LIVER CANCER EPIDEMIOLOGY AND RISK FACTORS IN SOUTH AFRICA

DANIEL WEI-HONG MAK

Thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

12 November 2019

DECLARATION

I, Daniel Wei-Hong Mak declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

…….

12th day of November 2019

This thesis is dedicated to my

MOTHER, FATHER, SISTER, BROTHER, PARTNER

my PAST and my FUTURE self

Keep Ithaka always in your mind.

Arriving there is what you are destined for.

But do not hurry the journey at all.

Better if it lasts for years,

so you are old by the time you reach the island,

wealthy with all you have gained on the way,

not expecting Ithaka to make you rich.

~ C.P. Cavafy

Papers and Presentations

The following published papers describe aspects of this work:

Mak, D., Sengayi, M., Chen, W.C., Babb de Villiers, C.B., Singh, E. and Kramvis, A. 2018. Liver cancer mortality trends in SA: 1999–2015. *BMC cancer*, *18*(1), p.798. **[Reproduced in Chapter 4]**

Mak, D., Babb de Villiers, C.B., Chasela, C., Urban, M.I. and Kramvis, A. 2018. Analysis of risk factors associated with hepatocellular carcinoma in black SAs: 2000–2012. *PloS one*, *13*(5), p.e0196057. **[Reproduced in Chapter 5]**

Aspects of this work have been presented in the following presentations:

Mak D. et al. Molecular Epidemiology of Hepatitis B Virus and Other Risk Factors Associated with Liver Cancer in South Africa. Abstract. HBV International Meeting, Taormina, Italy, October, 2018.

Mak D. et al. Epidemiology and Risk Factors associated with Liver Cancer in South Africa. CANSA Research in Action Conference. Poster presentation delivered at the CANSA Research in Action Conference, Pretoria, South Africa, July, 2018.

Mak D., Babb de Villiers C., Chasela C. Kramvis A. Case-control study of HBV/HIV/HCV mono and co-infection in black South Africans diagnosed with HCC from 2000-2012. Oral presentation delivered at the International Symposium: Hepatitis B Virus in Africa: No Room for Complacency, Johannesburg, South Africa, November, 2016.

Mak D., Babb de Villiers C., Chasela C. Kramvis A. Impact of Viral Hepatitis and Modifiable Risk Factors in a South African Cohort with Hepatocellular Carcinoma. Oral presentation delivered at the South Africa-China Bilateral Conference in Nanning, Guangxi, China, May, 2015.

Abstract

Primary liver cancer (LC) in South Africa (SA) is a public health concern; it is currently the seventh leading cause of death among SA men and women after colon cancer according to GLOBOCAN 2018. Despite this, publications of this disease remains scarce, if not absent.

Accordingly, trend analysis for LC incidence (from 1993-2012) and mortality (from 1999-2015) rates demonstrated an overall decrease in SA, and these trends varied between different sex-, age- and population-subgroups. Specifically, rates markedly increased in the middle-aged black African women in the recent years. LC risk was highest in black African men in their early 30s, and survival from this malignancy provided a dismal outlook as most patients were only diagnosed during autopsy rather than during hospital and/or clinic consultations. Barring this, these findings demonstrate the utility of mortality data in supplementing the cancer registries for cancer that progress quickly.

Liver cancer has several types, each originate from various type of cells that becomes cancerous. Hepatocellular carcinoma (HCC) accounts for the majority of liver cancers and originates from hepatocellular cells. The study of 150 HCC patients and 438 non-HCC cancer controls showed that risk factors that were significantly associated with HCC include: rural birthplace, male sex and living in an urban area for $\leq$14 years, Hepatitis B virus (HBV) DNA levels (>2000-$\geq$200,000 IU/ml) and antibodies against hepatitis C virus (anti-HCV) positivity. Interestingly, human immunodeficiency virus (HIV) infection alone did not confer any risk in developing HCC, nor did it synergistically interact with HBV infection. Comparably, lifestyle-related risk factors (alcohol consumption, tobacco smoking, number of sexual partners, diabetes and hormonal contraceptive use) were not associated with HCC risk.

Phylogenetic analyses of the HBV isolates from the HCC patients and non-HCC controls demonstrated that HBV subgenotypes included A1 (90.7%), A2 (2.3%) and A3 (7%). Core promoter mutations (T1753V, A1762T and G1764A), preS deletions, ps2F22L, and preS2 start codon mutations were associated with HCC development, and

the combination of which may potentially assist in the development of more targeted screening in high-risk groups.

In view of these findings, this work provides much needed analysis of LC trends in SA and provides a critical assessment of current control strategies, or gaps, in the prevention of and interventions for LC in SA. Moreover, this work pays particular attention to the consolidation of what is an overview of LC in SA by reporting various aspects: incidence, mortality, demographics, viral and non-viral risk factors and a combination of HBV mutations as biomarkers.

Preface

Accordingly, the aims of this study were to:

1. Address the gaps in research on primary LC (including HCC and cholangiocarcinoma) in South Africa (SA) by analysing incidence rates reported by the National Cancer Registry (NCR) of SA, and the mortality rates reported by Statistics SA (StatsSA), while taking sex and/or population (racial/ethnic) group variations into consideration.
2. Retrospectively describe the occurrence of viral (HBV, HCV and/or HCV infection) and non-viral (socio-demographics and lifestyle behaviours) risk factors associated with HCC, the most common type of LC, in patients presenting with and without HCC in public hospitals in the Johannesburg area, SA.
3. Characterise and identify, at the molecular level, a combination of possible HBV mutation biomarkers that are related to HCC development in the black African population group with HBV-related HCC.

This document is a thesis for a PhD by publication in the field of LC epidemiology, and in particular the risk factors that contribute to the pathogenesis of this malignancy. This document is presented as a set of published academic papers, with supporting chapters, providing both the necessary background and additional material. A list of references cited in the published papers appear within the reference section of the corresponding papers themselves, whereas those cited in the other chapters is given at the end of this document in the reference section.

This work was undertaken in the Hepatitis Virus Diversity Research Unit (HVDRU), Department of Internal Medicine, School of Clinical Medicine, Faculty of Health Sciences at the University of the Witwatersrand in Johannesburg, between January 2015 and July 2018.

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To my co-supervisor Professor Charles Chasela, your assistance with the statistical analysis has allowed me to complete my dissertation, and in addition for your guidance, encouragement and support, I thank you.

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List of Abbreviations

ε	Hepatitis B virus encapsidation signal
aa	Amino Acid
A	(Nucleotide) Adenosine
AAPC	Average annual percentage change
AFB1	Aflatoxin B_1
AFP	Alpha-fetoprotein
ALT	Alanine transaminase
Anti-HBc	Antibodies to HBV core antigen
Anti-HBe	Antibodies to HBV e antigen
Anti-HBs	Antibodies to HBV surface antigen
APC	Annual percentage change
APASL	Asian Pacific Association for the Study of the Liver
ART	Antiretroviral therapy
ASIR	Age-standardised incidence rate
ASpIR	Age-specific incidence rate
ASMR	Age-standardised mortality rate
AASLD	American Association for the Study of Liver Diseases
AST	Aspartate transaminase
BCP	Hepatitis B virus basic/basal core promoter
bp	Base pair
C	(Nucleotide) Cytidine
cccDNA	Covalently closed circular DNA
CHB	Chronic hepatitis B
CI	Confidence intervals
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
EASL	European Association for the Study of the Liver
ELISA	Enzyme-linked immunosorbent assay
EPI	Expanded Programme on Immunization
ER	Endoplasmic reticulum
g	grams
G	(Nucleotide) Guanosine
HAART	Highly active antiretroviral therapy
HBcAg	Hepatitis B virus core antigen
HBeAg	Hepatitis B virus e antigen
HBsAg	Hepatitis B virus surface antigen
HBV	Hepatitis B virus
HBx	Hepatitis B virus X protein
HCC	Hepatocellular carcinoma

HepB	Hepatitis B virus vaccine
HH	Hereditary haemochromatosis
HIC	High income country
HIV	Human immunodeficiency virus
IARC	International Agency for Research in Cancer
ICD-O	International Classification of Diseases for Oncology
JCS	Johannesburg Cancer Case-Control Study
JSH	Japan Society of Hepatology
kD	Kilodalton
KLCSG-NCC	Korean Liver Cancer study Group-National Cancer Centre Korea
LHBs	Hepatitis B virus large surface protein
LC	Liver cancer
LMIC	Low-middle income country
MHBs	Hepatitis B virus medium/middle surface protein
MV	Morphological Verification
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCR	National Cancer Registry
nm	Nanometer
nt	Nucleotide
OBI	Occult hepatitis B virus infection
OR	Odds Ratio
ORF	Open reading frame
PCR	Polymerase chain reaction
rcDNA	relaxed circular DNA
pgRNA	Pregenomic RNA
RNA	Ribonucleic acid
RR	Risk Ratio
SA	South Africa
SEER	Surveillance, Epidemiology and End Results
SHBs	Hepatitis B virus small surface protein
SSA	Sub-Saharan Africa
StatsSA	Statistics SA
SVP	Subviral particles
T	(Nucleotide) Thymidine
WHO	World Health Organization

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— CHAPTER 1 —

Literature Review on Liver Cancer

This chapter contains a short condensed literature review of the epidemiology of primary liver cancer (LC) worldwide as well as the underlying risk factors that is associated with the pathogenesis of this tumour. This also includes a review of hepatocellular carcinoma (HCC), the most common histological subtype of LC, and its clinical features, diagnostic modalities and disease surveillance.

1.1 Epidemiology

1.1.1 Global incidence of primary liver cancer

According to GLOBOCAN 2018, an estimated 841,080 new cases of LC was diagnosed worldwide in 2018 (Bray et al. 2018). Based on the number of new cases of diagnosed cancer in humans, LC has remained the sixth most commonly diagnosed cancer since 2008 (Ferlay et al. 2010) until the most recent available data in 2018 (Bray et al. 2018). The number of cases increased by 12.4% from 2008 (696,000) (Ferlay et al. 2010) to 2012 (782,000) (Ferlay et al. 2015), and 7.6% from 2012 to 2018 (841,080) (Bray et al. 2018). In 2012, LC constituted 9.1% of all newly diagnosed cancers (Ferlay et al. 2015), whereas this is reduced to 4.7% in 2018. Despite this, the public health concern regarding LC stems from the huge burden that this malignancy imposes by causing hundreds of thousand deaths (782,000) worldwide (Bray et al. 2018). This is a consequence of the dismal prognosis associated with LC, where untreated patients have a life expectancy of less than 6 months (Weledji et al. 2014). In particular, of the total LC cases that were diagnosed in 2018, as much as 93.9% died (781,631 deaths) as a consequence of this disease (Bray et al. 2018). Not only does this figure rank LC as the fourth leading cause of cancer-related death worldwide (8.2% of total cancer-related deaths in 2018), the annual fatality ratio (mortality-to-incidence ratio) of 0.93 is amongst the highest worldwide reported for a human cancer (Bray et al. 2018).

