma cell lines (Yan et al., 2015). The K127T (1753C) mutation has been described among HCC cases only and not in asymptomatic HBV carriers in South Africa (Baptista et al., 1999).

5.1.11.4. Drug resistance mutations

Despite a number of HIV/HBV co-infected HCC cases being on HAART that comprised lamivudine as the only anti-HBV drug, the frequency of drug-resistance mutations was low

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with only 3/43 (7%) having changes that are associated with drug resistance. Curiously, the most common mutation in the reverse transcriptase region was the L80I/V mutation that has been described as a compensatory mutation associated with resistance to lamivudine and telbivudine (Ogata et al., 1999, Warner et al., 2007). The mutation commonly develops after the M204V/I mutation restoring replication fitness by as much as 150% (Warner et al., 2007). The L80V occurred together with the M204V + L180M in an HIV-infected case while the L80I was in conjunction with the M204I + L180M in another HIV-infected patient. The development of these mutations is associated with HAART and indeed data showed that one of the HIV-infected patients was on lamivudine while drug therapy data for the other case was not available. The infrequency of resistance associated mutations in the HBV mono-infected patients is probably a reflection of the low proportion that are diagnosed in time to be placed on therapy. It may be expected that had more HBV-mono-infected HCC cases been diagnosed earlier, they would most likely have been on therapy with lamivudine and more resistance mutations would have been observed. Resistance to lamivudine has been described to reach as much 80% after 5 years on therapy among patients infected with HBV (Bartholomeusz and Locarnini, 2006, Ghany and Doo, 2009, Kamili et al., 2009).

5.1.11.5. Detection and vaccine escape mutations

Escape mutations in the surface antigen region of HBV, as annotated by the Max Planck Institute for Informatics, were detected in 14/43 (33%) of HCC samples. The most frequent of these mutations, P120T, has been associated with vaccine, therapy (immunoglobulin) and detection escape. The P120T sits in the major hydrophilic region 2 of the surface antigen covering amino acids 120-123 and mutations in this region may cause impaired detection in immunoassays (Tian et al., 2007). The P120 mutation was previously shown to markedly reduce the binding of antibodies mapped to amino acids 139-147 as well as to amino acid 122 (Wallace et al., 1994). The mutation is thought to arise due to immune pressure (Wallace et al., 1994, Sheldon and Soriano, 2008). More importantly in terms of pathogenesis, the P120T mutations is thought to cause impaired secretion of HBsAg from hepatocytes despite normal transcription levels (Amini-Bavil-Olyaee et al., 2010). It has been postulated that failure to secrete HBsAg leads to retention within hepatocytes leading to cellular stress which could be a trigger for carcinogenesis but this would have to be confirmed by further functional studies (Pollicino et al., 2014, Li et al., 2016b). Stop codon mutations other than the escape mutations were also detected in the surface antigen region and the results showed that these were part of mixed populations of viruses. The W182* mutation is associated with the V191I

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change in the overlapping reverse transcriptase region and leads to decreased viral replication (Yang et al., 2002a, Sheldon and Soriano, 2008). The V191I mutation has been described to occur in patients receiving therapy using any one of the drugs- adefovir, famciclovir, lamivudine and tenofovir (Seigneres et al., 2000, Wakil et al., 2002, Yang et al., 2002a, Sheldon et al., 2005, Sheldon and Soriano, 2008, Margeridon-Thermet et al., 2009). The L216* has also been described in samples from naïve and nucleotide reverse-transcriptase inhibitor (NRTI)-experienced patients and is associated with silent mutations within the reverse transcriptase region (Margeridon-Thermet et al., 2009). The significance of these mutations in pathogenesis of hepatitis B is not well understood and there is a need for further functional studies.

5.1.12. Occurrence of HCV infection on presentation of HCC

Exposure to HCV infection as evidenced by the presence of antibodies to HCV was 9.9% (95% CI: 4.1-15.7) in this study. The rate of active infection with HCV among HCC cases was lower with only 63% of the anti-HCV positive subjects having detectable HCV RNA translating to about 5% of the entire HCC cohort. The mean age ± standard deviation at presentation with HCC among those with HCV antibodies was older relative to those that were HCV negative being 57.5±16.4 years compared to 44.6±14.7 years respectively, p=0.01. The data is consistent with the known pathogenesis of HCV among African patients where cases present with HCV presented 20 years later compared to those that were not infected with HCV and were likely to be HBV-infected (Kew et al., 1997, de Martel et al., 2015, M'Bengue et al., 2015). The difference in age at presentation perhaps reflects the effect of the age at which one becomes infected with either HBV or HCV and also different rates of progression and pathogenesis of the infections (Kew et al., 1997, MacDonald et al., 2008). While hepatitis B in sub-Saharan Africa is traditionally spread from child to child through horizontal transmission, hepatitis C tends to be acquired later in life through iatrogenic transmission in resource-limited countries and through injecting drug in resource-rich countries (MacDonald et al., 2008). HCC in HCV-infected patients will only develop where there is underlying cirrhosis while this is not necessary for HBV-driven hepatocarcinogenesis (MacDonald et al., 2008). In addition, HBV integrates into the host genome causing genomic instability whereas HCV does not, underscoring the different pathogenic mechanisms in these two infections (MacDonald et al., 2008). The results showed a tendency towards greater exposure to HCV among males as only one female had evidence of HCV exposure which is

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in agreement with previous findings from other African countries (Andoulo et al., 2014). The results also show evidence of co-infection of HCV and HBV although the sample number was too low to draw meaningful conclusions.

South Africa is described as predominantly having HCV genotype 5a in circulation with prevalence ranging from 54% up to as much as 72% (Prabdial-Sing et al., 2008, Gededzha et al., 2012, Musyoki et al., 2015). This study detected genotype 1a and 1b in addition to 5a. However, the number of sequenced HCV isolates in this study is too low to make meaningful conclusions about the effect of these genotypes among South African HCC cases.

5.1.13. Limitations of the HCC Study

This current study only recruited HCC cases who were seen at the participating recruitment sites that are tertiary referral centres. As a result, many HCC cases would have been missed because of not getting to these referral centres thereby introducing recruitment bias into the study. Patients presenting with advanced HCC at primary health facilities may fail to live long enough to be seen at a referral centre. It is also plausible that a number of potential HCC cases could also have been missed through misdiagnosis and under-diagnosis at the primary health centres thus potentially excluding them from enrolment into the study. Sequencing of HBV was done in separate segments and it is thus impossible to determine whether the mutations affected virus fitness or not and if they were on the same virus particle. In addition, mutations were tested in viral DNA isolated from plasma and this may not necessarily reflect the diversity of HBV within the malignant hepatocytes. Collection of biopsy samples when diagnosing HCC and where it can be safely performed would enable sequencing of virus in malignant tissue and comparisons to be made as to whether the virus sequence is the same as that which is isolated in peripheral blood. More cases could also have been recruited from the participating sites in Gauteng if there were study nurses stationed at the recruiting units.

5.1.14. Implications of the HCC Study results

The first and best step approach to tackling HCC would be to increase awareness of hepatitis B among primary health care providers and to adopt a public health approach in the testing and diagnosing of viral hepatitis infections, similar to the approach toward HIV. It is currently not easy to get HBV mono-infected patients onto antiviral therapy in the state sector yet the same is readily available for HBV/HIV co-infected patients. There is therefore a need to recognize HBV infection as an important and independent disease entity with a significant

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effect on public health. Only then will it also become possible to allocate the necessary resources for screening, treatment and care of people with CHB. Importantly, accurate and complete data on the rate of diagnosis of hepatitis B is not widely available for African countries and this perpetuates the cycle of mostly diagnosing hepatitis B only after a diagnosis of HCC has been made. The HCC results also bring into question the suitability of current hepatitis B treatment and HCC screening and surveillance guidelines for the South African setting. Many of the guidelines work well in places where access to sophisticated equipment and testing are readily available but the results show that a sizeable number of HBV-infected patients develop HCC with no underlying cirrhosis. Therefore, using significant fibrosis or cirrhosis as checkpoints to begin therapy or start screening for HCC have been shown to be unsuitable for the South African setting. There is a need to identify patients with high HBV viral loads and those who are HBeAg positive as they are highly infectious and likely to be the source of ongoing infection within the communities.

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5.2. Liver Fibrosis and Immune Markers Study

Liver fibrosis refers to the excessive deposition of extracellular proteins including collagen, usually in the setting of a chronic liver disease (Bataller and Brenner, 2005). Chronic viral hepatitis due to HBV may cause liver fibrosis which may lead to cirrhosis and ultimately in the development of HCC (Pellicoro et al., 2012). Clearance of HBV and disease pathogenesis are mediated by the adaptive immune response (Guidotti and Chisari, 2006). Patients who are able to clear hepatitis B infection have a vigorous, polyclonal T-cell response to multiple antigenic epitopes (Chisari et al., 2010). Meanwhile, patients who develop chronic infection with HBV have a weak adaptive immune response that causes continuing hepatocyte injury, regeneration, non-resolving inflammation, DNA damage and dysregulation of cell growth genes that may lead to liver cirrhosis and HCC (Chisari et al., 2010). Liver fibrosis is reversible but may progress to cirrhosis and eventually to HCC if the underlying stimuli are not eliminated (Crane et al., 2012). The development of CHB is linked to acquisition of infection in early life thus implicating an immature immune system in enabling establishment of chronicity. Early life is characterized by an immune tolerant immune status and early infections prior to the switch to pro-inflammatory status gives viruses an advantage. However, the change to the immune clearance phase of HBV is associated with immune mediated liver damage that can be seen histologically on liver biopsy as necroinflammation with varying degrees of fibrosis and elevated liver transaminases (Chang, 2007).

5.2.1. Immunologic and virologic markers

It is well-known that HIV co-infection has an adverse impact on the natural progression of HBV infections. There is little data describing the effect of HBV infection on the progression of HIV. This Immune Study had a cross-sectional design where only HIV and HBV/HIV co-infected patients who were on HAART for at least three months were examined. HAART duration of at least three months was considered in order to eliminate extreme data variability due to uncontrolled HIV replication. HIV viraemia is known to decrease to <25 copies/ml following 8 weeks of treatment with combination therapy in adherent patients (Perelson et al., 1997). Clinic matched controls that were not infected with HIV or HBV were also recruited in order to establish baseline expression of immune markers.

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5.2.1.1. CD4 cell counts

The results showed that while on HAART administration, HBV/HIV co-infected patients had lower CD4 cell counts compared to those infected with HIV alone. This finding is consistent with results from elsewhere (De Luca et al., 2002, Idoko et al., 2009, Wandeler et al., 2013). The Swiss HIV Cohort Study reported that despite 3 years of HAART, HBV/HIV co-infected individuals had impaired CD4 cell count recovery relative to those with resolved HBV infection and HBV-uninfected patients (Wandeler et al., 2013). A study from Nigeria also showed lower CD4 cell counts in co-infected individuals compared to those with HIV mono-infection prior to and 48 weeks after HAART (Idoko et al., 2009). However, other studies have not detected a significant difference in CD4 cell counts between HIV-mono-infected and co-infected patients (Konopnicki et al., 2005, Hoffmann et al., 2008). It should be noted that this current study (Immune Study) had a small sample size compared to previous studies. Some of the reasons that have been postulated for the decreased immune recovery include upregulation of apoptotic pathways in co-infection also sequestration of lymphocytes within the spleen following HBV-driven liver fibrosis (Nebbia et al., 2012, Wandeler et al., 2013). Also, a lower initial CD4 count (<350 cells/µL) at initiation of therapy is indicative of greater T cell depletion and the restoration is incomplete compared to those patients that are stared on therapy earlier with a CD4 cell count above 350 cells/µL (Robbins et al., 2009). The CD4 cell count at HAART initiation was unknown but could also be a factor.

5.2.1.2. HIV RNA detection

Despite similar HIV treatment duration, the HBV/HIV group had a higher but non-significant proportion of individuals with persistent HIV RNAemia compared to the HIV group with prevalence rates of 27.8% and 18.1%, respectively. HIV viral loads above 1000 copies/ml were detected in 6/33 (18.1%) of co-infected and 3/33 (9.1%) of HIV mono-infected participants. This data is similar to results of a Chinese study that reported higher but non-significant detectable HIV RNA among co-infected patients compared to HIV-mono-infected after 37 months of HAART (Yang et al., 2014). The World Health Organization and the South African Department of Health defines HIV virological treatment failure as "a persistently detectable viral load exceeding 1000 copies/ml (that is, two consecutive viral load measurements within a 2-month interval, with adherence support between measurements) after at least six months of using antiretroviral drugs" (WHO, 2013). The study cannot determine whether the patients that had detectable HIV RNA were failing

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therapy or if this was due to non-adherence. The detection of HIV viremia among the participants in this study is a cause for concern. It has been reported that the effect of HBV co-infection in HIV participants on HAART becomes more apparent with longer duration of therapy causing more frequent detection of HIV RNA and accentuating the delayed recovery of CD4 cells (Yang et al., 2014). A decreased CD4/CD8 ratio despite effective HAART is associated with continuous immune dysregulation, immune senescence and is predictive of non-AIDS morbidity and mortality (Lu et al., 2015). The results showed that co-infected patients had significantly lower median CD4/CD8 ratio compared to those infected with HIV alone. This is explained by the fact that normalisation of CD4/CD8 ratio is solely based on the recovery of the CD4 cells (Lu et al., 2015).

5.2.1.3. HBV DNA detection

This study showed that HBV mono-infected subjects more frequently had detectable HBV DNA compared to the co-infected patients; 49% vs 73%, p=0.03. However, the carriage of HBeAg, although not statistically significant, was higher in the co-infected (28%) compared to the HBV-mono-infected patients (13%). HIV infection negatively impacts the natural progression of hepatitis B by increasing rates of HBV replication and reactivation of previous infection (Hoffmann and Thio, 2007). In addition co-infection decreases the chances of spontaneous clearance of HBsAg, causes higher occurrence of occult HBV, increases the rate of progression to cirrhosis and HCC and pushes up liver-related morbidity and mortality (Hoffmann and Thio, 2007, Puoti et al., 2008, Thio, 2009). HBV/HIV co-infection has also been shown to decrease response to treatment compared to persons with HBV mono-infection (Wandeler et al., 2013). Although the occurrence of HBV DNA in the HBV mono-infected subjects was not unexpected because not all the HBV-mono-infected subjects were on anti-HBV therapy, its presence among the co-infected patients who were on therapy with two drugs (tenofovir and emtricitabine) that are effective against HBV is a cause of concern. Questions may be justifiably raised as to whether patients are adherent to therapy especially in light of the non-detection of drug-resistance mutations which might have explained the detection of HBV viraemia. Also, the fact that almost 18% of co-infected individuals had detectable and quantifiable HBV DNA and HIV RNA is probably indicative of non-adherence to therapy. The low rate of drug-resistance mutations may be explained by the fact that all co-infected patients were on tenofovir and emtricitabine and also that Sanger sequencing was used for mutation detection as compared to deep sequencing that has higher

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sensitivity to detect minor variants when present. Therefore, low level drug-resistance cannot be entirely ruled out. Drug-resistance mutations in HBV infection have been detected by deep-sequencing where they were missed by Sanger sequencing (Margeridon-Thermet et al., 2009, Margeridon-Thermet et al., 2013, Aoudjane et al., 2014). Tenofovir has a higher genetic barrier to resistance compared to lamivudine or adefovir because it acts at three different sites of HBV replication (Marcellin et al., 2008).