LC as a primary liver cancer includes HCC, intrahepatic cholangiocarcinoma and other rare types, with the exclusion of secondary liver cancer. Globally, the distribution of LC is non-uniform and of all the new cases recorded in 2018, the vast majority occurred in regions such as East and south Eastern Asia and Western and Central Africa (Figure 1.1) (Ferlay et al. 2018). While numerous studies investigating LC epidemiology and aetiology have been published in Western and Asian countries (Goutté et al. 2017; Choo et al. 2016), there is little to no information investigating LC in the sub-Saharan African subcontinent, despite evidence also showing a medium to high incidence of this disease in this region. Even if information pertaining to LC incidence rate is available, as in this case, in the GLOBOCAN database, the lack of empirical data, for SA for example, about the levels of incidence experienced by adults unfortunately means that incidence rates can only be calculated using modelling of cancer registry data derived from

neighbouring countries (e.g. Zimbabwe). Not only is the generated LC incidence rate inaccurate, the implications of underestimating the true impact of this disease may lead to the neglect of the disease as a public health issue.

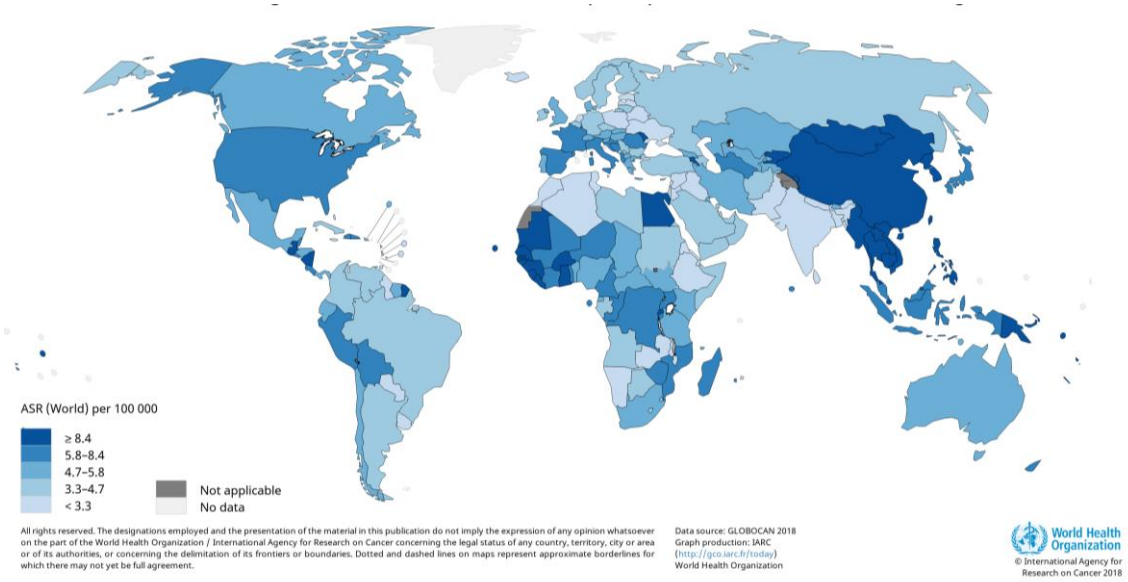

Figure 1.1 Liver cancer incidence rate in both sexes worldwide available from GLOBOCAN 2018 (http://gco.iarc.fr), accessed on 16 March 2019.

Age-standardised incidence rate (ASIR) are 2-fold greater among men in most regions in the world Figure 1.2, but the highest rates are observed mainly in lower Human Development Index (HDI) settings – a composite index of life expectancy, education, and gross national income (Ferlay et al. 2018). In particular, LC represents the most common cancer in 13 geographically diverse countries that include several in Northern and Western Africa (Burkina Faso, Egypt, the Gambia, Guinea, Guinea-Bissau, Mauritania, Niger) and Eastern and South-Eastern Asia (China, Cambodia, Laos, Mongolia, Thailand and Vietnam) (Bray et al. 2018).

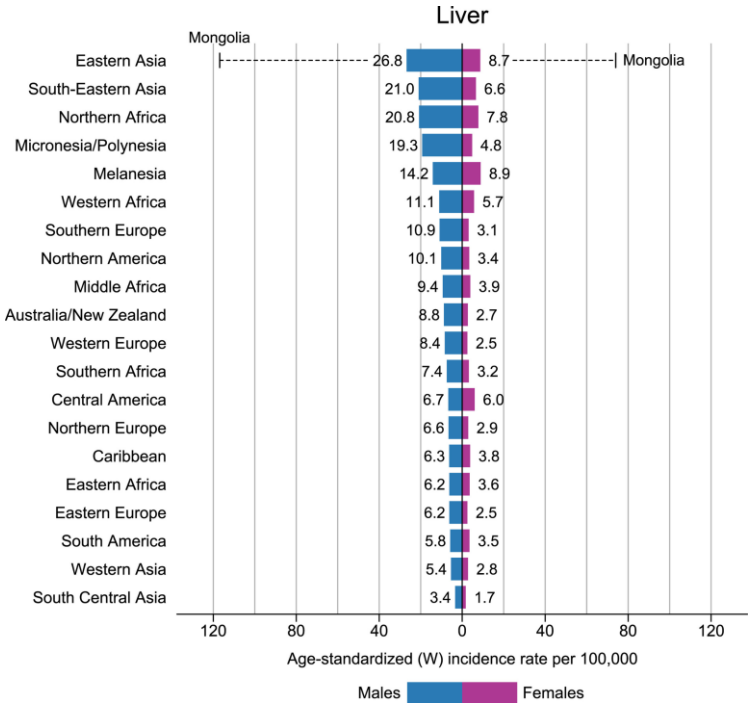

Figure 1.2 Region-specific incidence age-standardised rates by sex for liver cancer in 2018. Rates are shown in descending order of world age-standardized rates among men (Bray et al. 2018).

The ASIR for LC in both sexes can vary as much as 85-fold between high-risk (Mongolia: ASIR 93.7) and low-risk regions (Nepal: ASIR 1.1) and the rates are less than 5/100,000 persons/year in parts of Europe (Northern: 4.7; Central and Eastern: 4.0), South America (4.6) and Western Asia (or Middle East: 4.0) (Ferlay et al. 2018). In men, the ASIR in Mongolia (117) far exceed those of any other country (Figure 1.2), with estimated 2018 rates approximately 4 times higher than those estimated in The Gambia (36.5), China (27.6), Republic of Korea (27.7), and Ghana (24.4), for example (Ferlay et al. 2018).

1.1.2 Incidence of liver cancer in Africa

The continent of Africa encompasses a wide geographic area of 30.4 million square kilometres and is the world's second largest continent, making up 6% of the earth’s total surface and consisting of many low-middle income countries (LMICs). With just over 1 billion people (1.3 billion) (United Nations 2017), Africa is also the second most inhabited continent, and accounts for 16% of the global population. However, the

majority (83.6%; 1.1 billion (Anon 2017)) of the African population lives to the south of the Sahara Desert that covers the northern third of the continent. Although both northern African and sub-Saharan African countries share the same continent, they differ to some extent particularly in regards to climate, ethnic and cultural backgrounds of their populations. In particular, as a consequence of historical migrations, northern African countries are closely tied to the Middle East and parts of Europe (Shoup 2011). Therefore, almost every distinctive feature of cancer in Africa prevails predominantly in sub-Saharan Africa, and none more so than LC (Parkin et al. 2005).

In 2018, sub-Saharan Africa has the fourth highest number of diagnosed LC cases worldwide after south Eastern Asia, south Central Asia and North America, with more than 36,000 new cases of LC (Ferlay et al. 2018). The high fatality rate in sub-Saharan Africa black Africans suggests that the recorded annual death from the malignancy is virtually the same as the number of cases diagnosed in the population at that time, thus highlighting the inadequacy in the preventive, screening and treatment programs that are currently available on this subcontinent. The distribution of LC is not uniformly distributed in sub-Saharan Africa and varies geographically (Figure 1.3). LC ASIRs documented in the International Agency for Research In Cancer (IARC) registry in 2008 (Ferlay et al. 2010), 2012 (Ferlay et al. 2013) and 2018 (Ferlay et al. 2018) are depicted in Figure 1.3. Comparisons between these three years show that unlike more high income countries (HICs) show an increasing ASIRs for LC in each year, and opposite and more drastic decrease in observed in less developed/low income regions. In sub-Saharan Africa, the regions with decreases in rates occurred in western and Central Africa in both sexes. In contrast, an increase occurred in both sexes in eastern Africa and in southern African men only in the most recent year.

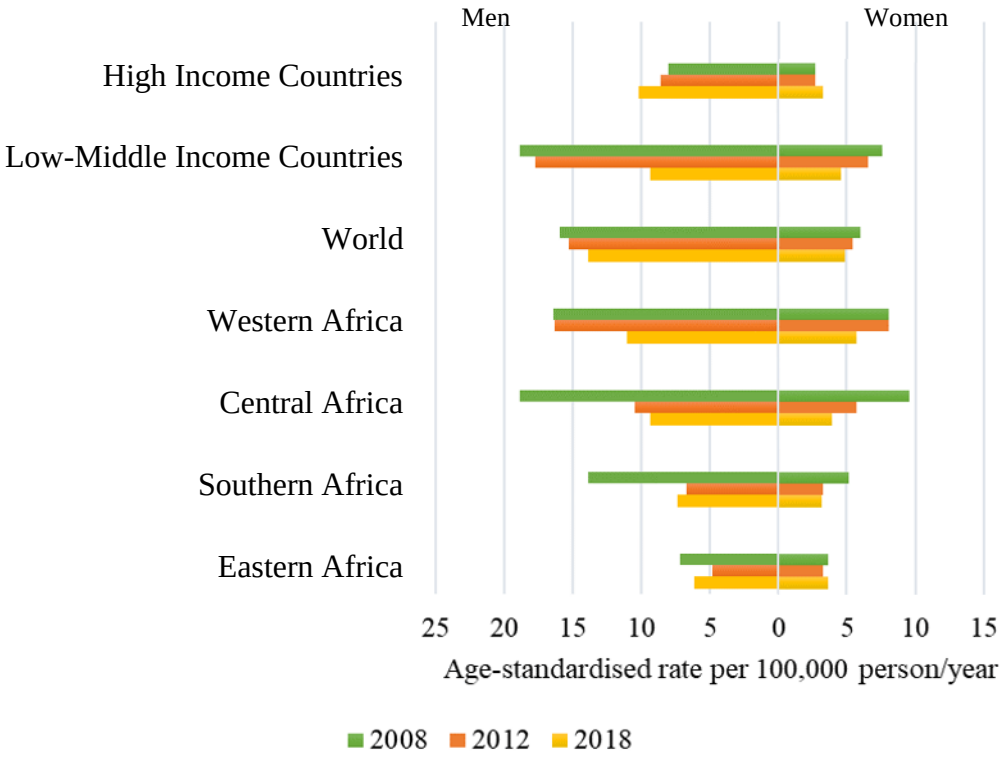

Figure 1.3 Liver cancer age-standardised incidence rate (ASIR) per 100,000 persons-year in 2008, 2012 and 2018, and compiled using data from (Ferlay et al. 2010), (Ferlay et al. 2013) and (Ferlay et al. 2018), respectively.

According to GLOBOCAN 2018, variations in the ASIR also occur between different countries in sub-Saharan Africa (Figure 1.4). Of the 11 western African countries, The Gambia and Guinea have high rates (ASIR > 20) and the remaining 9 (Benin, Burkina Faso, Cote d'Ivoire, Guinea Bissau, Ghana, Nigeria, Togo, Niger, Mali) have intermediate incidences (5 > ASIR < 20). In Central Africa, the incidence is intermediate in the Republic of Congo and low in the Cameroon and Gabon. Of the 11 eastern African countries, four (Kenya, Zimbabwe, Uganda and Rwanda) and seven (Malawi, Ethiopia, Tanzania, Mauritius, Zambia, Reunion, Mozambique) have intermediate and low incidences (ASIR < 5), respectively. Of the four southern African countries, two have intermediate incidences (Botswana and Swaziland) and the remaining (Namibia and the Republic of SA) have low incidence of LC.

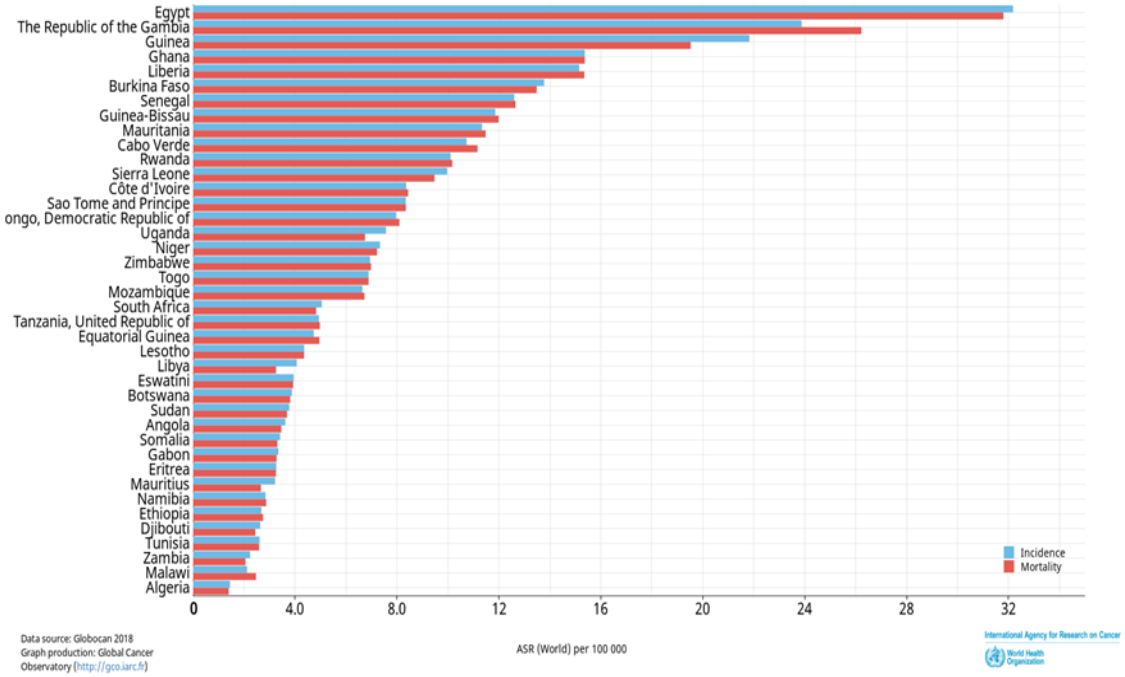

Figure 1.4 Liver cancer age-standardised incidence (blue) and mortality (red) rate per 100,000 persons-years in African countries in 2018 available from GLOBOCAN 2018 (http://gco.iarc.fr), accessed on 16 March 2019.

In other reports, the frequency of LC was expressed in another way: the tumour accounted for 1.9% of all malignant diseases in Nigeria (2009-2013) (Ekanem & Parkin 2016a), 2.2% in Malawi (2008–2010), 4% in Uganda (1991–2010) (Wabinga et al. 2014), 5% in Eastern Cape Province, SA (1998-2012) (Somdyala et al. 2015) and 7.6% in Zimbabwe (2006) (Chokunonga et al. 2013). In the Gambia 60% of all malignant diseases in males and 20% of those in females are LC (Bah et al. 2013). According to the National Cancer Registry (NCR) of The Gambia, covering the years 1990-2009, The Gambia, one of the poorest countries in the world (Ashta 2013), has the highest recorded incidence of LC among sub-Saharan Africa countries (31.6 and 16.9 per 100,000 persons/year in men and women, respectively (Bah et al. 2013), which is higher than that recorded by GLOBOCAN 2018 (Ferlay et al. 2018). This incidence rate is 3.4 and 5 times higher than that in white men and women in the United States, respectively (Islami et al. 2017).

Even within sub-Saharan Africa countries, the frequency of LC is not equally distributed. This phenomenon is most obvious in SA, where the tumour occurs

appreciably more often in rural inhabitants and at a younger age than in their urban counterparts (Kew 2013). It is likely that these variations at the subcontinental and local level reflect the disparities in the geographical distribution of certain environmental, viral and socioeconomic factors that all to some extent, play a contributory role in the development of LC.

Despite these findings, published incidences of LC in the sub-Saharan African subcontinent are believed to underestimate its true burden as a consequence of the many instances where the malignancy is either not definitively diagnosed or is not recorded in a cancer registry (Kew 2013). It has been suggested that the limited number of reliable cancer registries in sub-Saharan Africa and the challenges associated with diagnosing LC as a result of scarce resources are the major reasons for the underdiagnosis and under-reporting of LC. It is thus not surprising that many of these regions have a low score for data quality and the amount of information that is available (Ferlay et al. 2013).

1.2 Aetiology, Pathogenesis and Risk Factors for Hepatocellular Carcinoma

Depending on its cellular derivation, primary LC is comprised of a heterogenous group of tumours. The most common histological subtype worldwide is HCC which originates from the hepatocytes and constitutes 75%–90% of the total diagnosed LC cases. As HCC is a frequently diagnosed subtype, it is often used interchangeably to describe LC. The second leading subtype is intrahepatic cholangiocarcinoma that arises from the biliary epithelium and constitutes the remaining 15%-20% of diagnosed LC cases (Ananthakrishnan et al. 2006; Altekruse et al. 2009). Other rare forms that account for fewer than 1% of cases and this include angiosarcoma (inner lining of blood vessels), hepatoblastoma (hepatocyte precursors) and epitheloid hemangioendothelioma (endothelial cells) (Sia et al. 2017; Parkin et al. 2005; Ananthakrishnan et al. 2006).

Similar to most other cancers, HCC is a multistage disease that results from a culmination of pathologically induced damages that evolve over time. However, unlike most other cancers, HCC is unique in that it develops in the context of well-known and readily identifiable risk factors including host related factors (hereditary haemochromatosis), hepatitis B/C virus (HBV and HCV) and environmental agents

(dietary aflatoxin, alcohol exposure, iron overload, obesity and diabetes mellitus) (Yu & Yuan 2004; Kew 2013; Della Corte et al. 2013). Other risk factors that are still under investigation include: human immunodeficiency virus (HIV) infection, cigarette smoking and oral contraceptive drugs. HCC almost invariably occurs in the setting of histologically abnormal liver such as chronic hepatopathy and/or cirrhosis (Villanueva & Luedde 2016; Fattovich et al. 2004) or in association with genetic disorders such as haemochromatosis, Wilson's disease, alpha 1-antiprotease (antitrypsin) deficiency and cystic fibrosis (Kumar & Riely 1995). Interestingly, a proportion (10%-57%) of HCC cases may occur in the non-fibrotic or minimal fibrotic liver (Bralet et al. 2000; Young et al. 2012; Mittal et al. 2016), which may suggest a possible shift in the underlying aetiology of HCC particularly in developed regions of the world (Schlageter et al. 2014).

Table 1.1 Risk factors for Hepatocellular Carcinoma.

Viral	Environmental	Host Related
Hepatitis B Virus (HBV)	Alcohol exposure	Age
Hepatitis C Virus (HCV)	Aflatoxin B1 (AFB_1)	Male Sex
Adeno-associated Virus 2 (AAV2)	Iron Overload	Haemochromatosis
	Metabolic Syndrome	Wilson's Disease
	Aristolochic Acid	Alpha-1 Anti-Trypsin Disease

1.2.1 Cirrhosis

Cirrhosis or scarring of the liver is a direct result of chronic hepatic injury. Marked by the degeneration of the hepatocytes and impaired liver function, cirrhosis is an important risk factor for HCC that interconnects various hepatocarcinogens in the pathogenesis of this malignancy and is detected in 80% to 90% of autopsied HCC patients worldwide (Simonetti et al. 1991; Sanyal et al. 2010). Generally, cirrhosis provides a field effect that evolves to neoplasia (such as HCC) in a stepwise fashion through the dysplastic process that is characterised by continuous inflammation and regeneration of the hepatocytes (Ramakrishna et al. 2013). Specifically, the liver begins

to decompensate as it is unable to replace the damaged hepatocytes and scar tissue develops as a result. The loss of liver function is the eventual outcome in patients with advanced cirrhosis, ultimately resulting in hepatic failure and death. Greater exposure to common risk factors such as infection with HBV (30%) and/or HCV (27%) account for more than half of cirrhotic cases (57%) (Perz et al. 2006). Severe and repeated flares of HBV infection that are associated with an elevation of serum alpha-fetoprotein (AFP) coupled with continuous viral replication increase the likelihood of developing cirrhosis (Chang & Liaw 2014).