This study is not the first to report detectable HBV DNA in co-infected patients on therapy. Di Bisceglie et al. reported that after 6 months of HAART, only 7/18 (38.8%) of patients had suppressed HBV replication to undetectable levels, although it must be noted that the participants of that study were receiving lamivudine as the only drug effective against HBV (Di Bisceglie et al., 2010). A multinational study comparing HBV-active HAART regimens in co-infected patients reported that 63% of subjects receiving tenofovir with emtricitabine achieved HBV DNA levels <200 IU/ml after 24 weeks of therapy although it might be expected that the proportion of patients with detectable HBV DNA would be even higher if more sensitive assays were used (Thio et al., 2015). A study of Zambian and South African patients reported that only 15/21 (71%) of co-infected subjects achieved HBV suppression after 12 months using tenofovir plus lamivudine or emtricitabine (Hamers et al., 2013). It is recognized that not all HBV-infected patients on therapy will achieve virologic suppression especially those with high levels of replication such as in HIV co-infection and in those that are HBeAg positive who have been shown to have delayed viral suppression (Marcellin et al., 2008, Huang and Nunez, 2015, Thio et al., 2015).

5.2.1.4. HBeAg prevalence

The higher prevalence of HBeAg among co-infected subjects compared to HBV mono-infected subjects is a well-recognised effect of HIV infection (Oshitani et al., 1996, Hoffmann and Thio, 2007, Di Bisceglie et al., 2010, Andersson et al., 2013). Even with administration of an effective regimen such as tenofovir plus emtricitabine, not all patients will achieve HBeAg seroconversion. Kosi et al reported that after 5 years of continuous therapy HBeAg seroconversion was achieved by only 57% of HIV/HBV co-infected patients (Kosi et al., 2012). The higher prevalence of HBeAg among treated HBV mono-infected patients compared to those that were untreated in this study is probably a reflection of the reason why such patients had to be placed on therapy because they had active disease. It was beyond the scope of this study to determine the rate of HBeAg loss because of its cross-

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sectional nature. A longitudinal study starting from the time of therapy initiation would be able to determine the rate of HBeAg seroconversion to anti-HBe.

5.2.2. Non-invasive markers of liver fibrosis

Liver fibrosis was assessed using the Fibroscan 402 with a medium probe. In addition, APRI and FIB-4 scores were calculated using relevant laboratory tests and patients' ages.

5.2.2.1. Fibroscan

The results of transient elastometry (TE) showed that the HBV mono-infected group had the highest proportion of patients with significant or advanced fibrosis compared to all other groups. In addition, the median of TE values of the HBV mono-infected patients was higher than of the other groups. It was significantly higher than that of the HIV mono-infected group which was also significantly lower than that of the co-infected group. The results showing a higher proportion of patients with significant fibrosis among those with HBV/HIV coinfection compared to HIV mono-infected patients are consistent with findings from other groups where coinfection was associated with higher TE values (Castellares et al., 2008, Stabinski et al., 2011, Hawkins et al., 2013). HIV mono-infection has been described to independently increase liver fibrosis (Vlahakis et al., 2003, Tuyama et al., 2010). However, the finding of a higher proportion of HBV mono-infected individuals with advanced fibrosis compared to HIV mono-infected is not unexpected because of the fact that HBV is a hepatotrophic virus that drives liver pathogenesis. Furthermore, the majority of HBV mono-infected patients in this cohort were not on treatment allowing viraemia that drives fibrosis. Treatment of HBV infection has been shown to improve fibrosis and this probably explains the reason for lower TE values in the co-infected patients compared to those with HBV mono-infection (Marcellin et al., 2013). The study did not show a significant correlation between the TE scores and HBV viral loads in the HBV mono-infected group. However, the ungrouped analysis showed significant positive correlation between the median stiffness and both HBV and HIV viral loads. The fact that the same correlation was not observed in the grouped analyses is probably due to the effect of a larger sample size in the ungrouped analysis.

The results also showed that males had higher TE scores compared to females. This is an effect of gender that has been previously observed and a variety of reasons have been postulated (Corpechot et al., 2006, Roulot et al., 2008). It is thought that higher TE scores in

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males compared to females may be due to physiologic differences in the liver ECM (Wang et al., 1998). Mice models have shown that oestrogen inhibits HSCs from producing fibrotic collagen in dimethylnitrosamine-induced liver fibrosis (Yasuda et al., 1999). These differences may warrant an investigation of whether or not different cut-off values for the various fibrosis strata are needed according to gender.

5.2.2.2. APRI and FIB-4

Although the APRI and FIB-4 tests also showed the HBV mono-infected group with highest proportion of patients with moderate to advanced fibrosis, the differences between study groups were not statistically significant. All three non-invasive tests showed some degree of positive correlation, albeit poor. A study on Burkinabe patients evaluating these tests against the gold standard of liver biopsy reported that the APRI and FIB-4 performed poorly compared to the Fibroscan for the diagnosis of significant fibrosis (Bonnard et al., 2010). A separate study conducted in Senegal reported the superiority of the Fibroscan compared to the Fibrotest and Fibrosure among patients with CHB and normal ALT values (Mbaye et al., 2011). Other researchers have advised against the use of APRI for diagnosing advanced fibrosis in patients with HBV/HIV co-infection because of a poor positive predictive value despite the WHO recommending the use of APRI in resource-limited settings (Stockdale et al., 2016).

5.2.3. Microbial translocation markers

Primary HIV infection is known to cause damage of the defensive barriers of the gut-associated lymphoid tissue (GALT) leading to massive depletion of CD4+ T cells (Brenchley et al., 2006). Destruction of the GALT causes increased microbial translocation into the systemic circulation leading to systemic activation which is a hallmark of HIV infection (Brenchley et al., 2004, Brenchley et al., 2006, Brenchley and Douek, 2012). Microbial translocation has previously been measured using various biomarkers that include LPS, sCD14, LBP, or bacterial 16S ribosomal DNA (16S rDNA) (Abad-Fernandez et al., 2013).

CD14 plays a role in immunity and exists as membrane bound (mCD14) or soluble (sCD14) (Li et al., 2015). Soluble CD14 is a marker of monocyte/macrophage activation that is normally induced by exposure to LPS. The results from this study showed that co-infected patients had the highest plasma sCD14 levels compared to those infected with either HBV or HIV alone and also controls. The pattern of plasma sCD14 results suggest that HIV plays a

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more prominent role in shedding of sCD14 following activation by LPS as evidenced by HIV mono-infected patients having higher sCD14 quantities relative to those with HBV mono-infection. A study on patients from Thailand reported that co-infected patients that had not started HAART had higher plasma sCD14 than those with HBV mono-infection (Crane et al., 2014). Research in paediatric and older patients infected with HIV has shown that despite HAART, there is persistently higher immune activation punctuated by elevated levels of sCD14 and that this is not related to HIV viraemia (Wallet et al., 2010, Wallet et al., 2015). The results from this current study also support these findings because plasma sCD14 did not correlate with either plasma HBV DNA or HIV RNA in individual groups or total participants thus supporting the idea that gut microbial translocation is independent of current (on treatment) viral load. This also probably explains the reason for not detecting any differences in microbial translocation markers between HBV mono-infected patients that were on therapy and those that were not. A potential mechanism for continued monocyte activation may be due to blunted recovery of CD4 T cells during HAART (Dunham et al., 2014). However, this study cannot determine the rate of CD4 T cell recovery of the HIV-infected patients that were recruited because of its cross-sectional design.

Surprisingly, there was no difference between the controls and the HBV-mono-infected with regard to sCD14. This marker was expected to be significantly increased among the HBV-mono-infected patients compared to the controls. A role for sCD14 in pathogenesis of both acute and chronic hepatitis B has previously been described (Oesterreicher et al., 1995, Sandler et al., 2011, Li et al., 2015). The lack of a difference between the HBV mono-infected and controls is probably because hospital controls from the Gastroenterology Clinic were used and some of them may have had yet-undiagnosed inflammatory conditions associated with increased sCD14, such as inflammatory bowel disease (Pastor Rojo et al., 2007). It has been previously reported that patients with chronic viral hepatitis have significantly higher serum sCD14 levels compared to healthy controls (Oesterreicher et al., 1995, Steyaert et al., 2003). Interestingly, there was no correlation between Fibroscan values and sCD14 value for the entire cohort. No association of liver disease and sCD14 was reported previously (Crane et al., 2014). The significance of increased sCD14 in CHB has been questioned with suggestions that elevated levels of the marker (sCD14) may actually serve to protect liver function by downregulating the inflammatory cascade (Kitchens et al., 2001, Marchetti et al., 2012).

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LBP binds to LPS via the lipid A part of the endotoxin and transfers it to phagocytic cells through mCD14 or sCD14 (Park and Lee, 2013). The results from this study showed a trend towards increased plasma concentration of LBP in co-infected patients compared to those with mono-infection and controls. It has previously been reported that there was no significant difference in plasma LBP between patients with CHB and healthy controls (Steyaert et al., 2003). It is possible that the concentration of LBP may have been attenuated by HAART in the co-infected and HIV-mono-infected patients. In theory, HAART does not restore gut integrity and therefore continued elevations of LBP and sCD14 are expected in HIV-infected individuals despite HAART (Chen et al., 2014, Wada et al., 2015). A study in HCV/HIV co-infection reported that while sCD14 levels did not change with treatment duration, LBP was lowered by the administration of HAART and anti-HCV medication (Nystrom et al., 2015). The overall results from this study showed non-significant weak positive correlation between LBP and sCD14. This is consistent with other studies that have shown some degree of statistically significant and non-significant positive correlation between the two biomarkers in different disease entities (Redd et al., 2009, Abad-Fernandez et al., 2013, Gonzalez-Quintela et al., 2013). LBP and sCD14 were used in this current study rather than LPS because LPS has a short half-life in circulation and its measurement using the widely-utilized Limulus assay is subject to interference from other molecules in the blood where it may be bound to serum binding proteins (Ruiz et al., 2007). Measurement of sCD14 and LBP is considered as better than LPS because the two former biomarkers are more stable, long-lived and are markers of the body’s physiologic response to the latter (Ruiz et al., 2007). The overall finding from the microbial translocation markers and liver fibrosis markers would seem to suggest that gut translocation is not associated with liver stiffness in this treated cohort. Instead, viral replication is probably the driver of liver fibrosis according to the results of the ungrouped analysis.

5.2.4. Effect of HBV genotypes on liver fibrosis

Effect of HBV genotype on liver inflammatory activity and fibrosis has previously been shown for genotypes B and C where the latter was associated with worse liver disease and the former with development of HCC at a younger age (Kao et al., 2000). This study showed no significant association between liver fibrosis and HBV genotypes A, D and E using TE. There is little data from Africa reporting the effect of HBV genotypes on liver fibrosis apart from the association studies between genotype and replication as well as HCC development

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(Kew et al., 2005, Kramvis and Kew, 2005). Genotype D was previously described to be associated with more severe liver disease compared to genotype A among Indian patients but another study from the same country suggested that genotype D did not influence the progression of liver disease in CHB patients (Thakur et al., 2002, Gandhe et al., 2003). No clear association between HBV genotypes and liver diseases severity (determined using histologic examination of liver biopsy) was also observed from a French study (Halfon et al., 2006).

5.2.5. Cell-based immune activation markers

T lymphocytes are responsible for the pathogenesis as well as the clearance and resolution of CHB infections (Guidotti and Chisari, 2006, Chisari et al., 2010). However, very little is understood concerning the change of expression of activation markers by CD4+ T cells in either HIV infection or HBV/HIV co-infection. On the other hand, the percentage of CD8+ lymphocytes expressing CD38 and HLA-DR is well characterised, hence the discussion of activation markers is focussed on these cells (CD8+ T cells). CD8+ T lymphocytes mediate clearance of HBV infections from infected hepatocytes through cytolytic and cytokine-mediated non-cytolytic mechanisms (Thimme et al., 2003, Phillips et al., 2010). The results of the current showed that co-infected patients had highest percentage of CD4+ and CD8+ T lymphocytes expressing the immune activation markers HLA-DR and CD38. Furthermore, the results suggest that HIV infection is the driver of immune activation because HIV-mono-infected patients also had higher, although non-significant, percentage of cells expressing HLA-DR and CD38 on both CD4+ and CD8+ T lymphocytes compared to HBV-mono-infected patients. Interestingly, there was no correlation between the expression of immune markers of activation and liver stiffness as measured by any of the fibrosis markers. This may be due to the fact that the study measured immune activation in peripheral blood which may not be reflective of the hepatic environment. Research on the hepatic environment in HBV/HIV co-infected patients found no evidence of increased inflammation but rather apoptosis as the driver of liver disease (Iser et al., 2011). There is little data comparing the expression of the immune activation markers between co-infected and HBV mono-infected patients and where this was attempted, the researchers used subjects that were untreated for HIV. Significantly increased HLA-DR and CD38 among CHB patients compared to healthy controls has previously been described among Chinese patients (Cao et al., 2011).

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5.2.6. Cell-based immune exhaustion markers

It is known that chronic viral infections such as HIV, HCV and HBV cause a state of immune exhaustion in both CD8+ and CD4+ T lymphocytes as a way to protect from the effects of a continuous immune viraemia-associated activation leading to poor effector function (Zajac et al., 1998, Urbani et al., 2002, Day et al., 2006, Blackburn et al., 2009). Some of the markers that are upregulated in this state of immune exhaustion include CTLA-4, PD-1, Tim-3 and LAG-3 (Zajac et al., 1998, Urbani et al., 2002, Day et al., 2006, Blackburn et al., 2009). CD4 and CD8 T lymphocytes from co-infected and HIV mono-infected patients exhibited higher levels of immune exhaustion as shown by the percentage of cells staining positive for PD-1 compared to HBV mono-infected and control individuals. HBV co-infection seems to have a negligible effect on PD-1 expression in patients with HIV infection because there was no difference between the co-infected and HIV mono-infected subjects. However, there was a significant difference in expression of PD-1 on T lymphocytes of HIV mono-infected patients compared to controls. Surprisingly, expression of PD-1 on the CD8 T lymphocytes of controls was higher than that of the HBV mono-infected patients. This result may be reflective of underlying chronic inflammatory conditions among the hospital-based controls. Use of healthy controls might reflect a different result. There was a very low surface expression of the exhaustion marker CTLA-4 on T lymphocytes in this study. This is consistent with CTLA-4 being predominantly located in the intracellular compartments and barely detectable at the cell surface (Linsley et al., 1992, Leung et al., 1995, Valk et al., 2008). Detection of CTLA-4 might have been better investigated through intracellular staining or quantification of its mRNA transcripts (Valk et al., 2008).

5.2.7. Soluble immune markers

There were significantly higher systemic levels of a broad range of cytokines in the HBV mono-infected patients compared to the other groups. The effect of therapy within the HBV group was not apparent. This is most likely due to the fact that there was a wide range of treatment duration within the HBV group with individuals receiving tenofovir from between one week up to more than two years. Also, the sample size to compare individuals on therapy to those that were not on therapy was low. However, the finding that the HBV mono-infected group tended to have the highest serum concentration for the majority of cytokines is probably due to the fact that this group also had the highest (normal) CD4+ T cell counts and also the highest number of infected patients that were not on therapy. CD4+ T cells are the

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primary cytokine producers. Also, the HBV mono-infected group tended to have lower PD-1 expression on CD8 and CD4 T lymphocytes meaning that they had fewer cells with an exhausted phenotype. However, the levels of cytokines in HBV may be more indicative of "normal" rather than elevated and that the HIV-infected (mono-infected/co-infected) groups were suppressed.

The IL-1 family of cytokines (IL-1β and IL-1ra) were significantly increased in the plasma of HBV mono-infected patients compared to the HIV mono-infected and co-infected patients. The concentrations of IL-1β and IL-1ra had strong positive correlation with levels of IL-10, IL-12 and TNF-α. The concentrations of TNF-α, IL-4, IL-6, IL-10, IL-13, IL-17, Basic-FGF, G-CSF and IFN-γ were also highest in the HBV mono-infected group compared to all others. IL-1β is a mediator of inflammation that is produced by innate immune cells. It is important for host immunity in response to pathogens but its secretion in chronic disease such as CHB may worsen injury and also causes tissue damage in acute disease (Lopez-Castejon and Brough, 2011). IL-6 is an acute phase protein and a mediator of inflammation (Lan et al., 2015). IL-1ra is produced by a range of cells and is a natural inhibitor of the pro-inflammatory effects of IL-1β and IL-1α (Arend et al., 1998). The balance in tissues between IL-1b and IL-1ra is known to determine the outcome inflammatory disease. An excess of IL-1b relative to the antagonistic IL-1ra leads to structural damage (Arend et al., 1998). It was shown that an increased IL-1β/IL-1ra ratio in HCV infection was associated with increased liver fibrosis (Gramantieri et al., 1999). The role of IL-6 is HBV-driven liver disease is extensively described and its secretion follows after IL-1β and TNF-α induction (Lan et al., 2015, Schmidt-Arras and Rose-John, 2016). Levels of IL-6 have been shown to positively correlate with severity of hepatitis B with patients that have severe acute infection having higher concentrations compared to those with chronic active disease (Tan et al., 2010, Zhang et al., 2011a).