Cirrhosis can be classified according to the different anatomical structures: *micronodular*, characterised by nodule sizes up to 3 mm in diameter, with septa up to 2 mm in diameter; *macronodular*, characterised by the nodule sizes ranging from 3 mm to 3 cm in diameter, and *multilobular* with septa of irregular size. However, one anatomic form may not necessarily be isolated from another, and is named mixed *macronodular* and *micronodular* cirrhosis accordingly (Popper 1977). Whether the risk of developing HCC is uniformly associated with each anatomical type of cirrhosis is not clear, however, a surveillance program of a cohort of 313 Italian cirrhotic patients reported that late stage cirrhosis was associated with 3-fold increased risk of HCC (Bolondi 2001).

Table 1.2 shows the Child-Pugh Score and Prognosis, which is used to assess the prognosis of cirrhosis, which entails the measurement of five key clinical variables as shown in the table. Each measure correlates to a score from 1 to 3, and the sum of these scores are used to classify the prognosis of chronic liver disease into Child-Pugh class A to C.

Table 1.2 Child-Pugh Score and Prognosis

Child-Pugh Score			
Factor	1 point	2 point	3 point
Total bilirubin (μmol/L)	<34	34-50	>50
Serum albumin (g/L)	>35	28-35	<28
PT INR*	<1.7	1.71-2.30	>2.30
Ascites	None	Mild	Moderate to Severe
Hepatic encephalopathy	None	Grade I-II	Grade III-IV
	Class A	Class B	Class C
Total points	5-6	7-9	10-15
1-year survival	100%	80%	45%

*PR INR - prothrombin ratio and international normalized ratio

In a prospective study, consisting of 400 Chinese cirrhotic patients, Child-Pugh class B and C were independent prognostic factors for HCC accounting for 3- and 8-fold increased risk, respectively. Moreover, the authors reported a higher prevalence of Child-Pugh class C (15.0%) in HBV/HCV co-infected individuals than in those infected with HBV (3.4%) or HCV (3.6%) alone. Thus, patients with more advanced Child-Pugh scores are at a higher risk of HCC, that may be dependent on the underlying cause of the malignancy. This is supported by a previous study that reported the 5-year cumulative risk for HCC was higher in HBV-associated cirrhosis (17%-30%) than in HCV (10%-15%), while that for alcoholic and biliary cirrhosis was lower (8% and 4% respectively) (Fattovich et al. 2004). Cirrhosis can also be the consequence of other conditions including haemochromatosis, Wilson's disease, α_1-Antitrypsin deficiency, primary biliary cirrhosis, primary sclerosing cholangitis, autoimmune hepatitis (Fattovich et al. 2004).

1.2.2 Non-cirrhotic Hepatocellular Carcinoma

It is well established that neoplastic lesions usually originate on a bed of chronic necroinflammation that sequentially progresses from fibrosis to cirrhosis and finally culminates into HCC. However, a proportion of cases, ranging from 15%-20% of HCC patients, occur in the absence of cirrhosis (Simonetti et al. 1991; Evert & Dombrowski 2008). In Western countries, this progression towards the malignancy arises in 46% of

patients, with a history of anabolic steroid use and metabolic syndrome (obesity and diabetes mellitus) that is associated with non-alcoholic fatty liver disease (NAFLD) (Nzeako et al. 1996; Schütte et al. 2014; Evert & Dombrowski 2008). In less developed countries, non-cirrhotic HCC occurs in chronic viral hepatitis patients more frequently than in the more developed Western countries, 53% of the cases in SA (Kew 1989a) and only 9.5% in the United States (Chayanupatkul et al. 2017). While various reasons may account for this difference, geographic locations as an indicator of varying aetiological risk factors may be a possible explanation. Indeed, the literature has predicted that metabolic syndrome will become the most prominent risk factor for NAFLD-associated HCC in the coming decades (Margini & Dufour 2016), contributing to as much as 35% of the aetiological risks identified (Dyson et al. 2014) and it is estimated that 99% of NAFLD patients do not present signs of background cirrhosis (Dam-Larsen et al. 2004). This suggests that cirrhosis is not a condition *sine qua non* in HCC development.

Even though HCC patients are divided into cirrhotic and non-cirrhotic in some studies, the liver histology of non-cirrhotic HCC patients usually harbours some element of abnormality and is rarely 'normal'. In particular, the degree of fibrosis in the non-neoplastic liver may vary significantly from non-cirrhotic chronic HCC patients with minimal to severe fibrosis (Wang et al. 2013).

1.2.3 Hepatitis Viruses

Worldwide, hepatitis virus infections (HBV and HCV) are the most important causes of HCC development, with an attributable risk estimate of the combined effects of these two infections accounting for approximately 80% of cases worldwide (El-Serag 2012). According to WHO, an estimated 292 million people worldwide are living with chronic HBV (Polaris Observatory Collaborators 2018) (See Chapter 2 for literature review) or HCV infection in 2015, and is responsible for 1.34 million deaths (World Health Organization 2017b). Approximately 60% of cases worldwide, and 50% to more than 90% of cases in LMIC, are caused by these two viruses (de Martel et al. 2015), with 12- to 14-fold increased risk of developing HCC (Cho et al. 2011). Mono-infection from HBV and HCV are well-established independent risk factors for HCC and the relative importance of each virus varies globally (de Martel et al. 2015). It is reported that of the 770,000 cases of HCC that occurred worldwide in 2012, more than half (56%, 95% CI:

52-60) was attributable to HBV and 20% (95% CI: 18–22) to HCV (Maucort-Boulch et al. 2018). In Africa, HCV is the leading cause of HCC in Egypt (84%) whereas HBV is the leading cause in other African countries (55%) (Yang et al. 2017). Overall, only 3% of patients in Africa have evidence of HBV–HCV co-infection (Yang et al. 2017).

Accordingly, the growing concern regarding the lack of public awareness of HBV/HCV infection and the complication of eradicating this disease in both HIC and LMIC countries have led to the adoption of the first resolution on viral hepatitis by the World Health Assembly in 2010 (World Health Organization 2010). A second resolution in 2014 (World Health Organization 2014) further highlighted the importance of viral hepatitis and the impact it has on public health, and an advocacy policy document was issued in 2016 for the elimination of hepatitis infection by the year 2030.

1.2.4 Hepatitis C Virus (HCV)

HCV is a hepatotropic RNA virus of the family *Flaviviridae* and the genus *Hepacivirus* (Kim & Chang 2013). HCV a health problem of global importance with epidemiological studies having shown that an estimated 75% of cases of acute hepatitis C progress to chronic infection (Hajarizadeh et al. 2013), a quarter of which (25%) will develop complications of chronic liver disease including cirrhosis within two to three decades of disease onset (Ghany & Jake Liang 2016), and 1%–5% will develop LC (Westbrook & Dusheiko 2014). As a consequence, of more than 700,000 new cases of LC that occur each year, one third are attributable to HCV infection with 350,000 people die from HCV related diseases every year (Lee et al. 2014).

According to a comprehensive review of 138 countries worldwide, the total global HCV prevalence is estimated at 2.5%, affecting 177.5 million people in the world over the period of 2000-2015 (Figure 1.5) (Petruzziello et al. 2016). High prevalence rates of more than 3.5% are reported in Central Asia and Central Africa; moderate prevalence of 1.5%-3.5% are reported in East, South and Southeast Asia, West and East Africa, North Africa and Middle East, Southern and Tropical Latin America, Caribbean, Australasia, and Eastern Europe; low prevalence of less than 1.5% are reported in southern Africa, North America, Andean and Central Latin America, Pacific Asia and Western and Central Europe. The paradigm of HCV is affected by changes in policies concerning

competing causes of morbidity and mortality caused by other epidemics such as HIV, tuberculosis, bilharzia and malaria. These paradigms may have played a role in the decreasing trends of the respective diseases in Western Europe, Southern Africa and Australasia. In contrast, increasing trends are observed in Central Africa and Central Asia.

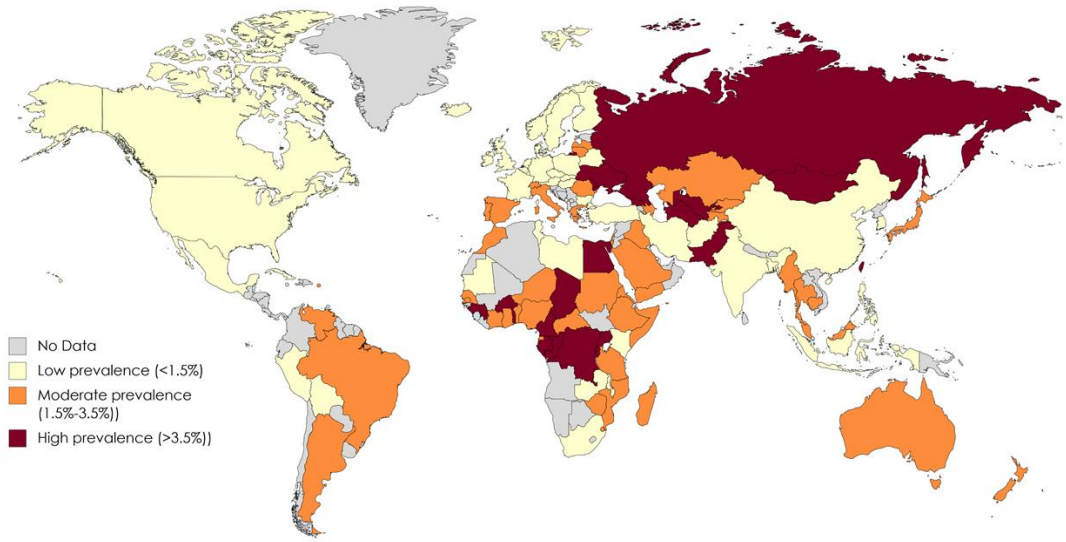

Figure 1.5 Global prevalence of Hepatitis C Virus infection, compiled using data from (Petruzziello et al. 2016). The map was created with https://mapchart.net.

In most regions worldwide HCV-related LCs are on the increase as a consequence of an epidemic of community-acquired infections and it is likely to worsen (Daw et al. 2016). Globally, from 1990 to 2013, the HCV-related LC age-standardised death rate (per 100,000) has increased by 125.4%, from 2.4 to 5.5, respectively (GBD 2013 Mortality and Causes of Death Collaborators). During this period, HCV has overtaken HBV as a leading cause of LC deaths in the western world (17.1% vs 38.9% in 1990 and 41.9% vs 36.7% in 2013, for HCV and HBV, respectively) (GBD 2013 Mortality and Causes of Death Collaborators).

Owing to the already higher prevalence of HCV, compared to HBV in Western countries, HCV constitutes the most important risk factor for HCC in these countries, including Japan, Egypt and Russia. Despite a 30% decrease since 2008, an Egyptian demographic health survey conducted in 2015 concluded that 10% the Egyptian population aged 15-59 are HCV antibody positive and 3.7 million persons in that age

group have chronic HCV infection, making this the highest prevalence in any population in the world (Kandeel et al. 2017). Whereas the HCV epidemic in Russia is attributed to intravenous drug addition (Paintsil et al. 2009), the epidemic in Egypt is attributed to the extensive iatrogenic transmission during the historical era of parenteral antischistosomal therapy, whereby only in the year 2001 was the broad infection control programs initiated by Egyptian Ministry of Health in the country (Mohamoud et al. 2013). The iatrogenic transmission of HCV infection included sharing or reuse of contaminated needles, inadequately sterilised surgical or dental equipment, and transfusions of contaminated blood (Mohamoud et al. 2013). Although an estimated 40% of patients may clear the virus spontaneously (Iqbal et al. 2017), the main health burden stems from the 60% of patients who develop chronic HCV. In this patient population, a lag phase of 20–30 years means that any clinical evidence of HCV infection will only become apparent several decades after infection (Elgharably et al. 2017). Of note, while HCV therapy with Sofosbuvir was launched in Egypt by Ministry of Health and the Egyptian National HCV Control Program in late 2015, there remains several challenges in combating this viral infection as a result of high costs associated with this treatment, limited resources, a lack of infection control practices, and barriers related to differences in socioeconomic and demographic characteristics (Elgharably et al. 2017). Positively, the Egyptian Government's broader strategy for reducing the price of the Sofosbuvir treatment has been successful, which makes it now more affordable to the average patient in Egypt.

HCV is associated with HCC development, mainly through indirect pathways such as chronic inflammation, cell apoptosis and proliferation. Up to 16% of HCV-infected persons develop cirrhosis 20 years after the infection (Thein et al. 2008). The annual incidence of HCC in HCV-induced cirrhosis is on average 2%–3% (Thrift et al. 2017). The natural history of HCV is highly variable with host and environmental factors both playing a role in progression to cirrhosis in patients chronically infected with this virus. This has been shown by the fact that the HCC ASIRs among Egyptians is higher in those living north of Cairo (56.8) than in the south (25.7), and in regions reaching the southern frontier of Egypt (33.2) (Ibrahim et al. 2014). A study conducted in Kalama, a village in the Nile Delta north of Cairo, has previously reported one of the highest HCV seroprevalences in the world (40% of the village residents) (Darwish et al. 2001). In

another study that was conducted in a similar geographical region, the authors reported that while HCV seroprevalence between HCC and cirrhotic patients did not differ statistically (95% and 90%, respectively), AFB_1 concentration in the serum was significantly higher in the former group and this was followed by the latter group when compared to healthy controls (Sharaf-Eldin et al. 2016). Thus, it is likely that exposure to AFB_1 may augment progression from HCV-related cirrhosis to HCC, resulting in the high incidence rates of this tumour in this region. On the other hand, older age (Freeman et al. 2001), older age at HCV acquisition (Freeman et al. 2001), male sex (Freeman et al. 2001), heavy alcohol intake (Mallat et al. 2008), tobacco smoking (Mallat et al. 2008), diabetes (Huang et al. 2014), obesity (Rao et al. 2017) and co-infection with HIV (Chen et al. 2014) or HBV (Zampino et al. 2015; L. Y. Cho et al. 2011) have all been implicated as factors that enhance development of cirrhosis in patients with chronic HCV-infection, and ultimately leading to the development of end stage liver disease such as HCC.

Of the total 180 million people in the world with HCV infection, Africa and Asia account for an estimated 138.5 million HCV infections, while in the Americas and Europe, approximately 25.8 million people are infected (Petruzziello et al. 2016). In the less developed countries, the higher number of HCV chronically infected individuals, the growing burden of HCC disease, and the absence of a vaccine all entail the growing importance of treatment as part of a disease control strategy. Meta-analyses have concluded that the eradication of HCV with antiviral therapies can be achieved, thereby resulting in a decrease in the risk of developing HCV-related HCC (Hosni et al. 2016) that may be independent of the fibrosis stage (Morgan et al. 2013). Despite the unprecedentedly high antiviral efficacy, the risk is not completely eliminated and the impact, effectiveness and outcomes of treatment in various groups remain uncertain (Baumert et al. 2017). This may be the result of the high drug cost of antiviral medications that range from US$ 83,320 to 150,000 for 3-months (in 2014) (Edlin 2016; Hafez 2018), thus rendering the access being limited to less than 1% of the total number of HCV-infected individuals who can afford it in resource limited countries (Hutchinson et al. 2015). With the 3–4 million newly infected cases each year (Baumert et al. 2017), the HCV-associated disease burden will remain high, if not higher, in the next decade.

1.2.5 Aflatoxin (AFB_1)

AFB_1 is a powerful hepatocarcinogen that contributes to high morbidity and mortality. Produced by the fungus *Aspergillus* species (*A. flavus* and *A. parasiticus*), AFB_1 is commonly detected in several food products including peanuts, grain, cereals, legumes, and corn (Mazumder & Sasmal 2001). In particular, AFB_1 is widespread in regions where food products are poorly stored in conditions that are unsanitary and high in temperature and humidity – mostly in tropical regions of sub-Saharan Africa and Southeast Asia, including China. It is estimated that 4.5 billion people are at risk chronic exposure to AFB_1-contaminated food (Hamid et al. 2013). Of the 550,000–600,000 new HCC cases worldwide each year, about 25,200–155,000 (4.6%-28.2%) may be attributable to aflatoxin exposure (Liu & Wu 2010).

When ingested, the process of metabolising AFB_1 produces an active substrate known as AFB_1-exo-8,9-epoxide by CP450 enzyme that plays an important role in the development of HCC. The substrate binds the guanine bases on the third base of codon 249 of the p53 gene to form aflatoxin-N7-guanine (AFB1-N7-Gua) (Aguilar et al. 1993; Smela et al. 2001), which can be detected in 36%–50% of HCC tumour samples collected from individuals in AFB_1-endemic areas, most of whom have HBV infections (Bressac et al. 1991; Huang et al. 2003; Kirk et al. 2000). Assays have been developed to detect and measure AFB_1 metabolites in urine (Qian et al. 1994), serum (Tang et al. 2009), and tissues (Mace 1997).

It has been reported that HBV infection is prevalent in regions with high AFB_1 contamination, including sub-Saharan Africa, Southeast Asia and China (Qian et al. 1994; Kew 2013). Although AFB_1 might have direct hepatocarcinogenic effects, its role in the pathogenesis of HCC is primarily as a co-carcinogen to that of chronic HBV infection (Kew 2003). For example, a previous study using transgenic mice demonstrated that HBV infection, specifically the HBV X protein, modulates the DNA GC → TC transversion at position 249 of the p53 gene mutation by as much as twofold when exposed to AFB_1 (Madden et al. 2002). In a prospective study in China, individuals exposed to AFB_1 have a 4-fold increased risk of developing HCC, and this risk increased to 60-fold in individuals who were also carriers of HBV (Qian et al. 1994).

1.2.6 Alcohol and Smoking

As the primary site of alcohol metabolism, the liver is a primary target for alcohol-related injury. Substantial epidemiological data have consistently shown that excessive consumption of alcohol is associated with increased risk of HCC development, particularly in European and American populations, where chronic HBV infection is less prevalent. For example, studies from the United States, Italy and Taiwan reported 32%, 45% and 21% of HCC cases are attributable to alcohol consumption, respectively (Hassan et al. 2002; Stickel et al. 2002). In a cross-sectional study that investigated the burden of alcohol consumption in SA, researchers observed relative risk of developing LC in both men and women ranging from 1.5 to 3.6 (Schneider et al. 2007). Furthermore, another South African study demonstrated a 4-fold increased risk of HCC after correcting for HBV infection (Mohamed et al. 1992). It has been suggested that alcohol may be associated with HCC only as a consequence of the development of cirrhosis (Donato et al. 2002). In addition, iIt has been suggested that daily ingestion of more than 80 g of alcohol for more than 10 years is generally required before there is significant risk of developing cirrhosis (Davis et al. 2008), and has been shown to have an equivalent increase risk of developing HCC (Donato et al. 2002). It should be noted that alcohol-induced HCC can develop in the absence of cirrhosis, and even if one abstains from alcohol consumption after cirrhosis has developed (Morgan et al. 2004). In SA, excessive alcohol consumption has been implicated as a major risk factor for HCC in older white (mean age 60 years) (Kew et al. 1984) and urban black African men (older than 40 years) (Mohamed et al. 1992).

In spite of this, the specific pathways by which alcohol causes HCC remain to be elucidated. Several mechanisms of pathogenesis have been proposed from animal studies including oxidative stress, alteration in DNA methylation reactions, reduced capacity for effective immune response and host genetic composition (Morgan et al. 2004). As with other risk factors, alcohol consumption may act as a co-carcinogen together with HBV infection in the development of HCC (Hassan et al. 2002).

Findings from earlier epidemiological studies that investigated the association between smoking and HCC are inconsistent and these inconsistencies varied between different population groups and in the presence of potential confounders (Gao et al. 2012). It has

been demonstrated in animal studies that N-Nitrosodimethylamine, a component in cigarette smoke, is hepatocarcinogenic (Arinç et al. 2007; Ma et al. 2015; Fadlallah et al. 1994). Moreover, The IARC has included LC in the list of carcinomas associated with smoking (World Health Organization 2004).

With regards to earlier cohort studies conducted in the United States (Hammond 1966), Japan (Hirayama 1989) Greece (Kuper et al. 2000), the reports all show evidences of increased risks of LC among smokers with some also reporting an increase in a dose-response relationship (Kuper et al. 2000; Tu et al. 1985). This is inconsistent other studies that found no risk (Austin et al. 1986) or were reported inconclusive evidence (Tanaka et al. 1992). In a previous meta-analysis of 38 cohort studies and 58 case–control studies from Europe (Norway, Sweden), North America (United States) and East Asia (China Japan, Taiwan, Korea, Philippines) (Lee et al. 2009), the authors reported that relative to never smokers, the adjusted relative risk was 1.51 [95% confidence interval (CI): 1.37–1.67] for current smokers and 1.12 (95% CI: 0.78–1.60) for former smokers. In addition, Wang *et al*. further reported that the risk of HCC development when exposed to smoking may be mediated by the reduced natural killer cell frequency in the peripheral blood in response to increased HBV viral load (Wang et al. 2018).