IL-4 is produced by T cells and its functions include the differentiation of naïve cells into Th2 cells, the regulation of humoral immunity and the activation of macrophages. In HBV infection, IL-4 has been associated with persistent replication of HBV and induction of tolerance through downregulation of IFN-γ production from Th1 cells (Li et al., 2016a). Higher serum levels of TNF-α, IL-4, IFN-γ and IL-2 have previously been described in CHB patients compared to controls (Jiang et al., 2010). Jiang et al also showed the manner in which cytokine secretion pattern switches from Th2 to Th1 following long term treatment with adefovir (Jiang et al., 2010). However, the effect of IL-4 is not as clear when one

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considers that treatment with adefovir was associated with increasing plasma concentration of the cytokine (Jiang et al., 2010) while another group treating CHB using pegylated IFN-α2a reported that the levels decreased in patients that exhibited virological response (Park et al., 2012). Using proteomic analysis, it was reported that IL-4 inhibited the expression of HBsAg and HBeAg in a "HBV stably transfected HCC cell line (HepG2.2.15)" (Yao et al., 2011). It has been hypothesized that IL-4 negatively regulates the immune system as a protective mechanism to reduce inflammation (Yao et al., 2011). Another Th2 cytokine is IL-13, which was significantly higher in the HBV mono-infected group compared to the HIV-mono-infected and controls. IL-13 shares the similar functional activities with IL-4 and the two cytokines engage with a common receptor α chain/signal transducer and activator of transcription protein 6 (STAT6) (Weng et al., 2009). Hepatic concentrations of IL-13 have been shown to positively correlate with amount of liver fibrosis in CHB and serum levels were also elevated in patients with hepatitis C compared to controls (Weng et al., 2009). The rate of detection of IL-13 was also reported to be higher in CHB patients (40%) compared to patients with occult HBV infection (8%) (Martin et al., 2009). This study had high detection rates for IL-13 compared to the report by Martin et al. The difference in detection rates of IL-13 and other cytokines is probably reflective of the variety of tests (Luminex vs ELISA vs qPCR) and matrices (hepatic vs serum) that different investigators have used.

The TNF family of cytokines is produced from T lymphocytes as well as macrophages. The cytokine is part of the acute phase proteins and has been associated with hepatic inflammation and fibrosis in CHB (Li et al., 2016a). Median plasma TNF-α concentration was significantly higher in the HBV mono-infected patients compared to those with HIV mono-infection. The levels of TNF-α were higher in the HBV mono-infected, but not significantly so when compared to the co-infected and control groups. Higher plasma levels of TNF-α and IFN-γ have been described in CHB patients compared to healthy controls (Zhang et al., 2011b). There was no significant difference between the HBV mono-infected and controls in this study because hospital controls were used who may not have been completely healthy although they did not have liver disease or symptoms of any infection. TNF-α is involved in the immunopathogenesis, inactivation and immune clearance of hepatitis B (Daniels et al., 1990, Suri et al., 2001, Phillips et al., 2010, Zhang et al., 2011b). TNF-α and IFN-γ were previously shown to be responsible for non-cytolytic control of HBV replication using various in-vitro models (Guidotti et al., 1996, Suri et al., 2001, Phillips et al., 2010). However, TNF-α and IFN-γ may also potentiate hepatic damage as demonstrated

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by the maintained secretion of the two cytokines in CHB patients with high HBV DNA and liver inflammation (Das et al., 2008).

IL-17 is the principle effector molecule produced from Th17 lymphocytes (Li et al., 2016a). The concentration of IL-17 was significantly higher in the HBV mono-infected group compared to the co-infected group only. Previous studies have described IL-17 being upregulated in CHB and has been linked with pathogenesis of liver cirrhosis and hepatocarcinogenesis (Wu et al., 2010b, Du et al., 2013). In light of these previous studies, the results of IL-17 in this study are puzzling because the HBV mono-infected and co-infected groups had higher proportions of patients with moderate to advanced liver fibrosis as determined by Fibroscan and levels of the cytokine would have been expected to be also high in the latter group. The effect of HAART on the expression of IL-17 in co-infected patients may provide clues and a longitudinal study tracking IL-17 levels in patients starting therapy may be useful in providing clarity.

Plasma levels IL-10 of the HBV-mono-infected group were significantly higher than that of the HIV mono-infected group but not of controls or the co-infected subjects. IL-10 is produced by Th2 lymphocytes and also macrophages. It has anti-inflammatory properties inhibiting the production of pro-inflammatory cytokines in hepatitis B hence its former name “cytokine synthesis inhibitory factor (Sabat et al., 2010, Li et al., 2016a). IL-10 is responsible for continual HBV replication and orchestrates liver fibrosis by inhibiting the secretion of IFN-γ (Li et al., 2016a). Increased serum IL-10 has been described in patients that were seropositive for HBeAg and HBV DNA with persistently elevated ALT compared to controls (Bozkaya et al., 2000, Das et al., 2012). No differences between controls and HBV mono-infected subjects were detected in this study probably due to the heterogeneity of the patients with HBV infection in terms of antiviral therapy and duration thereof.

Basic fibroblast growth factor (FGF) also referred to as FGF2 induces growth of fibroblasts and plays a role in angiogenesis during cancer development (Ribatti et al., 2007). The biomarker was significantly elevated in the plasma of HBV mono-infected patients compared to those with co-infection and HIV mono-infection. However, the levels in HBV-mono-infected patients were not significantly higher than in controls. Basic-FGF levels have been described to correlate with progression of HBV-driven liver disease and have highest expression in patients with HCC (Hsu et al., 1997, Jin-no et al., 1997).

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IP-10 is the only cytokine that had significant elevated expression in the co-infected group compared to all other groups. There was no significant difference in IP-10 expression between the mono-infected groups and the controls (refer to Figure 2-21). IP-10 is known to be associated with liver fibrosis progression in HCV infection (Diago et al., 2006, You et al., 2011). It appears that the increased concentration of IP-10 in this study was mainly due to HIV infection. IP-10 levels positively correlated with both HBV and HIV viral load in the co-infected group. IP-10 also had positive correlation with both LBP and sCD14 in the ungrouped analysis. There was significant positive correlation of IP-10 and expression of CD38 by CD8 lymphocytes in HIV-infected patients. HIV infection is known to be a potent inducer of IP-10 secretion from dendritic cells and monocytes and in turn IP-10 promotes HIV replication in macrophages and lymphocytes (Lane et al., 2003, Simmons et al., 2013). Increased IP-10 levels could thus be an indicator or biomarker of continued immune activation from viral replication and gut translocation in HBV/HIV co-infection.

Unexpectedly, TGF-β2 and TGF-β3 were detected at higher concentrations in the HIV-mono-infected group relative to the co-infected group. In addition, plasma levels of TGF-β2 in the HBV-mono-infected group were also higher than in the co-infected group. There was no significant difference in the plasma concentration of TGF-β1 across the four groups of subjects. These were unexpected findings as it was postulated that co-infected patients would have higher plasma TGF-β1 values compared to those with mono-infection and controls. TGF-β1 is known as a highly pro-fibrogenic cytokine that also has immunosuppressive properties (Cerwenka and Swain, 1999, Sacchi et al., 2015). However, the levels of TGF- β in this current study did not seem to correspond with the amount of liver fibrosis. The result may be due to HAART but the effect should also have been similar in the HIV-mono-infected group. In addition, other researchers have reported an inverse relationship between liver fibrosis and TGF-β1 levels in HCV-mono-infected and HCV/HIV co-infected patients giving rise to conclusions that high levels of the cytokine may actually be protective against liver fibrosis in HCV infections (Rallon et al., 2011, Sacchi et al., 2015). It would seem that the levels and effects of TGF-β1 in HBV infection is different from that in HCV infection because it has been reported that the cytokine occurs in greater amounts in patients infected with chronic HCV compared to those with CHB (Kirmaz et al., 2004). Furthermore, the results from this study suggest that TGF-β2 and TGF-β3 probably play a more immunosuppressive role in HIV infection than in HBV infection. Of the three TGF-β isoforms, the least is known about TGF-β2 and TGF-β3 with most literature having

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investigated the expression of TGF-β1. However, the concentrations at which TGF-β1 is detected are about 20-fold higher compared to those of TGF-β2 and –β3.

The chemokine macrophage inflammatory protein-1α (MIP-1α) was detected at significantly higher levels in the controls and HBV mono-infected compared to the HIV-mono-infected subjects. MIP-1α is one of the two isoforms of MIPs, the other being MIP-1β that also had a trend towards a higher concentration in controls and HBV-mono-infected subjects relative to those with HIV mono-infection. MIP-1α and MIP-1β have been described as CC chemokines that supress HIV replication and are produced by CD8 T cells (Cocchi et al., 1995, Vicenzi et al., 2000). MIP-1α has previously been demonstrated to be higher in HIV-infected patients compared to controls but decreasing with HAART administration (Ye et al., 2004). It would appear that MIP-1α levels in HIV-infected patients in this study were low because of normalization by the administration of HAART.

Some cytokines had poor detection rates across all groups. IL-15 was poorly expressed although the HBV-mono-infected group had the highest proportion of patients with detectable plasma concentration of the cytokine. Increased concentrations of IL-15 have previously been found in the liver environment of patients with active hepatitis attributed to infection to HCV infection (Kakumu et al., 1997, Golden-Mason et al., 2004). The cytokine is linked to the cytolytic activity of NK cells and the development of liver fibrosis and HCC (Kakumu et al., 1997, Zhang et al., 2011c).

The original hypothesis of the Liver Fibrosis and Immune Markers Study was that in patients infected with both HIV and HBV, HIV infection facilitates the development of liver fibrosis via immune activation in response to the leakage of gut microbial products into the systemic circulation. Instead, the study found that although there was increased microbial translocation (shown by increased sCD14) and elevated immune dysregulation (increased % CD8/CD38/HLA-DR and % CD8/PD-1) in HBV/HIV co-infected patients, the proportion of patients with moderate/advanced liver fibrosis (measured by Fibroscan) was higher in the HBV mono-infected group compared to those with co-infection. Also, the finding that there was no correlation between microbial translocation markers and liver fibrosis scores would seem to imply that microbial products are not the driver of fibrosis but rather viral replication. This is because median stiffness had significant positive correlation with both HIV and HBV viral loads in the ungrouped analysis. The relationship of active viral replication with fibrosis implicates active antigen-specific anti-viral responses as being important to pathogenesis of

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liver fibrosis. This would be probably be due to CTL destruction of hepatocytes, recruitment of macrophages promoting "healing" and stimulation of HSC-derived myofibroblasts to produce collagen.

5.2.8. Limitations of the study

The inability to obtain liver biopsies from the study participants makes it difficult to ascertain the accuracy of the results of the non-invasive tests for fibrosis although these have been suggested for use by the WHO. Fibroscan examinations could not be obtained on all subjects mainly due to visceral obesity. An effect of other undiagnosed infections such as cytomegalovirus on the expression of markers of immune activation cannot be ruled out although care was taken to exclude any patients with overt symptoms of infectious disease. The cross-sectional nature of the study makes it impossible to have a view of the temporal changes in the expression of immune markers and to determine how these are altered by duration on therapy. Another limitation is that the assessment of immune markers was not virus-specific. Use of viral antigen stimulation to assess T cell-specific responses of HBV- and HIV-specific cells may help in elucidation of the role of the specific viral infections. Immune activity in peripheral blood (cells and plasma) may not give a clear indication of events in the liver. Nevertheless, the data arising from this study is important in developing prospective studies to monitor prognosis of HBV infection, especially among the HBV-mono-infected patients. Most HBV-mono-infected patients were not on therapy because of being in the low replication phase of hepatitis B. However, there is still immune activity within the low replication phase that can lead to moderate fibrosis which may be a concern in the context of hepatocarcinogenesis. There is therefore a need for longitudinal African studies that will determine the clinical outcomes of patients that stay in the low replication phase for a long time vis-à-vis the finding that viraemia is the driver of fibrosis.

5.2.9. Implications of the Liver Fibrosis and Immune Markers Study

The results from the Immune Study highlight the under-recognition of hepatitis B as an independent disease that needs to be given more attention. The findings suggest that HBV-mono-infected patients seem to have worse clinical status than co-infected patients, with regards to liver fibrosis as measured using non-invasive tests, and this may be treatment related. HBV/HIV co-infected patients have easier access to effective treatment and monitoring than HBV-mono-infected patients. Such a situation leaves HBV-mono-infected patients at risk of disease progression such that they will only be seen at the clinic upon

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presentation with advanced disease such as liver cirrhosis and HCC. However, raising awareness may improve the diagnosis and care of patients infected with HBV who are usually asymptomatic until the infection has progressed substantially, hopefully allowing initiation of meaningful and life-saving therapy in time.

5.3. Options for the control of HBV-driven liver disease in Africa

It has been proposed that lessons learnt in responding to the HIV pandemic and also in also setting up initiatives such as the Global Alliance for Vaccines and Immunization could be put to use in advocating for lowering of prices of the diagnostic tests and medication for viral hepatitis infections (Lemoine et al., 2013). Another strategy that has been proposed is to merge HBV control programs with those for other infections (Mihigo et al., 2013). In the case of sub-Saharan Africa, the huge pool of HIV infected patients and the facilities and programs availed in response to the HIV pandemic offer an ideal opportunity for implementing HBV and HCC control programs alongside. Reports suggest that liver disease and HCC incidence have been rising among HIV-infected patients since the roll-out of HAART compared to the pre-HAART era (Brau et al., 2007, Rosenthal et al., 2009, Kew et al., 2010). HIV infected HCC cases are presenting at a younger age compared to those that are not infected with HIV (Puoti et al., 2004, Brau et al., 2007). The mechanism by which liver disease arises especially in HBV-infected patients in the context of HIV is still under-investigated. Most published research has focussed on HCV/HIV co-infected patients in developed nations while other research has focussed on the toxicity of antiretroviral medication on liver function and pathology. However, HCV is not as prevalent as HBV in sub-Saharan Africa and the pathogenesis of HCV is not similar to HBV (Mohd Hanafiah et al., 2013). It is therefore important to try and understand the pathogenesis of HBV-driven HCC in patients that are also infected with HIV.

5.3.1. Changing the epidemiology of HBV-driven HCC in sub-Saharan Africa

The motto “prevention is better than cure” is true and relevant for efforts to combat HCC. Although HBV is undoubtedly hepatocarcinogenic, infection with the pathogen is preventable by use of a vaccine which was first reported in 1976 and is recognized as the first cancer-preventing vaccine (Buynak et al., 1976, Beasley, 2009). It is estimated that achieving 90% HBV vaccination coverage with the first dose being given at birth would result in 84% decrease in mortality arising from HBV-related liver disease, which includes HCC,

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worldwide (Goldstein et al., 2005). In countries where HBV vaccine was first introduced such Taiwan, Thailand and The Gambia, a decrease in the incidence of HCC has already been observed (Beasley, 2009). In Taiwan, a 50% decrease in the incidence of HCC among children of the ages 6-14 years was seen after a period of just after 10 years following the introduction of infant vaccination (Chang et al., 1997). It is therefore of paramount importance to ensure good routine vaccination coverage for HBV within sub-Saharan Africa in order to prevent new HBV infections which may progress to HCC. Despite the WHO's recommendations for a birth dose, most African countries do not follow this because vertical transmission from mother to infant is thought to play a minor role in HBV transmission in the African setting. Early age of acquisition of HBV is the main risk factor for developing CHB, which poses the greatest risk for development of HCC with sub-Saharan Africa (Kiire, 1996). The majority of chronic HBV carriers in sub-Saharan Africa acquire the infection before the age of five years through horizontal transmission which may involve household contacts, playmates, cultural scarification rituals and unsafe medical procedures (Kiire, 1996, WHO, 2008). It is established that most HBV infections acquired in childhood progress to chronic infection due to the immaturity of the immune systems of children who fail to clear the virus thereby allowing chronicity to be established with a high risk of developing into cirrhosis and HCC (Pungpapong et al., 2007). In contrast, 95% of HBV infections acquired in adulthood resolve and do not establish chronicity (Tran, 2009). The fact that most chronic HBV infections found in adults within sub-Saharan Africa arise from horizontal transmission in childhood explains the reason why immunisation protocols in Africa are not designed to prevent vertical transmission (Burnett et al., 2005). However, there is evidence of decreased HBsAg carriage among vaccinated children which should eventually translate into decreased HCC incidence in adults over time (Tsebe et al., 2001, Viviani et al., 2008).

Despite relatively reasonable vaccine coverage rates of around 75% among infants within most of sub-Saharan Africa (rates according to 2014 WHO data on HepB3 immunisation coverage) , there remains a risk for vertical transmission from highly viraemic pregnant mothers as shown by data from Ghana and more recently South Africa (Candotti et al., 2007, Andersson et al., 2012, Andersson et al., 2013, Hoffmann et al., 2014). Data from South Africa suggests that more than 15% of HBsAg positive and HBeAg positive pregnant women regardless of their HIV status are at risk of transmitting infection to their infants (Andersson et al., 2012, Andersson et al., 2013). This risk will only be eliminated once a policy to actively screen all pregnant women for HBV infection is implemented. Until pregnant

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women that are infected with HBV are identified and treated with antivirals to reduce infectivity as well as having their babies vaccinated at birth and given prophylaxis, a pool of vertically-acquired infections will continue to exist which will become a source of horizontal transmission and later through sexual contact in adulthood to susceptible partners. Unfortunately, public health antenatal facilities in most African countries do not provide for HBsAg screening in pregnant women. It has been suggested that with the availability of a vaccine for HBV, maternal transmission has taken over as the "major source of HBV infection in the post-immunization era" (Chen et al., 2012). Breakthrough maternally-acquired infections are thought to result in chronic liver disease that has a higher risk of progressing to HCC compared to vertically-acquired HBV infections in non-vaccinated children (Chang et al., 2005).

CHB causes persistent inflammation which may progress to significant fibrosis but this can be stopped and even reversed by treating patients with CHB using drugs such as tenofovir and lamivudine (Dienstag et al., 2003, Hadziyannis et al., 2006, Mallet et al., 2007). However, the success of treatment hinges on being able to first diagnose people infected with HBV. It is not currently known what percentage of African patients with HBV are actually diagnosed but the findings from this study seem to suggest that most HBV infections in HCC cases are diagnosed too late, that is around the time that patients present with cancer showing that enough is not being done to ensure timely diagnosis and treatment of CHB. Treatment of HBV infection has been shown to improve prognosis of the infection and also to reduce the incidence of HCC.(Liaw et al., 2004, Yuen et al., 2007, Wu et al., 2014). Data on the efficacy of nucleos(t)ide treatment in preventing HCC among African patients is still lacking but would be extremely useful particularly taking into account the existence of other environmental and dietary carcinogens such as aflatoxin exposure and iron overload.

5.3.2. Improving diagnosis and prognosis of hepatitis B and liver disease in Africa

Early diagnosis and treatment of CHB and liver disease is a very important factor in determining outcome and in also changing the epidemiology of HBV. Identification of persons infected with CHB not only helps in getting them into care thereby preventing or at least delaying the onset of hepatic disease. Safe and effective options for treating CHB are available with antivirals such as tenofovir, entecavir and lamivudine that are effective in the treatment of HBV and can be used in the context of HBV/HIV co-infection. Early diagnosis

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of infections is also useful for breaking transmission clusters as susceptible contacts within households and also susceptible sexual partners can be vaccinated (Weinbaum et al., 2009). Current international guidelines suggest that all Africans with CHB above the age of 20 years should be screened for HCC at regular intervals to enable early diagnosis (Bruix and Sherman, 2005). A randomized trial performed in China showed that surveillance leads to detection of cancers at early stages thereby allowing for curative options such as surgical resection and also reduces mortality (Zhang et al., 2004). Despite the availability of these guidelines and the benefits that are expected, implementation remains a challenge because a huge proportion of patients infected with CHB are not diagnosed in time to be placed under HCC screening programmes. The reason why many people do not get diagnosed is because chronic viral hepatitis usually causes subclinical disease so that infected individuals will not have a reason to seek medical care until signs and symptoms of chronic viral hepatitis and its sequelae are seen later on in life (Weinbaum et al., 2008). This is particularly relevant in HBV-driven HCC occurring in Africa where as many as 55% of patients may develop HCC without any underlying cirrhosis (Paterson et al., 1985, Kew, 1989, Yoshida et al., 1994). This current study also shows a low proportion of only 23% of HCC cases presenting with concomitant cirrhosis. As a result of HCC arising in the absence of cirrhosis, opportunities for surveillance and early diagnosis are lost. It was recently reported that screening of HIV/HBV co-infected patients leads to HCC being diagnosed at an earlier stage and is associated with improved survival compared to unscreened patients that present with symptoms of the malignancy (Aytaman et al., 2014). This again reiterates the need to have pro-active screening programs for HCC diagnosis and also raising awareness on CHB. WHO guidelines suggest that HBV serology should be performed at diagnosis of HIV infection and before the administration of HAART but this is rarely followed in routine clinical practice.

5.3.3. Control of other risk factors for HCC

Suffice to say, it is not only the control of HBV that will change the epidemiology of HCC in sub-Saharan Africa. HCV is clearly a significant risk factor in some countries within sub-Saharan Africa as was shown in this study. It is therefore important to ensure that people already infected with HCV get diagnosed and be provided with optimal treatment to curb progression of chronic infection into HCC. Also it will be imperative that awareness be raised on how new infections may be avoided. Other steps that should also be considered include ensuring that donated blood products and tissue are safe. Furthermore, practices such as reuse

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of syringes in health care facilities should be outlawed while traditional healers should be educated on safe hygienic procedures when performing cultural scarification rituals including circumcisions. With regards to aflatoxin exposure, small-scale farmers mostly within the rural areas will need to be educated on better methods of grain harvesting and storage to discourage growth of the *Aspergillus* fungi.

Alcohol misuse is a significant risk factor for the development of alcoholic liver disease which can result in the development of cirrhosis and ultimately HCC (World Health Organization, 2011). Besides being highly endemic for HBV, many sub-Saharan Africa countries within the WHO Africa region have the highest rates for heavy episodic alcohol drinking/capita/year (World Health Organization, 2011). The Global Burden of Disease for 2010 results rank as alcohol as being responsible for the third highest cause of liver cirrhosis in Southern sub-Saharan Africa after HBV and HCV (IHME, 2013). Excessive alcohol consumption by individuals with HBV/HIV co-infection serves to only worsen the prognosis as data from the Multicenter AIDS Cohort Study showed that in addition to a low CD4 cell nadir, increased weekly alcohol consumption of ≥210g was significantly associated with liver-related mortality in HBV/HIV co-infected men-who-have-sex-with-men (Thio et al., 2002). Education and advocacy against excessive alcohol consumption therefore also need to be considered when designing prevention, diagnosis and treatment efforts for liver disease among HIV-infected patients in order to curb the incidence and improve the prognosis of HCC. Similarly, other diseases such as non-alcoholic steatohepatitis, obesity and diabetes will also need to be controlled in order to have a holistic approach to HCC control. In order to curb the HCC epidemic in Africa, implementing the WHO proposed action plan will be vital. The action plan comprises of some of the following processes: a) a consistent and feasible HBV vaccination strategy for children throughout Africa; b) a reduction in dietary exposure to aflatoxin; c) knowledge of the epidemiology of HCV in Africa and its relation with HIV/AIDS and; d) early diagnosis of malignancies and their complete registration as a means of formulating the appropriate clinical interventions (Hainaut and Boyle, 2008).

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Chapter six

6. Conclusions and future work

The HCC Study component of this study has shown that active hepatitis B continues to be the most prevalent cause of HCC among patients in South Africa as 64% (95% CI: 59-77) of cases were positive for HBsAg. In addition, rates of exposure to HBV among HCC patients are very high as 83% (95% CI: 76-90) of subjects were anti-HBc seropositive. In addition, this study has shown that despite HCC being non-AIDS defining, there is a high prevalence of HIV and HBV co-infection among HCC patients in South Africa. HIV infection seems to have an effect on early presentation of malignancy. There is a trend towards younger age at diagnosis of HCC among HIV-positive women compared to those who are HIV-negative. The results suggest that this early presentation may be linked to modification of the carcinogenic process and not due to early detection because cases presented late with malignancy regardless of their HIV infection status. The fact that HIV-infected HCC cases presented with equally advanced malignancy as the HIV-uninfected HCC cases might suggest HIV infection having an influence on the carcinogenesis process in addition to increased longevity associated with HAART allowing for the development and presentation of HCC. The effect of HIV infection on the age at presentation among male HCC cases was not so apparent. The fact that the modal age occurred between 30-40 years supports the calls for HCC screening of HBV-infected Africans to be started at 20 years of age (Bruix and Sherman, 2005). Larger multi-centred studies are needed to more accurately evaluate the impact of HIV infection on the epidemiology of HCC among sub-Saharan populations. To this end, collaborative multinational studies may also be necessary to fully understand the epidemiology of HCC in the context of HIV infection. Another area of research is to evaluate the implementation of recommended guidelines for diagnosing and monitoring of patients with hepatitis B. Hepatitis B treatment and HCC screening guidelines will not serve any purpose if they are not put into practice because patients will continue to present late with advanced malignancy due to untreated underlying HBV infections, thereby resulting in poor survival rates.

Experimental studies are also needed to determine whether HIV and HBV proteins have a synergistic effect on the expression of key cell growth and signalling molecules that are involved in the development of HCC. Such a synergistic effect might explain the rapid development of HCC in HBV/HIV co-infected patients. Based upon the differences in the age at presentation of HCC among cases infected with HBV genotypes A and D, an experimental

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approach to the effect of genotypes on carcinogenesis may also be appropriate. This could be achieved using transgenic animal models of the different HBV genotypes and observing the time to cancer development. Alternatively, cell culture models could also be used to determine effect of genotypes on cell-signalling and repair pathways.

The Immune Study component of this study shows that despite receiving HAART, HBV/HIV co-infected patients have persistent T-lymphocyte dysregulation and exhibit delayed immune recovery. In addition, co-infected patients also have increased gut microbial translocation as compared to mono-infected patients. However, immune dysregulation and gut translocation do not appear to be associated with severity of liver fibrosis in this cohort. The fact that HIV-infected individuals continue to have detectable HIV and HBV viraemia also strengthens the call to ensure complete adherence to therapy in order to achieve viral suppression in order to prevent development of drug-resistance variants of HIV and HBV. The results show that a greater proportion of HBV mono-infected patients have moderate/advanced liver fibrosis compared to HAART-experienced HBV/HIV co-infected patients. The finding is contrary to the original study hypothesis that increased microbial translocation in HBV/HIV co-infection is the driver of liver fibrosis. Instead, viral replication seems to be the driver of liver fibrosis with a significant association observed between HBV and HIV viral loads and liver stiffness. Uncontrolled replication supports the finding of a higher proportion of advanced liver fibrosis among the HBV mono-infected patients who were frequently not on therapy. These results are indicative of inadequate access to screening and treatment of HBV within much of South Africa where a majority of HBV mono-infected patients are also undiagnosed. It is these undiagnosed patients that are unlikely to be treated or placed under HCC surveillance programs and will only likely present late with advanced malignancy when options for therapy are limited.

Based on the finding that viral (HBV and HIV) replication appears to be the key factor in liver fibrosis, future work will need to examine the association between antigen-specific responses and hepatocyte damage/death due to the effect of CTLs. Another aspect to be considered is change in liver fibrosis scores with duration of therapy. As the period of therapy gets longer, it may be expected that rates of viral suppression will improve and confirming the findings from this current study, an improvement in fibrosis scores should be seen. It is also necessary to continue evaluating and monitoring adherence to anti-retroviral therapy from a public health point of view especially when considering the number of subjects that do not achieve viral suppression. If viral detection is due to non-adherence and not resistance to antivirals

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then patients can be appropriately counselled and the unnecessary switching to second-line HAART avoided.

Overall, the results strengthen the call to address the problem of HBV infection within sub-Saharan Africa countries. Childhood-acquired HBV infections frequently become chronic and are the greatest risk factor for developing HCC within the sub-Saharan Africa. HBV birth dose vaccination is the cornerstone of the HBV prevention strategy and should be available to all, in particular to infants born in high prevalence countries. Meanwhile, better strategies for diagnosis and treatment of hepatitis B are needed. In addition, screening and management strategies for HCC need to be developed for sub-Saharan African populations who present with HCC early in life.

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Chapter seven

7. References

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Appendices

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Appendix 1 HCC consent form

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT: HIV-associated hepatocellular carcinoma – prevalence, presentation, survival and risks

REFERENCE NUMBER:

PRINCIPAL INVESTIGATOR: Dr Monique Andersson

ADDRESS:
Division of Medical Virology, Dept of Pathology
Stellenbosch University, Faculty of Health Sciences
Francie van Zijl Ave, 8th Floor, Room 8078
PO Box 19063, Tygerberg Campus, 7505
Western Cape Province
SOUTH AFRICA

CONTACT NUMBER: 082 709 6152

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

- *This study will be conducted in a number of hospitals in South Africa and one hospital in Malawi in the departments of Oncology and/or Gastroenterology. We will be recruiting a total of about 300 people with liver cancer.*
- *We want to know who is getting liver cancer and hope to better understand why liver cancer is developing*

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- *We will be asking you some questions about risk factors, perform a routine medical examination to look for signs of liver disease, we will take a blood sample and a urine test*
- *The blood tests will tell us about any changes in the blood that may be associated with developing liver cancer and any risk factors for liver disease eg cirrhosis*

Why have you been invited to participate?

- *You have been invited to participate in this study because you have been diagnosed with liver cancer.*

What will your responsibilities be?

- *We would like you to consent to taking part on the study which will involve answering some questions, allowing a doctor to examine you, donate a blood (two 5ml tubes) sample and some urine.*
- *If you have cancer we will keep in touch with you and your family to follow you up. A nurse from the department where you have been seen will contact you or your family by telephone.*

Will you benefit from taking part in this research?

- *There will be no benefit to you, but this study will help us be able to help others in the future.*

Are there in risks involved in your taking part in this research?

- *There is some pain associated with taking blood. We will try to take this sample when you are having blood taken anyway.*

If you do not agree to take part, what alternatives do you have?

- *This will not affect your care in any way.*

Who will have access to your medical records?

- *Only those people who are directly involved with the study or with your clinical care*

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

- *It is unlikely that you experience any major injury as a result of taking part in the study. As a result of having blood taken you may experience some pain or bruising, which will be minor in nature.*

Will you be paid to take part in this study and are there any costs involved?

- *No you will not be paid to take part in the study, but we will cover the costs of your transport to the clinic if you need a follow up appointment for the study*

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Is there anything else that you should know or do?