In SA, even though anti-smoking legislation and increased prices of cigarettes have contributed to a decreased consumption by 33% from 1993 to 2003 (van Walbeek 2014), several studies conducted prior to this legislation found little or no support for causal relation between cigarette smoking and HCC development (Kew et al. 1985; Kew, Song, et al. 1990; Mohamed et al. 1992).

1.2.7 Non-alcoholic Liver Disease and Diabetes

In a study that selected a total sample size of more than 8.5 million cases from 22 countries, it was reported that the global prevalence rate of NAFLD was estimated to be 25.2% (95% CI: 22.1-28.6%) (Younossi et al. 2016). The authors also showed that the highest prevalence occurred in the Middle East, followed by South America, Asia, United States, and Europe, and the lowest in Africa. These findings were reported despite the apparent paucity of studies investigating NAFLD as shown in Figure 1.6

(Younossi et al. 2016). While NAFLD and its inflammatory component non-alcoholic steatohepatitis (NASH) are responsible for the third leading cause of death in developed regions of the world (Adams et al. 2005), HCC remains the primary cause of death in this group (Yatsuji et al. 2009).

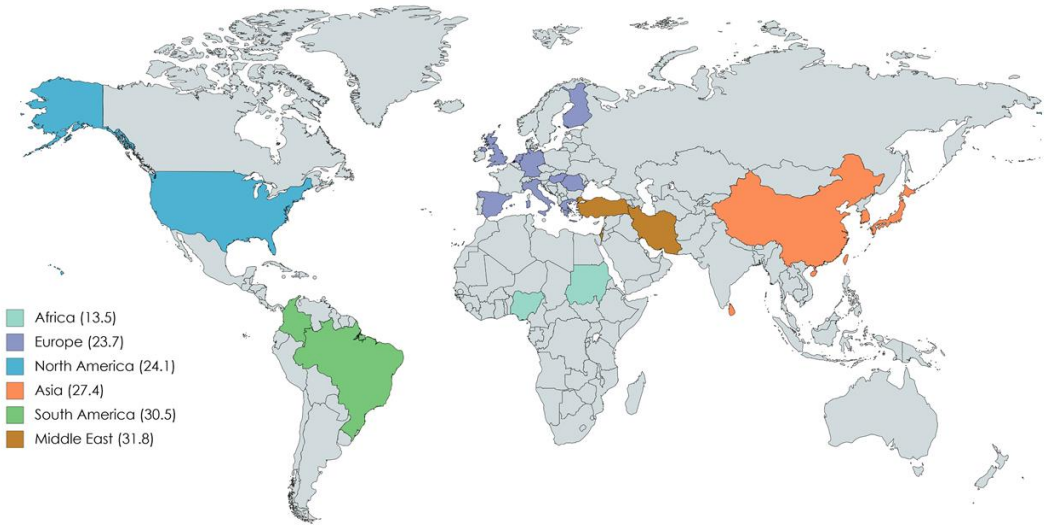

Figure 1.6 Non-alcoholic Liver Disease and Diabetes (NAFLD) prevalence stratified by region, data obtained from (Younossi et al. 2016) and compiled with https://mapchart.net.

In NAFLD/NASH related HCC, several risk factors have been identified including metabolic syndrome and insulin resistance, which lead to changes in the inflammatory cytokines and/or adipokines (Charrez et al. 2016), persistent inflammation, and altered gut microflora and bile composition (Jiang et al. 2014; Nagaoki et al. 2012; Fingas et al. 2016). In a meta-analysis of cohort studies from mainly developed countries (Europe, USA, Japan and Korea), the summary relative risk of LC was 1.89 (95% CI: 1.51–2.36) for those who were obese compared with persons of normal weight (Larsson & Wolk 2007). It is likely that NASH may play a role in the increased risk of developing this malignancy. It is further estimated that in the United States, 5%-7% of the population are affected by this liver disease and as much as 40% present abnormal signs of elevated liver enzymes (Pereira et al. 2015). Caused by the build-up of fat in the liver, NASH is the inflammation of the liver and 9% of patients with this disease progress to cirrhosis (Ong & Younossi 2007; Starley et al. 2010). Of these patients with NASH-related cirrhosis, an estimated 40%-62% died as a result of liver failure, including HCC, over a

period of 5-7 years of follow-up (Starley et al. 2010). A systematic review suggested that the cumulative annual incidence rate for developing HCC in cases of NASH-cirrhosis is 2.4%-12.8% (White et al. 2012). While it has been suggested that NAFLD can progress to HCC in the absence of NASH or cirrhosis, these patients have been reported to present with less aggressive tumours (Cholankeril et al. 2017).

1.2.8 Hereditary Haemochromatosis and Iron Overload

Hereditary haemochromatosis (HH) is an autosomal recessive disorder common among people of North European origin that leads to the accumulation of iron in various organs of the body, including the liver (Merryweather-Clarke et al. 1997). This pathophysiologic predisposition of iron overload may lead to the development of cirrhosis and ultimately HCC. The mutated human haemochromatosis gene, located on the short arm of chromosome 6 at position at location 6p21.3, is responsible for HH, which is principally caused by a two mutations: a single amino acid substitution of tyrosine for cysteine at position 282 (C282Y) and aspartate to histidine at amino acid position 63 (H63D). The C282Y mutation accounts for 80% to 90% of HH cases, while H63D accounts close to 20% of the cases with HH (Beutler et al. 1996; Bacon et al. 2011). These mutations lead to increased iron absorption and progressive iron storage that results in liver damage and fibrosis, which may eventually lead to HCC (George et al. 1998). Numerous earlier studies have established HH as a causal link to the development of HCC, with estimated risk as high as 200-fold (Bradbear et al. 1985; Strohmeyer et al. 1988), however, this may likely be based on the evidence that the HCC incidence of the study cohorts was approximately 8%–10%. A subsequent Swedish study involving a cohort of 1847 patients also showed a strong relationship between HH and HCC, wherein the risk of HCC was reported to be 20-fold (Elmberg et al. 2003).

In contrast, HH is uncommon in Africans (Merryweather-Clarke et al. 1997) but has been reported in Cameroonians (Nouedoui et al. 2003), SAs (Mandishona et al. 1998) and African Americans (McLaren & Gordeuk 2009). Studies in African populations find that patients with iron overload are not associated with the C282Y mutation as reported in Northern Europeans (Mcnamara et al. 1998), but is instead a consequence of drinking traditional beer containing dissolved iron that occurs from using iron drums

and cans in which the beer is brewed (Kew & Asare 2007). Mandishona *et al.* reported a relative risk of HCC of 10.6 (95% CI: 1.5-76.8) when compared to individuals with normal iron status and a population attributable risk of 29 in rural South African Blacks, after adjusting for the confounding effects of HBV/HCV infection and exposure to AFB_1 (Mandishona et al. 1998). Although the possible causal role of cirrhosis could not be determined in this study because the histological material was obtained by percutaneous biopsy, previous studies have suspected that the deposited iron in hepatocytes and macrophages may play a role in liver organ damage, and thus, leading to the development of cirrhosis (Yun and Vincelette 2015).

The almost universal association between cirrhosis and the subsequent onset of HCC strongly suggests that chronic necroinflammatory hepatic disease as a result of excess hepatic iron indirectly causes malignant transformation of hepatocytes, as it does with most other causes of HCC. On the other hand, evidence from other studies have shown a direct causation of HCC in patients with HH without cirrhosis (Britto et al. 2000; Goh et al. 1999; Thompson et al. 1995; Chung 2003; von Delius et al. 2006; Köhler et al. 1999; Hiatt et al. 2007). Furthermore, studies investigating dietary iron overload in southern African black populations demonstrated increased risk of HCC development even after adjusting for the confounding effect of cirrhosis (Gordeuk et al. 1996; Moyo et al. 1998). Despite these findings, HCC is known to develop in a number of iron-loading conditions not complicated by cirrhosis, such as thalassemia (Rotaru et al. 2014; Al Qasem et al. 2018), sideroblastic anaemias (Barton & Lee 2006), and spherocytosis (Barry et al. 1968; Zhao et al. 2010).

1.2.9 Hormonal Contraception

Based on existing evidence, the IARC concluded that hormonal contraception use is a recognised human carcinogen, however, whether there was an increase in risk of LC was not specified (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans & International Agency for Research on Cancer 1999). Oral contraceptives are widely used worldwide and may have a considerable impact on public health. Since the earliest study by Baum *et al*. in 1973 (Baum et al. 1973), numerous epidemiologic studies have investigated the association between oral contraceptive use and LC risk but the findings have been inconsistent. The risk is not uniform across the world, with

evidence from earlier studies in Britain reporting increased risk of developing HCC (4.4- and 7.2-fold, $p<0.01$) (Neuberger et al. 1986), whereas this was not the case in SA (Kew, Song, et al. 1990).

In a meta-analysis by Yu and Yuan (Yu & Yuan 2004), 8 case–control studies of Caucasian women from the United States and Europe showed a summary OR of 2.5 (95% CI: 1.7–3.5) in ever- versus never-users of oral contraceptives and a summary OR of 5.8 (95% CI: 3.0–11.0) for the longest duration of use. This finding was not supported in another meta-analysis (14 of 17 studies are case–control studies) (An 2015) of studies conducted in various countries including the UK, Egypt, USA, China, Germany, France, Italy, Greece, Spain, Serbia, Chile, Colombia, Kenya, Israel, Nigeria, Philippines, South Africa, and Thailand. Accordingly, it was shown that there was no statistically significant association between oral contraceptives use and LC risk (Risk Ratio of 1.23, 95% CI: 0.93-1.63) (An 2015).

Studies that investigated LC risk with oral contraceptives use in high-risk Asian and African women uniformly yielded inconclusive or null results (Kew, Song, et al. 1990; Maheshwari et al. 2007; An 2015). In these regions, it is difficult to explain whether these apparently disparate findings can be explained as the non-synergistic effect of both viral (HBV) and hormonal factors combined, given that detecting the risk associated with oral contraceptive use (2- to 3-fold) against the very high background relative risk (20-fold) in HBV carriers may be particularly difficult (Yu & Yuan 2004).