- *You can contact Dr Andersson at tel 082 709 6152 if you have any further queries or encounter any problems.*
- *You can contact the Health Research Ethics Committee at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.*
- *You will receive a copy of this information and consent form for your own records.*

Declaration by participant

By signing below, I ……………………………………..………… agree to take part in a research study entitled *HIV-associated hepatocellular carcinoma – prevalence, presentation, survival and risks*

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) ....…........…………... on (*date*) …………....……2012.

....	....
Signature of participant	**Signature of witness**

Declaration by investigator

I *(name)* ……………………………………………..……… declare that:

- I explained the information in this document to ……………………………………..
- I encouraged him/her to ask questions and took adequate time to answer them.

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- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use an interpreter. (*If an interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) ....…........……………….. on (*date*) …………....… 2012.

....
Signature of investigator **Signature of witness**

Declaration by interpreter

I *(name)* ……………………………………………..……… declare that:

- I assisted the investigator (*name*) ………………………………………. to

  explain the information in this document to (*name of participant*)

  ……………..…………………………….. using the language medium of

  Afrikaans/Xhosa.

- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) ....…........……………….. on (*date*) …………....………………..

....
Signature of interpreter **Signature of witness**

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Appendix 2 HCC Case record form

HEPATOCELLULAR CARCINOMA STUDY

Patient has diagnosis of HCC? Yes ☐ Diagnosis: **AFP** **Imaging** **Clinical** **Histology**

Informed consent completed? Yes ☐ If no, please complete before proceeding

Site: Hospital/Clinic Name (PLACE STICKER HERE)

Hospital number PLEASE CIRCLE AS APPROPRIATE

DOB Sex M F

Nationality Race B W I C other ____________

Monthly Income ZAR _________ Education: Primary Secondary Tertiary

Country of birth Area Born: Urban ☐ Rural ☐

Other areas for lived >12months

Dates	Area/City/Town	Urban/Rural

Family History of liver disease YES ☐ NO ☐ Give Details____________________

Family History of HCC YES ☐ NO ☐ Give Details____________________

HIV status POS ☐ NEG ☐ UNKNOWN ☐

Date HIV diagnosed ________________

If HIV positive, CD4 current __________________ Nadir CD4 _____________________

On HAART YES ☐ NO ☐

Length of time on HAART ____________

Current HAART *(please ring)* tenofovir lamivudine efavirenz nevirapine AZT stavudine DdI lopinavir/ritonovir atazanavir other____________________

Previous HAART *(please ring)* tenofovir lamivudine efavirenz nevirapine AZT stavudine DdI lopinavir/ritonovir atazanavir other____________________

Cirrhosis NO ☐ YES ☐ Date diagnosed

Cause of cirrhosis KNOWN ☐ (detail) ______________________________ UNK ☐

Liver biopsy NO ☐ YES ☐ Date *Please attach copy of report*

Ever alcohol dependence/misuse (loss job, breakdown relationships) YES ☐ NO ☐

Consumed *(please ring)*: >28 Units per week for ______years OR Average between 10 and 28 Units OR Less than 10 units (0.5l beer=2.5U, 750ml wine = 9U)

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Type of alcohol consumed (please ring) Beer Spirits Wine Other please detail_______________

History of eating peanuts? YES ☐ NO ☐

Source of peanuts? Pre-packed ☐ Grows own ☐ Buys from market ☐ Other(detail) ______

Dietary Iron exposure – home brewed beer from iron pots ***'Umqombothi'*** YES ☐ NO ☐

Duration of dietary iron exposure____________ years

Oral contraception NO ☐ YES ☐ name______________ duration _____yrs

Smoker NO ☐ YES ☐ Pack years_____________

CLINICAL PRESENTATION

Ascites YES ☐ NO ☐

Ankle Oedema YES ☐ NO ☐

Abdominal mass YES ☐ NO ☐

Liver decompensation (ever) YES ☐ NO ☐

Childs-Pugh classification A B C

Weight __________________kg Height _____________________________cm

Evidence of chronic liver disease – please ring positives

clubbing, leukonychia, spider naevi, gynaecomastia, caput medusa, bruising, jaundice, splenomegaly, ascites, liver flap, Dupuytren's contracture, testicular atrophy, parotid enlargement, hepatomegaly, hepatic arterial liver bruit

USS OR MRI OR CTscan (please tick) – ***please attach copy of report and please ring positives***

shrunken liver, enlarged liver, splenomegaly, ascites, multiple liver lesions, single liver lesion,

evidence of hypervascularity

date of scan: ______/______/201__

Planned therapy

Surgical ☐ Liver transplant Y N

Medical ☐

Palliative ☐

Name

Sign Date

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HEPATOCELLULAR CARCINOMA STUDY – RESULTS SHEET

PLEASE TICK UNKNOWN IF THE TEST HAS NOT BEEN PERFORMED

Site: Hospital/Clinic Name (PLACE STICKER HERE)

Hospital number

DOB Sex

HBV status				
	HBsAg	POS ☐	NEG ☐	UNKNOWN ☐
	antiHBc	POS ☐	NEG ☐	UNKNOWN ☐
	eAg	POS ☐	NEG ☐	UNKNOWN ☐
	anti-e	POS ☐	NEG ☐	UNKNOWN ☐

HBV viral load (if >1 give peak level) ____________________copies/ml OR IU/ml

HCV antibody	POS ☐	NEG ☐	UNKNOWN ☐
HCV PCR	POS ☐	NEG ☐	UNKNOWN ☐

AFP __________________

FBC Hb MCV

Platelets

eGFR HbA1c

	Enrolment	peak value
ALT		
Bilirubin (conj)		
Bilirubin (unconj)		
Alk Phos		
Albumin		
INR		

NAME

Signed Date

Stellenbosch University https://scholar.sun.ac.za

Appendix 3 Stellenbosch University HCC Study HREC approval

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot • your knowledge partner

05 October 2011 **MAILED**

Dr M Andersson
Department of Medical Virology
8th Floor, Clinical building
Stellenbosch University
Tygerberg campus
7505

Dear Dr Andersson

HIV-associated hepatocellular carcinoma – prevalence, presentation, survival and risks.

ETHICS REFERENCE NO: N11/09/284

RE : APPROVAL

It is a pleasure to inform you that a review panel of the Health Research Ethics Committee has approved the above-mentioned project on 5 October 2011, including the ethical aspects involved, for a period of one year from this date.

This project is therefore now registered and you can proceed with the work. Please quote the above-mentioned project number in ALL future correspondence. You may start with the project. Notwithstanding this approval, the Committee can request that work on this project be halted temporarily in anticipation of more information that they might deem necessary.

Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly and subjected to an external audit.

Translations of the consent document in the languages applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Please note that for research at primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Hélène Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

05 October 2011 15:56 Page 1 of 2

Fakulteit Gesondheidswetenskappe · Faculty of Health Sciences

Verbind tot Optimale Gesondheid · Committed to Optimal Health
Afdeling Navorsingsontwikkeling en -steun · Division of Research Development and Support
Posbus/PO Box 19063 · Tygerberg 7505 · Suid-Afrika/South Africa
Tel.: +27 21 938 9075 · Faks/Fax: +27 21 931 3352

Stellenbosch University https://scholar.sun.ac.za

Approval Date: 5 October 2011 Expiry Date: 5 October 2012

Yours faithfully

MS CARLI SAGER
RESEARCH DEVELOPMENT AND SUPPORT
Tel: +27 21 938 9140 / E-mail: carlis@sun.ac.za
Fax: +27 21 931 3352

Stellenbosch University https://scholar.sun.ac.za

Appendix 4 University of Cape Town HCC Study HREC approval

UNIVERSITY OF CAPE TOWN

Faculty of Health Sciences
Faculty of Health Sciences Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6338 • **Facsimile** [021] 406 6411
e-mail: sumayah.ariefdien@uct.ac.za

08 December 2011

HREC REF: 544/2011

Prof M Kew
Department of Medicine
J-floor
OMB

Dear Prof Kew

PROJECT TITLE: HIV-ASSOCIATED HEPATOCELLULAR CARCINOMA-PREVALENCE, PRESENTATION, SURVIVAL AND RISKS

Thank you for addressing the comments raised by the committee.

It is a pleasure to inform you that the Ethics Committee has **formally approved** the above-mentioned study including the following documentation:-

- Informed Consent Form HREC General ICF Version 2, July 2009

Approval is granted for one year till the 15 December 2012.

Please submit a progress form, using the standardised Annual Report Form (FHS016), if the study continues beyond the approval period. Please submit a Standard Closure form (FHS010)if the study is completed within the approval period.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

pp

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

Federal Wide Assurance Number: FWA00001637.

sAriefdien

Stellenbosch University https://scholar.sun.ac.za

Appendix 5 University of Witwatersrand HCC Study HREC approval

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

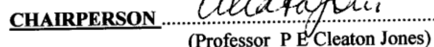
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Anna Sparaco

CLEARANCE CERTIFICATE	M111189
PROJECT	HIV Associated Hepatocellular Carcinoma-Prevalence, Presentation, Survival, Survival and Risks
INVESTIGATORS	Dr Anna Sparaco
DEPARTMENT	Department of Surgery
DATE CONSIDERED	25/11/2011
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 25/04/2012 CHAIRPERSON ....



(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr TE Luvhengo

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Stellenbosch University https://scholar.sun.ac.za

Appendix 6 University of Pretoria HCC Study HREC approval

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

2/05/2013

Approval Notice
New Application

Ethics Reference No.: 108/2013

Title: Hepatocellular Carcinomain the context of HIV – incidence, presentation, risks and survival

Dear Prof. LM Dreosti

The **New Application** for your research received on the 2/04/2013, was approved by the Faculty of Health Sciences Research Ethics Committee on the 2/05/2013

Please note the following about your ethics approval:

- Ethics Approval is valid for 3 years.
- Please remember to use your protocol number (108/2013) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
Standard Conditions:

- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

We wish you the best with your research.

Yours sincerely

DR R SOMMERS; MBChB; MMed(Int); MPharmMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee
University of Pretoria

012 354 1677 | 0866516047 | deepeka.behari@up.ac.za | http://www.healthethics-up.co.za
Private Bag X323, Arcadia, 0007 - 31 Bophelo Road, HW Snyman South Building, Level 2, Room 2.33, Gezina, Pretoria

Stellenbosch University https://scholar.sun.ac.za

Appendix 7 Immune Study HREC approval

Approval Notice
New Application

04-Mar-2014
Maponga, Tongai TG

Ethics Reference #: S13/04/072

Title: **HIV-associated gut microbial translocation and immune dysregulation: a risk factor for the development of hepatic fibrosis in HBV infection**

DearMr. Tongai Maponga,

The **New Application** received on **18-Apr-2013**, was reviewed by members of **Health Research Ethics Committee 1** via Minimal Risk Review procedures on **04-Mar-2014** and was approved.
Please note the following information about your approved research protocol:

Protocol Approval Period: **04-Mar-2014 -04-Mar-2015**

Please remember to use your **protocol number** (**S13/04/072**) on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:
Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.
Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Helene Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.
For standard HREC forms and documents please visit: www.sun.ac.za/rds

If you have any questions or need further assistance, please contact the HREC office at 0219389156.

Included Documents:
CV TALJAARD
DEC LETTERS
IC FORM

Stellenbosch University https://scholar.sun.ac.za

Appendix 8 Immune Study Case record form

LIVER FIBROSIS STUDY CASE RECORD FORM

Please do not proceed without participant's consent.

Informed consent forms signed? Yes ☐

DEMOGRAPHIC DATA

Race (*please circle*): B W C I Other ______________

Gender: Male ☐ Female ☐

Age: _____

Weight *(in kilos)* Height *(in metres)*

RISK FACTORS		
1. Current alcohol consumption	**YES**	**NO**
2. How many UNITS of alcohol do you drink on a typical day when you are drinking? Pint of regular beer/lager/cider = 2 units ________UNITS Can of lager = 1.5 units ________UNITS Glass of wine (175ml) = 2 units ________UNITS Single measure of spirits = 1 unit ________UNITS Bottle of wine = 9 units ________UNITS		
3. Family history of liver disease *If yes to family history of liver disease, please provide details*	**YES**	**NO**
4. Smoking history (*please tick appropriate response*) Current Past Never		
5. If yes to smoking, please indicate number of pack years* _____pack years ***Number of pack years = (packs smoked per day) × (years as a smoker)***		
6. Use of herbal medicine (please circle appropriate response) Current Past Never *If yes to herbal drugs, please provide details*		
7. Known diagnosis of diabetes	**YES**	**NO**
8. Known current diagnosis of TB disease or medication for TB.	**YES**	**NO**
9. History of liver biopsy *If yes, please provide details*	**YES**	**NO**
10. Known diagnosis of any known cancer	**YES**	**NO**
11. HIV status (please tick appropriate response) ***If response is negative skip to 20***	**POS**	**NEG**

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12. If positive, state date diagnosed		
13. Current CD4		
14. Nadir CD4		
15. HIV viral load		
16. HAART (please tick appropriate response) ***If response is no, please skip to 20***	**POS**	**NEG**
17. Period of time on HAART		
18. If on HAART, please tick applicable below Tenofovir Lamivudine Efavirenz Nevirapine AZT Stavudine DdI Lopinivir/ritonavir Azatanavir Other (please state)		
19. If on previous HAART, please tick applicable below Tenofovir Lamivudine Efavirenz Nevirapine AZT Stavudine DdI Lopinivir/ritonavir Azatanavir Other (please state)		
20. If HIV negative, treatment for HBV	**YES**	**NO**
21. If receiving HBV treatment, please state regimen		

Please thank participant and collect blood in 1 SST tubes (yellow top) and 1 EDTA tube (purple top) and 1 Heparin tube (green top).

Interviewer name:

Signature: Date:

Blood collected by:

Signature:

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Appendix 9 Correlation matrix (controls)

Correlation matrix for controls calculated using Spearman test. Values in red font represent statistically significant correlation.

Infection status=Control Spearman Rank Order Correlations Marked correlations are significant at p <.05000

	APRI Score	CD4 count	AST	CD4/CD8 ratio	CD8 count	Median stiffness	FIB-4 SCORE	ALT	sCD14	LBP	CD4/CD38-	CD4/CD38+/HLAD	CD4/CD38+/HLAD	CD4/CD38	CD4/HLADR	CD8/CD38-	CD8/CD38+/HLAD	CD8/CD38+/HLAD	CD8/CD38	CD8/HLADR	CD4/PD1+/CTLA4	CD4/PD1-/CTLA4+	CD4/PD1+	CD4/CTLA4+	CD8/PD1+/CTLA4-	CD8/PD1+/CTLA4	CD8/PD1-/CTLA4+	CD8/PD1+	CD8/CTLA4+
APRI Score	1.00	-0.24	0.78	-0.06	-0.16	0.04	0.57	0.67	0.05	0.17	-0.07	0.01	-0.08	-0.12	-0.02	-0.07	-0.05	-0.21	-0.26	0.11	-0.43	-0.07	-0.14	-0.15	-0.32	-0.33	-0.28	-0.29	-0.42
CD4 count	-0.24	1.00	-0.21	0.31	0.36	0.06	0.00	-0.27	-0.04	-0.03	-0.12	-0.45	0.10	0.03	-0.33	-0.08	-0.37	0.16	-0.18	-0.37	-0.19	-0.16	-0.06	-0.16	0.20	0.23	0.04	0.22	0.05
AST	0.78	-0.21	1.00	-0.22	0.11	0.03	0.57	0.84	0.17	0.38	-0.13	-0.01	-0.21	-0.21	-0.05	-0.05	-0.11	-0.31	-0.31	0.09	-0.19	0.17	0.09	0.10	-0.21	-0.27	-0.24	-0.20	-0.38
CD4/CD8 ratio	-0.06	0.31	-0.22	1.00	-0.70	-0.03	-0.15	0.00	-0.08	0.15	0.08	-0.28	-0.18	-0.23	-0.15	0.00	-0.29	0.11	-0.28	-0.38	-0.01	-0.33	0.07	-0.23	0.16	0.12	0.01	0.16	0.04
CD8 count	-0.16	0.36	0.11	-0.70	1.00	0.03	0.20	-0.10	0.01	-0.15	-0.04	-0.03	0.16	0.16	0.00	0.01	0.05	-0.16	0.09	0.16	-0.17	0.05	-0.07	0.00	0.06	0.12	0.03	0.07	0.03
Median stiffness	0.04	0.06	0.03	-0.03	0.03	1.00	0.25	0.05	-0.06	-0.30	0.12	0.24	-0.19	-0.15	0.11	0.09	0.29	0.16	0.19	0.19	-0.12	0.19	-0.24	0.13	-0.32	0.20	0.34	-0.31	0.34
FIB-4 SCORE	0.57	0.00	0.57	-0.15	0.20	0.25	1.00	0.53	0.06	0.20	-0.09	0.18	-0.07	-0.09	0.14	0.04	0.13	-0.39	-0.06	0.44	-0.21	-0.06	-0.31	-0.09	-0.40	-0.01	0.08	-0.38	0.00
ALT	0.67	-0.27	0.84	0.00	-0.10	0.05	0.53	1.00	0.16	0.35	-0.17	-0.02	-0.23	-0.23	-0.17	-0.07	-0.02	-0.38	-0.25	0.06	-0.22	0.07	-0.06	0.05	-0.24	-0.30	-0.31	-0.22	-0.38
Soluble CD14	0.05	-0.04	0.17	-0.08	0.01	-0.06	0.06	0.16	1.00	-0.15	-0.45	-0.13	0.36	0.36	-0.39	-0.35	-0.03	0.05	0.01	-0.25	0.12	0.30	-0.10	0.28	-0.10	0.16	0.28	-0.07	0.24
LBP	0.17	-0.03	0.38	0.15	-0.15	-0.30	0.20	0.35	-0.15	1.00	0.05	-0.20	-0.14	-0.15	-0.01	0.31	-0.23	-0.19	-0.19	0.19	-0.06	-0.33	0.24	-0.31	0.15	-0.39	-0.37	0.11	-0.41
CD4/CD38-/HLADR+	-0.07	-0.12	-0.13	0.08	-0.04	0.12	-0.09	-0.17	-0.45	0.05	1.00	0.41	-0.42	-0.39	0.85	0.64	0.21	-0.20	-0.04	0.47	-0.13	-0.33	0.10	-0.29	0.16	0.04	-0.07	0.12	-0.06
CD4/CD38+/HLADR+	0.01	-0.45	-0.01	-0.28	-0.03	0.24	0.18	-0.02	-0.13	-0.20	0.41	1.00	0.09	0.21	0.77	0.09	0.84	-0.10	0.55	0.61	0.13	0.11	0.16	0.14	-0.10	0.07	0.09	-0.11	0.12
CD4/CD38+/HLADR-	-0.08	0.10	-0.21	-0.18	0.16	-0.19	-0.07	-0.23	0.36	-0.14	-0.42	0.09	1.00	0.98	-0.24	-0.33	0.19	0.30	0.47	-0.03	-0.05	0.16	-0.15	0.12	-0.25	0.05	0.29	-0.22	0.25
CD4/CD38	-0.12	0.03	-0.21	-0.23	0.16	-0.15	-0.09	-0.23	0.36	-0.15	-0.39	0.21	0.98	1.00	-0.16	-0.32	0.29	0.28	0.54	0.03	0.00	0.20	-0.11	0.17	-0.23	0.06	0.29	-0.20	0.26
CD4/HLADR	-0.02	-0.33	-0.05	-0.15	0.00	0.11	0.14	-0.17	-0.39	-0.01	0.85	0.77	-0.24	-0.16	1.00	0.43	0.55	-0.20	0.28	0.65	-0.04	-0.19	0.15	-0.14	0.06	0.01	-0.05	0.03	-0.03
CD8/CD38-/HLADR+	-0.07	-0.08	-0.05	0.00	0.01	0.09	0.04	-0.07	-0.35	0.31	0.64	0.09	-0.33	-0.32	0.43	1.00	-0.07	-0.43	-0.37	0.64	0.01	-0.15	-0.06	-0.15	-0.05	-0.08	-0.14	-0.09	-0.16
CD8/CD38+/HLADR+	-0.05	-0.37	-0.11	-0.29	0.05	0.29	0.13	-0.02	-0.03	-0.23	0.21	0.84	0.19	0.29	0.55	-0.07	1.00	-0.11	0.70	0.57	0.20	0.18	0.18	0.26	0.02	0.12	0.10	0.03	0.14
CD8/CD38+/HLADR-	-0.21	0.16	-0.31	0.11	-0.16	0.16	-0.39	-0.38	0.05	-0.19	-0.20	-0.10	0.30	0.28	-0.20	-0.43	-0.11	1.00	0.52	-0.46	0.03	0.23	-0.02	0.20	-0.20	0.01	0.35	-0.17	0.30
CD8/CD38	-0.26	-0.18	-0.31	-0.28	0.09	0.19	-0.06	-0.25	0.01	-0.19	-0.04	0.55	0.47	0.54	0.28	-0.37	0.70	0.52	1.00	0.18	0.16	0.21	0.09	0.26	-0.08	0.07	0.27	-0.05	0.26
CD8/HLADR	0.11	-0.37	0.09	-0.38	0.16	0.19	0.44	0.06	-0.25	0.19	0.47	0.61	-0.03	0.03	0.65	0.64	0.57	-0.46	0.18	1.00	0.11	0.03	0.04	0.05	-0.15	-0.13	-0.11	-0.18	-0.14
CD4/PD1+/CTLA4+	-0.43	-0.19	-0.19	-0.01	-0.17	-0.12	-0.21	-0.22	0.12	-0.06	-0.13	0.13	-0.05	0.00	-0.04	0.01	0.20	0.03	0.16	0.11	1.00	0.64	0.55	0.76	0.32	0.31	0.10	0.33	0.13
CD4/PD1-/CTLA4+	-0.07	-0.16	0.17	-0.33	0.05	0.19	-0.06	0.07	0.30	-0.33	-0.33	0.11	0.16	0.20	-0.19	-0.15	0.18	0.23	0.21	0.03	0.64	1.00	0.11	0.95	-0.18	0.23	0.34	-0.13	0.31
CD4/PD1+	-0.14	-0.06	0.09	0.07	-0.07	-0.24	-0.31	-0.06	-0.10	0.24	0.10	0.16	-0.15	-0.11	0.15	-0.06	0.18	-0.02	0.09	0.04	0.55	0.11	1.00	0.30	0.79	0.10	-0.33	0.79	-0.25
CD4/CTLA4+	-0.15	-0.16	0.10	-0.23	0.00	0.13	-0.09	0.05	0.28	-0.31	-0.29	0.14	0.12	0.17	-0.14	-0.15	0.26	0.20	0.26	0.05	0.76	0.95	0.30	1.00	0.00	0.30	0.28	0.05	0.28
CD8/PD1+/CTLA4-	-0.32	0.20	-0.21	0.16	0.06	-0.32	-0.40	-0.24	-0.10	0.15	0.16	-0.10	-0.25	-0.23	0.06	-0.05	0.02	-0.20	-0.08	-0.15	0.32	-0.18	0.79	0.00	1.00	0.18	-0.41	0.99	-0.31
CD8/PD1+/CTLA4+	-0.33	0.23	-0.27	0.12	0.12	0.20	-0.01	-0.30	0.16	-0.39	0.04	0.07	0.05	0.06	0.01	-0.08	0.12	0.01	0.07	-0.13	0.31	0.23	0.10	0.30	0.18	1.00	0.73	0.24	0.82
CD8/PD1-/CTLA4+	-0.28	0.04	-0.24	0.01	0.03	0.34	0.08	-0.31	0.28	-0.37	-0.07	0.09	0.29	0.29	-0.05	-0.14	0.10	0.35	0.27	-0.11	0.10	0.34	-0.33	0.28	-0.41	0.73	1.00	-0.34	0.98
CD8/PD1+	-0.29	0.22	-0.20	0.16	0.07	-0.31	-0.38	-0.22	-0.07	0.11	0.12	-0.11	-0.22	-0.20	0.03	-0.09	0.03	-0.17	-0.05	-0.18	0.33	-0.13	0.79	0.05	0.99	0.24	-0.34	1.00	-0.23
CD8/CTLA4+	-0.42	0.05	-0.38	0.04	0.03	0.34	0.00	-0.38	0.24	-0.41	-0.06	0.12	0.25	0.26	-0.03	-0.16	0.14	0.30	0.26	-0.14	0.13	0.31	-0.25	0.28	-0.31	0.82	0.98	-0.23	1.00

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Appendix 10 Correlation matrix for HIV mono-infected subjects

Correlation matrix for HIV mono-infected subjects. Significant correlations marked in red font.

Infection status=HIV Mono Spearman Rank Order Correlations Marked correlations are significant at $p < .05$

	APRI Score	CD4 count	AST	HIV viral	CD4/CD8 ratio	CD8 count	Median stiffness	FIB-4 SCORE	ALT	Length on HAART	sCD14	LBP	CD4/CD38-	CD4/CD38+/HLAD
APRI Score	1.00	-0.26	0.76	0.34	0.08	-0.41	0.47	0.79	0.73	0.21	-0.04	-0.49	-0.06	0.05
CD4 count	-0.26	1.00	-0.04	-0.10	0.51	0.27	-0.24	-0.33	0.03	0.33	-0.16	-0.10	-0.29	-0.29
AST	0.76	-0.04	1.00	0.16	-0.04	-0.14	0.48	0.47	0.89	0.20	0.05	-0.26	-0.20	0.22
HIV viral load	0.34	-0.10	0.16	1.00	-0.09	0.04	-0.20	0.17	0.18	-0.30	-0.10	0.01	-0.08	-0.03
CD4/CD8 ratio	0.08	0.51	-0.04	-0.09	1.00	-0.56	-0.10	-0.09	0.12	0.33	-0.17	-0.35	-0.44	-0.43
CD8 count	-0.41	0.27	-0.14	0.04	-0.56	1.00	-0.10	-0.30	-0.19	-0.26	-0.11	0.21	0.15	0.14
Median stiffness	0.47	-0.24	0.48	-0.20	-0.10	-0.10	1.00	0.51	0.26	0.07	-0.07	-0.01	0.03	0.24
FIB-4 SCORE	0.79	-0.33	0.47	0.17	-0.09	-0.30	0.51	1.00	0.42	0.21	-0.04	-0.34	0.16	0.13
ALT	0.73	0.03	0.89	0.18	0.12	-0.19	0.26	0.42	1.00	0.31	-0.02	-0.15	-0.31	0.06
Length on HAART	0.21	0.33	0.20	-0.30	0.33	-0.26	0.07	0.21	0.31	1.00	-0.23	-0.12	-0.03	-0.17
Soluble CD14	-0.04	-0.16	0.05	-0.10	-0.17	-0.11	-0.07	-0.04	-0.02	-0.23	1.00	0.30	0.22	0.34
LBP	-0.49	-0.10	-0.26	0.01	-0.35	0.21	-0.01	-0.34	-0.15	-0.12	0.30	1.00	0.02	0.20
CD4/CD38-/HLADR+	-0.06	-0.29	-0.20	-0.08	-0.44	0.15	0.03	0.16	-0.31	-0.03	0.22	0.02	1.00	0.37
CD4/CD38+/HLADR+	0.05	-0.29	0.22	-0.03	-0.43	0.14	0.24	0.13	0.06	-0.17	0.34	0.20	0.37	1.00
CD4/CD38+/HLADR-	-0.04	0.34	0.19	0.16	0.33	0.04	0.00	-0.14	0.25	0.01	-0.14	0.09	-0.75	-0.11
CD4/CD38	0.09	0.15	0.35	0.21	0.11	0.01	0.06	-0.03	0.38	0.01	0.08	0.18	-0.58	0.29
CD4/HLADR	0.08	-0.39	0.02	0.04	-0.54	0.13	0.17	0.26	-0.12	-0.11	0.32	0.09	0.86	0.73
CD8/CD38-/HLADR+	-0.24	-0.02	-0.36	-0.21	-0.18	0.15	0.08	0.08	-0.33	0.14	-0.10	-0.04	0.57	-0.15
CD8/CD38+/HLADR+	-0.21	-0.12	0.20	0.18	-0.27	0.21	-0.06	-0.37	0.09	-0.24	0.31	0.36	0.00	0.69
CD8/CD38+/HLADR-	0.05	0.34	0.03	0.10	0.50	-0.11	0.01	-0.19	0.21	-0.08	-0.13	-0.02	-0.68	-0.23
CD8/CD38	0.10	-0.05	0.32	0.25	-0.01	-0.01	0.00	-0.21	0.31	-0.22	0.27	0.23	-0.40	0.44
CD8/HLADR	-0.17	-0.31	-0.02	-0.02	-0.54	0.17	0.04	-0.06	-0.14	-0.04	0.28	0.33	0.63	0.50
CD4/PD1+/CTLA4+	-0.39	-0.43	-0.37	0.00	-0.49	0.22	0.01	-0.30	-0.34	-0.27	0.16	0.31	0.20	0.17
CD4/PD1-/CTLA4+	-0.33	-0.22	-0.40	-0.06	-0.31	0.16	-0.14	-0.27	-0.24	-0.09	-0.02	0.20	0.02	-0.14
CD4/PD1+	-0.02	-0.51	0.12	-0.03	-0.55	0.13	0.41	0.04	0.04	-0.17	0.12	0.25	0.12	0.44
CD4/CTLA4+	-0.36	-0.34	-0.42	0.00	-0.40	0.20	-0.09	-0.31	-0.30	-0.17	0.04	0.26	0.10	-0.03
CD8/PD1+/CTLA4-	-0.25	-0.20	-0.18	-0.03	-0.26	0.20	0.09	-0.04	-0.16	0.02	-0.12	0.22	0.09	-0.08
CD8/PD1+/CTLA4+	-0.04	-0.36	-0.14	-0.26	-0.09	-0.12	-0.08	0.08	-0.08	-0.06	-0.04	-0.03	0.10	-0.10
CD8/PD1-/CTLA4+	0.22	-0.32	-0.10	0.02	0.10	-0.28	-0.13	0.21	0.03	-0.18	0.02	-0.11	-0.03	-0.18
CD8/PD1+	-0.24	-0.22	-0.19	-0.04	-0.26	0.20	0.08	-0.02	-0.15	0.02	-0.13	0.20	0.09	-0.09
CD8/CTLA4+	0.14	-0.37	-0.14	-0.12	0.03	-0.23	-0.13	0.23	-0.03	-0.13	-0.01	-0.08	0.03	-0.15