It should be taken into consideration that changes in the composition of oral contraceptives have taken place since their early use in the 1960s. Compared to the original contraceptive pills, formulations of these pills today generally contain lower dosages of oestrogen and a greater variety of progestins (Bassuk & Manson 2015; Marnach et al. 2013). Despite this, some biological and experimental evidence suggests a possible role of oral contraceptives in hepatocarcinogenesis. In *in vitro* experiments, oestrogen was reported to stimulate the cellular proliferation of human LC cells HepG2 (Brandt et al. 2004; Guo et al. 2009) and the rate of spontaneous mutation (De Benedetti et al. 1996). In patients, increased risk of liver disease associated with oral contraceptive use was observed in pills containing more than 50 μg of oestrogen, and regression in disease was noted after cessation of contraceptive use (Lindberg 1992; Hannaford et al.

1997; Fiel et al. 1998). These findings all suggest that contraceptive use may have potential hepatocarcinogenic effects, of which, may be dependent on various factors that include the period of exposure, dosage levels and the influence arising from confounding factors. As such, results from various studies may vary on the risk of developing HCC, and the same would apply for other lifestyle-related risk factors such as smoking.

1.3 Clinical Features of Hepatocellular Carcinoma

Although it is widely known that early detection and diagnosis of LC provides a beneficial impact in treatment outcome, it has nonetheless proven a major challenge as no pathognomonic symptoms or signs are attributable to this malignancy. Instead, LC usually runs an indolent course in the early stages of the disease. The difficult anatomical positioning of the liver (beneath the lower ribs) makes it relatively inaccessible for physical examination. On the other hand, the considerable functional reserve that the liver possesses means that various clinical presentations of hepatic dysfunction (i.e. jaundice) generally do not emerge until a significant portion of the organ has been replaced by the tumour. As a result, the clinical picture of HCC is extremely variable and can range from asymptomatic presentation with no physical signs other than of cirrhosis, to spontaneous rupturing of the tumour mass with haemoperitoneum, a contour protrusion, and portal vein thrombosis (Kim et al. 1998; Choi et al. 2013; Zhu 2012; Ishihara et al. 1997; Kawai et al. 2016).

HCC typically presents in different manners in cirrhotic (90%) and non-cirrhotic (20%) patients (Gaddikeri et al. 2014). In the former group, patients tend to have lower tolerance for malignant infiltration within the liver and majority present the tumour in advanced stages of the disease with symptoms and manifestations suggestive of liver decompensation and cirrhosis including: elevated serum α-fetoprotein and portal vein thrombosis (35% of HCC cases) (Chawla & Bodh 2015). As a consequence, the prognosis in these patients is generally poor even with treatment - median survival rate of 4 to 17 months, depending on severity of the disease (Greten et al. 2005). Common symptoms include chronic hepatitis, with pain in the right superior quarter of the abdomen, palpable hepatomegaly and weight loss. Other symptoms include jaundice, variceal bleeding, ascites, anasarca (Bialecki & Di Bisceglie 2005; Lam et al. 2004; Qin

2003). Excess vasoactive intestinal peptide and gastrin hormone production can lead to watery diarrhoea, which occurs mainly in patients with underlying cirrhosis as a result of their secretion characteristics (Luo et al. 2002; Bruix et al. 1990; Bialecki & Di Bisceglie 2005). In contrast, non-cirrhotic patients have a distinct clinical feature such as a large tumour that is able to grow without restriction as seen in sub-Saharan Africa (Kew 1989b) and even in low incidence areas (Taie et al. 2015). A hard, nodular liver with palpable irregular mass is typically described in the sub-Saharan Africa populations as a result of the very late stage of disease presentation (Umoh et al. 2011). This is in contrast to the clinical picture presented in the white population group in the United States with non-cirrhotic HCC, where few presented with jaundice, ascites, right upper quadrant tenderness on palpation, signs of cachexia, or cardiovascular shock (Smalley et al. 1988). Hepatic bruits, due to hypervascularization of the tumour can be detected in up to 10%-20% of patients (El-Serag et al. 2008).

The Budd-Chiari syndrome is characterised by hepatic venous outflow obstruction at various levels including the inferior vena cava and hepatic veins, and have been reported to be associated with HCC (Liu et al. 2013). Paraneoplastic syndromes manifested in HCC patients have also been documented, and one of these include hypercalcaemia, which is caused by osteolytic metastasis (Sultan-e-Rome et al. 2013; Kim et al. 2015). Another paraneoplastic manifestation is erythrocytosis, caused by the increase in erythropoietin production in HCC patients (Kew & Fisher 1986). Hypoglycaemia is a well-established paraneoplastic symptom of HCC, however, it is rarely found as a primary presentation of the malignancy (van den Berg & Krol 2017; Jha & Borpujari 2012). Other symptoms include: arterial hypertension (Kew 1989a), hyperammonemia (Bender et al. 2015), hypercholesterolemia (Sohda et al. 2013), thrombocytosis (Hwang 2004), thyrotoxicosis (Helzberg et al. 1985) and polycythaemia (Ke et al. 2017).

Various cutaneous features are well described in HCC including the dermatomyositis, which is the manifestation skin rash (Miyata et al. 2016), and Leser-Trélat sign, which is the appearance of multiple seborrheic keratoses. *Porphyria cutanea tarda,* also a skin disorder, has been associated with HCC patients with chronic HCV (Berkowitz) and in

the South African black population (Bialecki & Di Bisceglie 2005; Berkowitz et al. 1989).

1.4 Diagnosis

Dramatic improvements in the detection of HCC from using dynamic imaging series over the last 2 decades, have generally led to the replacement of more invasive procedures (e.g., angiography, exploratory laparotomy, and percutaneous biopsy) as the preferred tools of identifying hepatic tumours. As a result, HCC is one of the few cancers that can be diagnosed on radiological criteria alone, in the absence of histology. The application of the various diagnostic methods, varies depending on the size of the nodule, and between Asian and Western institutions. However, serum tumour markers and histological evaluation (liver biopsy) continue to have important roles, particularly in the setting of small (Tzartzeva et al. 2018) or atypical hepatic lesions (Jain 2014).

According to the American Association for the Study of Liver Diseases (AASLD) (Bruix et al. 2011) and the Korean Liver Cancer study Group-National Cancer Centre Korea (KLCSG-NCC) (Korean Liver Cancer Study Group (KLCSG) & National Cancer Center, Korea (NCC) 2015) guidelines, small lesions of <1 cm in size are recommended to be screened every three to six months as further imaging tests may not be reliable for the diagnosis. For lesions of >1 cm in size, contrast-enhanced imaging with either multiphasic computed tomography, magnetic resonance imaging (MRI) or contrast-enhanced ultrasound (CEUS) are usually performed as diagnostic tests. A different (3-way) algorithm is presented in the European Association for the Study of the Liver (EASL) (European Organisation for Research and Treatment of Cancer 2012) guidelines, which includes ultrasound imaging of nodules with diameters of < 1 cm, 1–2 cm, and >2 cm. In terms of diagnostic performance for 1-2 cm nodules, a multimodality approach is often required (Khalili et al. 2011). In contrast, guidelines from the Asian Pacific Association for the Study of the Liver (APASL) (Omata et al. 2010) and the Japan Society of Hepatology (JSH) (Kudo et al. 2014) ignore the size of the liver lesion.

Unlike the other European, American and Asian algorithms, the JSH guidelines suggest examining high-risk patients with chronic hepatitis B, chronic hepatitis C or non-viral cirrhosis with a combination of ultrasound and three tumour markers [AFP proteins

induced by vitamin K absence (PIVKA-II), and AFP-L3] every 6–12 months, whereas a 3-4 month screening interval is recommended for the extremely high-risk patients with hepatitis B or C cirrhosis. Moreover, adolinium-ethoxybenzyl-diethylenetriamine penta-acetic acid-enhanced MRI is recommended as a first line of surveillance and diagnostic tool for HCC.

Histological confirmation by means of percutaneous fine needle aspiration biopsy or core biopsy has higher sensitivity and specificity than non-invasive techniques. However, this procedure is not required if patients whose lesion fulfils the criteria with a dynamic imaging technique: one or more mass-lesions in the liver showing a typical hypervascularity profile in the form of arterial phase enhancement, followed by washout with loss of enhancement during portal venous phases on CT or has increased T2 signal intensity on MRI. There are however, situations where biopsy may or can be useful such as in the patient whose suspicious lesion does not necessarily meet the characteristic radiographic or laboratory features of HCC, or indeterminate lesions without specific features (Bialecki & Di Bisceglie 2005).

AFP is a serum marker still used for the diagnosis and surveillance of HCC in some guidelines (APASL and JSH), with a cut-off value of 17.23 IU/ml considered to be the optimal in suspecting the development of HCC in the setting of chronic liver disease (Sarwar et al. 2014). Despite this, AFP has been considered as a poor biomarker for surveillance of HCC. For instance, inconsistencies in tumour AFP levels pose a challenge in effectively diagnosing HCC based on this serum marker level alone – 32%–59% of HCC patients have normal levels, whereas non-tumour-related AFP elevations may occur in patients with cirrhosis or chronic hepatitis (Biselli et al. 2015). AASLD and EASL-EORTC algorithms no longer include measurement of serum AFP in the diagnostic algorithm for hepatic nodules found on surveillance programs and instead recommend surveillance with ultrasound alone.

1.5 HCC disease Surveillance

The incidence-to-mortality rate ratios of HCC between developing and developed regions in the world generally overlap (Wong et al. 2017), which suggests that majority of patients who are diagnosed with this malignancy do so during advanced stages of the

disease. This invariably reduces the potential number of cases that can be adequately treated and thus cured of the disease.

A number of staging systems that aims to assess and describe the extent of HCC burden in the liver and it's spread throughout the body have been proposed. These staging systems plays an important role in the management of the disease itself and therapeutic decisions. According to an analysis in the United States that compared the predictive power of 6 HCC prognostic systems for patient survival in a cohort of 244 patients of any stage, Barcelona-Clinic Liver Cancer (BCLC) staging classification showed the best independent predictive power (Marrero et al. 2005). BCLC classify stages of HCC into five subgroups (0, A, B, C and D) which are associated with a specific therapy and prognosis (Llovet et al. 1999).