	CD4/CD38+/HLAD	CD4/CD38	CD4/HLADR	CD8/CD38-	CD8/CD38+/HL	CD8/CD38+/HLAD	CD8/CD38	CD8/HLADR	CD4/PD1+/CTLA4	CD4/PD1-/CTLA4+	CD4/PD1+	CD4/CTLA4+	CD8/PD1+/CTLA4-	CD8/PD1+/CTLA4	CD8/PD1-/CTLA4+	CD8/PD1+	CD8/CTLA4+
APRI Score	-0.04	0.09	0.08	-0.24	-0.21	0.05	0.10	-0.17	-0.39	-0.33	-0.02	-0.36	-0.25	-0.04	0.22	-0.24	0.14
CD4 count	0.34	0.15	-0.39	-0.02	-0.12	0.34	-0.05	-0.31	-0.43	-0.22	-0.51	-0.34	-0.20	-0.36	-0.32	-0.22	-0.37
AST	0.19	0.35	0.02	-0.36	0.20	0.03	0.32	-0.02	-0.37	-0.40	0.12	-0.42	-0.18	-0.14	-0.10	-0.19	-0.14
HIV viral load	0.16	0.21	0.04	-0.21	0.18	0.10	0.25	-0.02	0.00	-0.06	-0.03	0.00	-0.03	-0.26	0.02	-0.04	-0.12
CD4/CD8 ratio	0.33	0.11	-0.54	-0.18	-0.27	0.50	-0.01	-0.54	-0.49	-0.31	-0.55	-0.40	-0.26	-0.09	0.10	-0.26	0.03
CD8 count	0.04	0.01	0.13	0.15	0.21	-0.11	-0.01	0.17	0.22	0.16	0.13	0.20	0.20	-0.12	-0.28	0.20	-0.23
Median stiffness	0.00	0.06	0.17	0.08	-0.06	0.01	0.00	0.04	0.01	-0.14	0.41	-0.09	0.09	-0.08	-0.13	0.08	-0.13
FIB-4 SCORE	-0.14	-0.03	0.26	0.08	-0.37	-0.19	-0.21	-0.06	-0.30	-0.27	0.04	-0.31	-0.04	0.08	0.21	-0.02	0.23
ALT	0.25	0.38	-0.12	-0.33	0.09	0.21	0.31	-0.14	-0.34	-0.24	0.04	-0.30	-0.16	-0.08	0.03	-0.15	-0.03
Length on HAART	0.01	0.01	-0.11	0.14	-0.24	-0.08	-0.22	-0.04	-0.27	-0.09	-0.17	-0.17	0.02	-0.06	-0.18	0.02	-0.13
Soluble CD14	-0.14	0.08	0.32	-0.10	0.31	-0.13	0.27	0.28	0.16	-0.02	0.12	0.04	-0.12	-0.04	0.02	-0.13	-0.01
LBP	0.09	0.18	0.09	-0.04	0.36	-0.02	0.23	0.33	0.31	0.20	0.25	0.26	0.22	-0.03	-0.11	0.20	-0.08
CD4/CD38-/HLADR+	-0.75	-0.58	0.86	0.57	0.00	-0.68	-0.40	0.63	0.20	0.02	0.12	0.10	0.09	0.10	-0.03	0.09	0.03
CD4/CD38+/HLADR+	-0.11	0.29	0.73	-0.15	0.69	-0.23	0.44	0.50	0.17	-0.14	0.44	-0.03	-0.08	-0.10	-0.18	-0.09	-0.15
CD4/CD38+/HLADR-	1.00	0.82	-0.59	-0.43	0.11	0.75	0.44	-0.53	-0.32	-0.24	-0.19	-0.28	-0.06	-0.31	-0.13	-0.09	-0.23
CD4/CD38	0.82	1.00	-0.27	-0.60	0.45	0.56	0.74	-0.28	-0.18	-0.15	-0.06	-0.18	-0.18	-0.35	-0.17	-0.21	-0.26
CD4/HLADR	-0.59	-0.27	1.00	0.34	0.33	-0.63	-0.08	0.73	0.22	-0.04	0.33	0.06	0.08	0.03	-0.12	0.08	-0.06
CD8/CD38-/HLADR+	-0.43	-0.60	0.34	1.00	-0.41	-0.60	-0.85	0.52	0.08	0.04	-0.13	0.05	0.31	0.18	-0.09	0.33	0.01
CD8/CD38+/HLADR+	0.11	0.45	0.33	-0.41	1.00	-0.06	0.72	0.44	0.13	-0.04	0.18	0.02	-0.21	-0.28	-0.29	-0.23	-0.30
CD8/CD38+/HLADR-	0.75	0.56	-0.63	-0.60	-0.06	1.00	0.50	-0.79	-0.29	-0.18	-0.17	-0.22	-0.22	-0.22	0.12	-0.22	-0.05
CD8/CD38	0.44	0.74	-0.08	-0.85	0.72	0.50	1.00	-0.15	-0.02	-0.08	0.14	-0.05	-0.36	-0.34	-0.10	-0.38	-0.22
CD8/HLADR	-0.53	-0.28	0.73	0.52	0.44	-0.79	-0.15	1.00	0.30	0.09	0.22	0.16	0.13	0.02	-0.26	0.13	-0.16
CD4/PD1+/CTLA4+	-0.32	-0.18	0.22	0.08	0.13	-0.29	-0.02	0.30	1.00	0.85	0.59	0.95	0.43	0.45	0.23	0.45	0.35
CD4/PD1-/CTLA4+	-0.24	-0.15	-0.04	0.04	-0.04	-0.18	-0.08	0.09	0.85	1.00	0.26	0.96	0.27	0.37	0.20	0.29	0.29
CD4/PD1+	-0.19	-0.06	0.33	-0.13	0.18	-0.17	0.14	0.22	0.59	0.26	1.00	0.42	0.52	0.44	0.20	0.53	0.30
CD4/CTLA4+	-0.28	-0.18	0.06	0.05	0.02	-0.22	-0.05	0.16	0.95	0.96	0.42	1.00	0.36	0.42	0.22	0.39	0.33
CD8/PD1+/CTLA4-	-0.06	-0.18	0.08	0.31	-0.21	-0.22	-0.36	0.13	0.43	0.27	0.52	0.36	1.00	0.60	0.12	1.00	0.33
CD8/PD1+/CTLA4+	-0.31	-0.35	0.03	0.18	-0.28	-0.22	-0.34	0.02	0.45	0.37	0.44	0.42	0.60	1.00	0.74	0.64	0.91
CD8/PD1-/CTLA4+	-0.13	-0.17	-0.12	-0.09	-0.29	0.12	-0.10	-0.26	0.23	0.20	0.20	0.22	0.12	0.74	1.00	0.15	0.94
CD8/PD1+	-0.09	-0.21	0.08	0.33	-0.23	-0.22	-0.38	0.13	0.45	0.29	0.53	0.39	1.00	0.64	0.15	1.00	0.38
CD8/CTLA4+	-0.23	-0.26	-0.06	0.01	-0.30	-0.05	-0.22	-0.16	0.35	0.29	0.30	0.33	0.33	0.91	0.94	0.38	1.00

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Appendix 11 Correlation matrix for HBV mono-infected subjects

Correlation matrix for HBV mono-infected subjects. Significant correlations marked in red font.

HBV Mono-infected subjects: Spearman Rank Order Correlations Marked correlations are significant at p <.05

	APRI Score	CD4 coun	AST	HBV DNA load	CD4/CD8 ratio	CD8 count	Median stiffness	FIB-4 SCORE	ALT	sCD14	LBP	CD4/CD38-/HLADR+	CD4/CD38+/HLADR+	CD4/CD38+/HLADR-	CD4/CD38
APRI Score	1.00	-0.26	0.81	0.47	-0.18	-0.07	0.65	0.75	0.59	0.16	0.18	-0.20	-0.07	-0.08	-0.24
CD4 count	-0.26	1.00	-0.03	-0.24	0.31	0.62	-0.24	-0.43	0.02	-0.06	-0.29	0.11	-0.19	0.10	-0.05
AST	0.81	-0.03	1.00	0.32	-0.12	0.01	0.43	0.49	0.79	0.33	0.03	-0.06	0.03	-0.13	-0.24
HBV DNA load	0.47	-0.24	0.32	1.00	-0.19	-0.06	0.26	0.58	0.22	0.10	0.10	0.06	0.19	-0.28	-0.27
CD4/CD8 ratio	-0.18	0.31	-0.12	-0.19	1.00	-0.50	-0.08	-0.02	-0.19	-0.30	-0.22	-0.02	-0.29	0.36	0.18
CD8 count	-0.07	0.62	0.01	-0.06	-0.50	1.00	-0.13	-0.38	0.12	0.07	-0.06	0.08	0.01	-0.19	-0.14
Median stiffness	0.65	-0.24	0.43	0.26	-0.08	-0.13	1.00	0.34	0.43	0.00	0.29	-0.05	-0.11	-0.04	-0.18
FIB-4 SCORE	0.75	-0.43	0.49	0.58	-0.02	-0.38	0.34	1.00	0.25	0.03	0.04	0.11	0.24	-0.13	-0.23
ALT	0.59	0.02	0.79	0.22	-0.19	0.12	0.43	0.25	1.00	0.23	0.03	0.10	0.01	-0.01	-0.11
Soluble CD14	0.16	-0.06	0.33	0.10	-0.30	0.07	0.00	0.03	0.23	1.00	0.27	-0.05	0.00	-0.31	-0.37
LBP	0.18	-0.29	0.03	0.10	-0.22	-0.06	0.29	0.04	0.03	0.27	1.00	-0.09	0.00	-0.07	0.00
CD4/CD38-/HLADR+	-0.20	0.11	-0.06	0.06	-0.02	0.08	-0.05	0.11	0.10	-0.05	-0.09	1.00	0.65	-0.47	-0.29
CD4/CD38+/HLADR+	-0.07	-0.19	0.03	0.19	-0.29	0.01	-0.11	0.24	0.01	0.00	0.00	0.65	1.00	-0.35	0.03
CD4/CD38+/HLADR-	-0.08	0.10	-0.13	-0.28	0.36	-0.19	-0.04	-0.13	-0.01	-0.31	-0.07	-0.47	-0.35	1.00	0.82
CD4/CD38	-0.24	-0.05	-0.24	-0.27	0.18	-0.14	-0.18	-0.23	-0.11	-0.37	0.00	-0.29	0.03	0.82	1.00
CD4/HLADR	-0.23	-0.01	-0.09	0.10	-0.12	0.07	-0.11	0.10	0.04	-0.08	-0.04	0.93	0.85	-0.47	-0.13
CD8/CD38-/HLADR+	-0.29	0.20	-0.03	-0.08	-0.04	0.22	-0.17	-0.17	0.16	-0.03	-0.07	0.52	0.17	-0.37	-0.28
CD8/CD38+/HLADR+	0.06	-0.11	-0.05	0.11	-0.14	-0.03	-0.01	0.29	-0.02	-0.12	-0.07	0.31	0.65	-0.15	0.11
CD8/CD38+/HLADR-	0.09	-0.09	-0.04	-0.17	0.23	-0.22	0.16	-0.12	-0.22	-0.06	-0.13	-0.58	-0.60	0.50	0.23
CD8/CD38	0.11	-0.13	-0.11	-0.13	0.20	-0.28	0.16	0.10	-0.17	-0.16	0.00	-0.20	0.02	0.39	0.38
CD8/HLADR	-0.16	-0.03	-0.03	0.08	-0.12	0.02	-0.06	0.08	0.20	-0.05	0.14	0.68	0.64	-0.40	-0.11
CD4/PD1+/CTLA4+	0.23	-0.13	0.14	-0.02	-0.46	0.23	0.06	-0.05	0.06	0.04	0.07	-0.01	0.09	-0.14	-0.07
CD4/PD1-/CTLA4+	0.06	0.10	-0.02	-0.21	-0.25	0.35	-0.03	-0.32	-0.01	0.03	0.21	-0.14	-0.16	-0.01	-0.06
CD4/PD1+	0.29	-0.58	0.19	0.09	-0.35	-0.29	0.09	0.35	0.05	0.02	-0.20	0.17	0.28	-0.23	-0.14
CD4/CTLA4+	0.11	0.04	0.06	-0.16	-0.34	0.35	-0.01	-0.28	0.03	0.04	0.16	-0.13	-0.11	-0.06	-0.09
CD8/PD1+/CTLA4-	0.09	-0.17	0.04	-0.17	0.21	-0.34	0.24	0.22	0.14	0.08	-0.03	0.02	0.04	0.09	0.04
CD8/PD1+/CTLA4+	0.19	0.30	0.27	0.15	0.14	0.17	0.09	0.06	0.25	0.19	-0.30	0.09	-0.01	-0.25	-0.36
CD8/PD1-/CTLA4+	-0.02	0.32	0.18	0.17	0.09	0.29	-0.08	-0.19	0.23	0.15	-0.19	-0.05	-0.10	-0.16	-0.19
CD8/PD1+	0.10	-0.16	0.05	-0.15	0.25	-0.35	0.24	0.24	0.15	0.07	-0.04	0.01	0.04	0.10	0.04
CD8/CTLA4+	0.01	0.35	0.19	0.17	0.16	0.25	-0.04	-0.14	0.20	0.16	-0.25	-0.03	-0.09	-0.16	-0.24

	CD4/HLADR	CD8/CD38-/HLADR+	CD8/CD38+/HLADR+	CD8/CD38+/HLADR-	CD8/CD38	CD8/HLADR	CD4/PD1+/CTLA4+	CD4/PD1-/CTLA4+	CD4/PD1+	CD4/CTLA4+	CD8/PD1+/CTLA4-	CD8/PD1+/CTLA4+	CD8/PD1-/CTLA4+	CD8/PD1+	CD8/CTLA4+
APRI Score	-0.23	-0.29	0.06	0.09	0.11	-0.16	0.23	0.06	0.29	0.11	0.09	0.19	-0.02	0.10	0.01
CD4 count	-0.01	0.20	-0.11	-0.09	-0.13	-0.03	-0.13	0.10	-0.58	0.04	-0.17	0.30	0.32	-0.16	0.35
AST	-0.09	-0.03	-0.05	-0.04	-0.11	-0.03	0.14	-0.02	0.19	0.06	0.04	0.27	0.18	0.05	0.19
HBV DNA load	0.10	-0.08	0.11	-0.17	-0.13	0.08	-0.02	-0.21	0.09	-0.16	-0.17	0.15	0.17	-0.15	0.17
CD4/CD8 ratio	-0.12	-0.04	-0.14	0.23	0.20	-0.12	-0.46	-0.25	-0.35	-0.34	0.21	0.14	0.09	0.25	0.16
CD8 count	0.07	0.22	-0.03	-0.22	-0.28	0.02	0.23	0.35	-0.29	0.35	-0.34	0.17	0.29	-0.35	0.25
Median stiffness	-0.11	-0.17	-0.01	0.16	0.16	-0.06	0.06	-0.03	0.09	-0.01	0.24	0.09	-0.08	0.24	-0.04
FIB-4 SCORE	0.10	-0.17	0.29	-0.12	0.10	0.08	-0.05	-0.32	0.35	-0.28	0.22	0.06	-0.19	0.24	-0.14
ALT	0.04	0.16	-0.02	-0.22	-0.17	0.20	0.06	-0.01	0.05	0.03	0.14	0.25	0.23	0.15	0.20
Soluble CD14	-0.08	-0.03	-0.12	-0.06	-0.16	-0.05	0.04	0.03	0.02	0.04	0.08	0.19	0.15	0.07	0.16
LBP	-0.04	-0.07	-0.07	-0.13	0.00	0.14	0.07	0.21	-0.20	0.16	-0.03	-0.30	-0.19	-0.04	-0.25
CD4/CD38-/HLADR+	0.93	0.52	0.31	-0.58	-0.20	0.68	-0.01	-0.14	0.17	-0.13	0.02	0.09	-0.05	0.01	-0.03
CD4/CD38+/HLADR+	0.85	0.17	0.65	-0.60	0.02	0.64	0.09	-0.16	0.28	-0.11	0.04	-0.01	-0.10	0.04	-0.09
CD4/CD38+/HLADR-	-0.47	-0.37	-0.15	0.50	0.39	-0.40	-0.14	-0.01	-0.23	-0.06	0.09	-0.25	-0.16	0.10	-0.16
CD4/CD38	-0.13	-0.28	0.11	0.23	0.38	-0.11	-0.07	-0.06	-0.14	-0.09	0.04	-0.36	-0.19	0.04	-0.24
CD4/HLADR	1.00	0.45	0.49	-0.67	-0.15	0.75	0.04	-0.15	0.19	-0.12	0.00	0.04	-0.03	-0.01	-0.03
CD8/CD38-/HLADR+	0.45	1.00	-0.12	-0.65	-0.77	0.68	-0.15	-0.06	-0.11	-0.06	-0.06	0.04	0.08	-0.06	0.07
CD8/CD38+/HLADR+	0.49	-0.12	1.00	-0.37	0.45	0.51	0.00	-0.21	0.24	-0.19	0.21	0.19	-0.09	0.23	-0.03
CD8/CD38+/HLADR-	-0.67	-0.65	-0.37	1.00	0.55	-0.85	0.05	0.17	0.05	0.12	0.25	-0.08	-0.21	0.23	-0.16
CD8/CD38	-0.15	-0.77	0.45	0.55	1.00	-0.29	0.08	0.01	0.22	-0.03	0.35	-0.11	-0.33	0.34	-0.28
CD8/HLADR	0.75	0.68	0.51	-0.85	-0.29	1.00	-0.07	-0.20	0.12	-0.17	0.04	-0.06	-0.10	0.05	-0.11
CD4/PD1+/CTLA4+	0.04	-0.15	0.00	0.05	0.08	-0.07	1.00	0.77	0.49	0.88	-0.15	-0.06	0.05	-0.16	-0.01
CD4/PD1-/CTLA4+	-0.15	-0.06	-0.21	0.17	0.01	-0.20	0.77	1.00	0.15	0.97	-0.14	-0.07	0.13	-0.15	0.06
CD4/PD1+	0.19	-0.11	0.24	0.05	0.22	0.12	0.49	0.15	1.00	0.25	0.36	-0.11	-0.43	0.34	-0.40
CD4/CTLA4+	-0.12	-0.06	-0.19	0.12	-0.03	-0.17	0.88	0.97	0.25	1.00	-0.21	-0.08	0.14	-0.22	0.07
CD8/PD1+/CTLA4-	0.00	-0.06	0.21	0.25	0.35	0.04	-0.15	-0.14	0.36	-0.21	1.00	0.30	-0.29	1.00	-0.14
CD8/PD1+/CTLA4+	0.04	0.04	0.19	-0.08	-0.11	-0.06	-0.06	-0.07	-0.11	-0.08	0.30	1.00	0.63	0.34	0.78
CD8/PD1-/CTLA4+	-0.03	0.08	-0.09	-0.21	-0.33	-0.10	0.05	0.13	-0.43	0.14	-0.29	0.63	1.00	-0.25	0.97
CD8/PD1+	-0.01	-0.06	0.23	0.23	0.34	0.05	-0.16	-0.15	0.34	-0.22	1.00	0.34	-0.25	1.00	-0.10
CD8/CTLA4+	-0.03	0.07	-0.03	-0.16	-0.28	-0.11	-0.01	0.06	-0.40	0.07	-0.14	0.78	0.97	-0.10	1.00

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Appendix 12 Correlation matrix for HBV/HIV co-infected subjects

Correlation matrix for HBV/HIV co-infected subjects. Significant correlations marked in red font.

Co-infected subjects Spearman Rank Order Correlations Marked correlations are significant at p <.05

	APRI Score	CD4 count	AST	HBV DNA	HIV viral load	CD4/CD 8 ratio	CD8 count	Median stiffness	FIB-4 SCORE	ALT	Length on HAART	sCD14	LBP
APRI Score	1.00	-0.04	0.77	0.26	0.12	0.07	-0.10	0.40	0.85	0.48	0.08	-0.31	-0.18
CD4 count	-0.04	1.00	-0.13	-0.34	-0.45	0.52	0.35	-0.20	0.02	-0.20	0.02	-0.04	-0.07
AST	0.77	-0.13	1.00	0.47	0.18	-0.03	-0.04	0.20	0.55	0.72	-0.04	0.12	-0.05
HBV DNA load	0.26	-0.34	0.47	1.00	0.42	-0.34	-0.02	0.17	0.11	0.32	-0.37	0.28	0.22
HIV viral load	0.12	-0.45	0.18	0.42	1.00	-0.58	0.18	0.28	0.03	0.10	-0.16	-0.14	-0.11
CD4/CD8 ratio	0.07	0.52	-0.03	-0.34	-0.58	1.00	-0.58	-0.27	0.10	-0.04	0.27	-0.20	-0.18
CD8 count	-0.10	0.35	-0.04	-0.02	0.18	-0.58	1.00	0.02	-0.09	-0.07	-0.13	0.14	0.09
Median stiffness	0.40	-0.20	0.20	0.17	0.28	-0.27	0.02	1.00	0.36	0.10	-0.14	-0.28	-0.14
FIB-4 SCORE	0.85	0.02	0.55	0.11	0.03	0.10	-0.09	0.36	1.00	0.20	0.11	-0.33	-0.09
ALT	0.48	-0.20	0.72	0.32	0.10	-0.04	-0.07	0.10	0.20	1.00	-0.07	0.16	0.00
Length on HAART	0.08	0.02	-0.04	-0.37	-0.16	0.27	-0.13	-0.14	0.11	-0.07	1.00	-0.34	-0.24
Soluble CD14	-0.31	-0.04	0.12	0.28	-0.14	-0.20	0.14	-0.28	-0.33	0.16	-0.34	1.00	0.20
LBP	-0.18	-0.07	-0.05	0.22	-0.11	-0.18	0.09	-0.14	-0.09	0.00	-0.24	0.20	1.00
CD4/CD38-/HLADR+	0.10	-0.36	0.27	0.19	0.19	-0.19	-0.01	-0.09	0.09	0.34	0.08	0.02	0.17
CD4/CD38+/HLADR+	0.06	-0.54	0.11	0.46	0.42	-0.44	-0.03	0.24	0.04	0.16	0.01	0.08	0.07
CD4/CD38+/HLADR-	-0.10	0.42	-0.23	-0.25	-0.30	0.16	0.09	0.01	-0.17	-0.28	-0.09	-0.07	-0.14
CD4/CD38	-0.02	0.11	-0.26	-0.03	-0.05	0.00	0.01	0.21	-0.02	-0.30	-0.05	-0.20	-0.18
CD4/HLADR	0.13	-0.48	0.19	0.33	0.35	-0.26	-0.07	0.06	0.16	0.26	0.12	-0.04	0.07
CD8/CD38-/HLADR+	0.07	0.18	0.14	-0.05	-0.19	0.16	0.05	-0.33	0.11	0.10	0.03	0.01	0.25
CD8/CD38+/HLADR+	0.08	-0.48	0.10	0.32	0.46	-0.38	-0.02	0.43	0.07	0.23	-0.09	0.09	-0.09
CD8/CD38+/HLADR-	-0.01	0.30	-0.05	-0.14	-0.35	0.24	-0.09	0.10	-0.17	-0.11	-0.09	0.06	-0.08
CD8/CD38	0.01	-0.28	-0.04	0.16	0.26	-0.19	-0.10	0.38	-0.06	0.05	-0.11	0.06	-0.22
CD8/HLADR	0.08	-0.40	0.07	0.22	0.38	-0.32	0.06	0.04	0.17	0.18	0.05	-0.02	0.04
CD4/PD1+/CTLA4+	-0.17	-0.50	-0.03	0.05	0.33	-0.53	0.21	-0.11	-0.34	0.17	0.01	0.10	0.15
CD4/PD1-/CTLA4+	-0.10	-0.03	-0.06	-0.15	0.00	-0.26	0.21	-0.21	-0.26	-0.04	0.02	0.03	0.30
CD4/PD1+	0.05	-0.66	0.31	0.37	0.38	-0.30	-0.18	0.01	-0.12	0.55	-0.03	0.21	0.08
CD4/CTLA4+	-0.20	-0.29	-0.09	-0.03	0.20	-0.42	0.22	-0.15	-0.37	0.04	0.01	0.07	0.22
CD8/PD1+/CTLA4-	-0.31	-0.41	-0.12	-0.11	0.21	-0.18	-0.08	-0.23	-0.37	0.03	0.02	0.02	-0.14
CD8/PD1+/CTLA4+	-0.26	-0.26	-0.11	-0.28	0.29	-0.21	0.02	-0.16	-0.39	0.04	0.16	0.00	-0.19
CD8/PD1-/CTLA4+	-0.08	0.02	0.07	-0.15	-0.03	-0.11	0.12	-0.18	-0.13	0.03	0.12	0.17	0.05
CD8/PD1+	-0.34	-0.41	-0.14	-0.15	0.20	-0.15	-0.11	-0.25	-0.40	0.02	0.05	0.03	-0.17
CD8/CTLA4+	-0.20	-0.13	-0.02	-0.21	0.13	-0.17	0.05	-0.17	-0.29	0.04	0.11	0.12	-0.06

	CD4/CD3 8-	CD4/CD38 +/HLADR+	CD4/CD38 +/HLADR-	CD4/ CD38	CD4/HL ADR	CD8/CD38- /HLADR+	CD8/CD38 +/HLADR+	CD8/CD38+ /HLADR-	CD8/ CD38	CD8/HL ADR	CD4/PD1 +/CTLA4+	CD4/PD1- /CTLA4+	CD4/P D1+	CD4/CTLA 4+	CD8/PD1 +/CTLA4-	CD8/PD1 +/CTLA4	CD8/PD1- /CTLA4+	CD8/PD 1+	CD8/CT LA4+
APRI Score	0.10	0.06	-0.10	-0.02	0.13	0.07	0.08	-0.01	0.01	0.08	-0.17	-0.10	0.05	-0.20	-0.31	-0.26	-0.08	-0.34	-0.20
CD4 count	-0.36	-0.54	0.42	0.11	-0.48	0.18	-0.48	0.30	-0.28	-0.40	-0.50	-0.03	-0.66	-0.29	-0.41	-0.26	0.02	-0.41	-0.13
AST	0.27	0.11	-0.23	-0.26	0.19	0.14	0.10	-0.05	-0.04	0.07	-0.03	-0.06	0.31	-0.09	-0.12	-0.11	0.07	-0.14	-0.02
HBV DNA load	0.19	0.46	-0.25	-0.03	0.33	-0.05	0.32	-0.14	0.16	0.22	0.05	-0.15	0.37	-0.03	-0.11	-0.28	-0.15	-0.15	-0.21
HIV viral load	0.19	0.42	-0.30	-0.05	0.35	-0.19	0.46	-0.35	0.26	0.38	0.33	0.00	0.38	0.20	0.21	0.29	-0.03	0.20	0.13
CD4/CD8 ratio	-0.19	-0.44	0.16	0.00	-0.26	0.16	-0.38	0.24	-0.19	-0.32	-0.53	-0.26	-0.30	-0.42	-0.18	-0.21	-0.11	-0.15	-0.17
CD8 count	-0.01	-0.03	0.09	0.01	-0.07	0.05	-0.02	-0.09	-0.10	0.06	0.21	0.21	-0.18	0.22	-0.08	0.02	0.12	-0.11	0.05
Median stiffness	-0.09	0.24	0.01	0.21	0.06	-0.33	0.43	0.10	0.38	0.04	-0.11	-0.21	0.01	-0.15	-0.23	-0.16	-0.18	-0.25	-0.17
FIB-4 SCORE	0.09	0.04	-0.17	-0.02	0.16	0.11	0.07	-0.17	-0.06	0.17	-0.34	-0.26	-0.12	-0.37	-0.37	-0.39	-0.13	-0.40	-0.29
ALT	0.34	0.16	-0.28	-0.30	0.26	0.10	0.23	-0.11	0.05	0.18	0.17	-0.04	0.55	0.04	0.03	0.04	0.03	0.02	0.04
Length on HAART	0.08	0.01	-0.09	-0.05	0.12	0.03	-0.09	-0.09	-0.11	0.05	0.01	0.02	-0.03	0.01	0.02	0.16	0.12	0.05	0.11
Soluble CD14	0.02	0.08	-0.07	-0.20	-0.04	0.01	0.09	0.06	0.06	-0.02	0.10	0.03	0.21	0.07	0.02	0.00	0.17	0.03	0.12
LBP	0.17	0.07	-0.14	-0.18	0.07	0.25	-0.09	-0.08	-0.22	0.04	0.15	0.30	0.08	0.22	-0.14	-0.19	0.05	-0.17	-0.06
CD4/CD38-/HLADR+	1.00	0.34	-0.70	-0.73	0.73	0.50	0.20	-0.61	-0.30	0.63	0.33	0.04	0.52	0.21	0.39	0.12	0.03	0.39	0.04
CD4/CD38+/HLADR+	0.34	1.00	-0.55	0.11	0.79	-0.38	0.84	-0.46	0.55	0.63	0.45	0.02	0.54	0.33	0.31	0.06	-0.16	0.28	-0.07
CD4/CD38+/HLADR-	-0.70	-0.55	1.00	0.57	-0.82	-0.18	-0.46	0.69	-0.01	-0.70	-0.39	0.10	-0.54	-0.21	-0.26	-0.07	0.02	-0.27	-0.01
CD4/CD38	-0.73	0.11	0.57	1.00	-0.27	-0.66	0.14	0.40	0.53	-0.31	-0.16	-0.08	-0.38	-0.07	-0.22	-0.13	-0.22	-0.23	-0.16
CD4/HLADR	0.73	0.79	-0.82	-0.27	1.00	-0.01	0.66	-0.72	0.22	0.81	0.41	-0.07	0.54	0.26	0.33	0.03	-0.17	0.32	-0.11
CD8/CD38-/HLADR+	0.50	-0.38	-0.18	-0.66	-0.01	1.00	-0.53	-0.37	-0.93	0.21	-0.05	0.10	0.05	-0.04	0.09	-0.18	-0.04	0.06	-0.14
CD8/CD38+/HLADR+	0.20	0.84	-0.46	0.14	0.66	-0.53	1.00	-0.40	0.73	0.57	0.35	-0.06	0.42	0.21	0.17	0.11	-0.13	0.17	-0.04
CD8/CD38+/HLADR-	-0.61	-0.46	0.69	0.40	-0.72	-0.37	-0.40	1.00	0.20	-0.93	-0.26	0.11	-0.35	-0.10	-0.30	0.01	0.24	-0.29	0.18
CD8/CD38	-0.30	0.55	-0.01	0.53	0.22	-0.93	0.73	0.20	1.00	0.04	0.13	-0.12	0.15	0.07	-0.02	0.19	0.04	0.00	0.13
CD8/HLADR	0.63	0.63	-0.70	-0.31	0.81	0.21	0.57	-0.93	0.04	1.00	0.31	-0.14	0.46	0.12	0.32	0.03	-0.19	0.31	-0.13
CD4/PD1+/CTLA4+	0.33	0.45	-0.39	-0.16	0.41	-0.05	0.35	-0.26	0.13	0.31	1.00	0.60	0.65	0.92	0.51	0.41	0.04	0.51	0.22
CD4/PD1-/CTLA4+	0.04	0.02	0.10	-0.08	-0.07	0.10	-0.06	0.11	-0.12	-0.14	0.60	1.00	0.05	0.82	0.23	0.45	0.38	0.23	0.41
CD4/PD1+	0.52	0.54	-0.54	-0.38	0.54	0.05	0.42	-0.35	0.15	0.46	0.65	0.05	1.00	0.42	0.51	0.23	-0.05	0.51	0.10
CD4/CTLA4+	0.21	0.33	-0.21	-0.07	0.26	-0.04	0.21	-0.10	0.07	0.12	0.92	0.82	0.42	1.00	0.44	0.45	0.17	0.44	0.32
CD8/PD1+/CTLA4-	0.39	0.31	-0.26	-0.22	0.33	0.09	0.17	-0.30	-0.02	0.32	0.51	0.23	0.51	0.44	1.00	0.54	0.03	0.99	0.30
CD8/PD1+/CTLA4+	0.12	0.06	-0.07	-0.13	0.03	-0.18	0.11	0.01	0.19	0.03	0.41	0.45	0.23	0.45	0.54	1.00	0.68	0.59	0.90
CD8/PD1-/CTLA4+	0.03	-0.16	0.02	-0.22	-0.17	-0.04	-0.13	0.24	0.04	-0.19	0.04	0.38	-0.05	0.17	0.03	0.68	1.00	0.09	0.92
CD8/PD1+	0.39	0.28	-0.27	-0.23	0.32	0.06	0.17	-0.29	0.00	0.31	0.51	0.23	0.51	0.44	0.99	0.59	0.09	1.00	0.36
CD8/CTLA4+	0.04	-0.07	-0.01	-0.16	-0.11	-0.14	-0.04	0.18	0.13	-0.13	0.22	0.41	0.10	0.32	0.30	0.90	0.92	0.36	1.00