Stage 0 is defined as very early stage disease with patients presenting a well-preserved liver function and diagnosed with a single nodule $\leq$ 2 cm without tumour metastasis into surround tissue. Stage A is defined as an early stage disease whereby the patient has Child-Pugh A or B score, a single tumour with variable size or with maximum 3 nodules each $\leq$ 3 cm. Both stage 0 and A patients are optimal candidates for effective curative therapies(resection and ablation, liver transplantation; or percutaneous treatments). Stage B is defined as an intermediate disease that occurs in an asymptomatic patient with preserved liver function, and the tumour is multinodular without the characteristics of macrovascular invasion or extrahepatic spread. Stage B patients may be treated with transarterial chemoembolization. The advanced stage as classified as stage C refers to patients with macrovascular invasion or extrahepatic spread. Lastly, end stage disease which is classified under stage D, is used to classify patients presenting advanced liver failure, tumour growth with macrovascular invasion, extrahepatic spread or physical impairment. Accordingly, stage C and D treatment entails any specific cancer therapy and supportive care (Llovet et al. 1999).

As the prognosis of HCC is affected by the staging of the tumour and the effects of cirrhosis on liver's ability to function normally, this means that surveillance of at-risk populations has an important role to play in the detection of early tumours, particularly when they are still amenable for curative therapies such as resection, ablation and/or transplantation. While mass screening of the general population for HCC is in general

costly (Kuo et al. 2016), the surveillance of high- and intermediate-risk patients with cirrhosis have proved to be more cost-effective than non-stratified biannual ultrasound in all patients with cirrhosis (Goossens et al. 2017). Currently, the AASLD and the EASL recommend surveillance for patients with cirrhosis of any aetiology and for selected HBV carriers. On the other hand, the APASL and JSH guidelines include surveillance for cirrhotic patients with hepatitis virus (HBV/HCV) infection. In Western countries, however, increasing metabolic diseases that are related the burgeoning prevalence of diabetes and obesity have been recognised to be causally related to HCC as well, and a large proportion of which develop this tumour in the absence of cirrhosis. Thus, not surprisingly, these patients are less likely to be detected during surveillance that initially screen for cirrhosis, and thus are more likely to have more advanced liver disease when compared to their cirrhotic counterparts (Ascha et al. 2010; Schütte et al. 2014; Kolly & Dufour 2016).

Table 1.3 Groups in whom HCC screening and surveillance are recommended (El-Serag et al. 2008).

HBV carriers	Non-hepatitis B cirrhosis
● Asian males >40 years ● Asian females >50 years ● All cirrhotic hepatitis B carriers ● Family history of HCC ● Africans over age 20 years	● Hepatitis C ● Alcoholic cirrhosis ● Genetic haemochromatosis ● Primary biliary cirrhosis ● Possibly; non-alcoholic steatohepatitis, autoimmune hepatitis, alpha 1-antitrypsin deficiency ● Wilson's Disease

While it is acknowledged that there is currently a large burden of LC in sub-Saharan Africa (Ojo 2017), it is necessary to first understand that unlike many of developed countries in the world, the availability of cancer data in sub-Saharan African is generally sparse, if not absent. Founded in 2012, The African Cancer Registry Network (AFCRN) (www.afcrn.org) currently consists of 30 cancer registries from 22 countries that cover close to 10% of the total population in sub-Saharan Africa (99.4 million). The cancer registries may either be population-based or pathology-based. Population-based cancer registries systematically collect information on all reportable neoplasms

occurring in a geographically defined population from multiple sources whereas a pathology-based cancer registry collects information from neoplasms that have been histologically identified in one or more laboratories.

The population size covered by The African Cancer Registry Network ranges from 90,000 in Seychelles to 56.5 million in the South African NCR and the number of cancer cases reported ranged from 234 to 74,519 per year, respectively (Figure 1.7 and Table 1.4). This coverage is in stark contrast to the Surveillance, Epidemiology and End Results (SEER) program of the National Cancer Institute that collects data on cancer diagnoses for approximately 30% of the United States population (Duggan et al. 2016), while 200 population-based cancer registries that are active in Europe cover about 60% of the European population (Forsea 2016). Consequently, not only is the lack of coverage in sub-Saharan Africa a cause for concern, it also suggests that the burden of LC incidence and mortality may in reality be much higher. Furthermore, given the lack of effective treatment for LC as discussed in the sections above, it may further suggest the implementation of adequate surveillance be made a priority and a target for intervention as it forms a fundamental part of any rational programme for cancer control.

Figure 1.7 Map the 23 countries with cancer registries that are members of The African Cancer Registry Network (AFCRN, http://afcrn.org). Map was created with https://mapchart.net/africa.html.

Table 1.4 Information of African cancer registries including the year founded, surveillance area and number of cases registered from (Gakunga et al. 2015) with updates from http://afcrn.org.

Country	Cancer Registry	Year Founded	Area Covered by Population Based Cancer Registry	Staff	Methods of Registration	Reported Population (Nearest 1000)	Cases Registered	Year Cases were Registered	Case per Year
The Republic of Benin	Cotonou CR	2013	Cotonou City	5	-	679,000	-	-	
Botswana	Botswana NCR	1999	Botswana (national)	5	Active	2,025,000	8,938	2005-2010	1,490
Congo	R. des C. de Brazzaville	1995	Brazzaville city	11	Active	1,376,000	495	2011	495
Côte d'Ivoire	R. des C. d'Abidjan	1994	Abidjan (city and environs)	5	Active	3,772,000	2815	1995-1997	938
Ethiopia	Addis Ababa CR	2011	10 sub cities of Addis Ababa	4	Active	3,049,000	5701	2011-2014	1,425
Gambia	Gambia NCR	1986	Gambia (national population)	8	Active	1,728,000	4375	2000-2009	438
Ghana	Kumasi CR	2004	Kumasi city (Ashanti Region)	8	-	2,035,000	231	2012	231
Guinea	R. de C. de Guinea	1990	Conakry (city and environs)	3	Active	1,090,000	3146	2006-2010	629
Kenya	Eldoret CR	1999	Uasin Gishu county	3	-	894,000	5,366	1999-2006.	671
	Nairobi CR	2001	Nairobi county	7	-	2,788,000	8,982	2004–2008	1,796
Mali	R. des C. du Mali	1986	Bamako City	-	-	1,810,000	-	-	-
Malawi	Malawi CR	1989	Blantyre (Urban and Rural districts)			1,000,000	618	2015	618
Mauritius	NCR of Mauritius	1989	Republic of Mauritius (Islands of Mauritius and Rodrigues)	-	-	1,185,000	7685	2006-2010	1,537
Mozambique	R. de C. de Beira	2005	Beira city	2		443,000	1229	2009-2011	410
	R. de C. da Cidade de Maputo	2014	Maputo City	-	Active and Passive	1,226,000	12,674	1991-2008	704
Namibia	Namibian CR	1995	Namibia (national)	3	Passive	2,105,000	11248	2010-2014	2,250
Nigeria	Abuja CR	2009	Abuja municipality	2	Active and Passive	1,600,000	490	2011	490

	Calabar CR	2004	Calabar Municipality & Calabar South LGAs (Cross-River State)	4	Active	2,893,000	128	2014	128
	Ibadan CR	1960	Ibadan and environs	8	Active	2,549,000	3921	2009-2010	1,961
Niger	CR of Niger	1992	Niamey City		Active	521,000	-	-	-
Seychelles	Seychelles NCR	2008	Seychelles (national)	1	Active and Passive	90,000	234	2015	234
SA	Eastern Cape Province C.R.	1980s	8 magisterial districts in Eastern Cape province (rural)	7	Active and Passive	1,100,000	2808	2003-2007	562
	NCR-SA	1986	SA (National)	12	Passive	56,500,000	74519	2012	74,519
Swaziland	Swaziland N.C.R	2015	Swaziland (National)	3	Active	1,250,000	1426	2014-2015	713
The Réunion	R. des C. de la Réunion	1989	The Réunion (National)	3	Active	850,000	5,280	2006-2008	1,760
Uganda	Kampala CR	1954	Kyadondo County (Kampala city & environs)	3	-	2,010,000	4216	2007-2009	1,405
	Gulu CR	2014	Northern Uganda districts of Gulu, Nwoya, Omoro and Amuru	2	Active	762,000	1660	2013-2016	415
Zambia	Zambia NCR	1977	Lusaka District	4	Active	1,747,000	12891	1990-2009	645
Zimbabwe	Zimbabwe NCR	1985	Harare city	7	Active and Passive	1,469,000	5549	2010-2012	1,850

"-" indicates information not specified

The reasons for the underdiagnosis of LC cases in sub-Saharan Africa are complex and multifactorial. One of these factors is the reportedly lack of direct funding to registries. This has been estimated to be only $US 8-9 per case, which is significantly less when compared to €50 (~$70) per case in Europe (Gakunga et al. 2015). Moreover, many sub-Saharan Africa countries depend on foreign aid in order to meet their health care needs and goals (Stefan 2015). As documented in 2010 (Novignon et al. 2012), annual health expenditure in sub-Saharan Africa was low and was estimated at only $85 per capita, more than two thirds (67%) of the amount spent in the private sector. In contrast, the annual health expenditure per capita was reported to be $500 in the East Asia and Pacific region and $8050 in North America, and 28.6% and 47.1% of health expenditure

was spent on the private sector, respectively. Despite an observed decline in poverty in sub-Saharan Africa over a period of four decades, the proportion of population living below the poverty line (living on less than $1.25 a day standard) remains staggeringly high, at close to 50% (Fosu 2014). Given these significant financial constraints, it is not surprising that most, if not all, the resources (physical and human) are devoted to other priorities other than LC screening and surveillance. This provides little in supporting the disease prevention strategies, interventions, treatments, monitoring and future planning that are necessary (Gakunga et al. 2015). As a consequence, poor survival estimates are unfortunately not surprising given these limitations. In countries where mortality data is available, information such as this may be utilised by cancer registries in order to determine the accuracy and completeness of the incidence data.

1.6 Aims and Objectives

With previous reports indicating changes in LC incidence, risk factors and management in various HICs and LMICs in the world, the aims of this study is to:

- Evaluate the current trends in the LC incidence and mortality rates in SA, a upper-middle income country that is characterised by a diverse racial/ethnic population, as well as compare these trends to neighbouring Southern African countries.

Moreover, the objectives of this study is to:

- Characterise the overall and most recent incidence and mortality trends of LC in SA, by using Joinpoint regression, stratified by age, sex and population groups.
- Determine the mortality-incidence rate ratio by using data collected from the SA NCR and StatsSA in which the rates were reported for the different population groups by sex.
- Determine the proportion of the different subtypes of LC in SA stratified by sex and population group.
- Compute age-period-cohort of the LC incidence in SA between black African and white population groups (coloured and Asian population groups were excluded because of the low numbers).

This study was divided into Chapter 3 and Chapter 4, which analyses the incidence and mortality data that was provided by the South African NCR and StatsSA, respectively.

— CHAPTER 2 —

Literature Review of Hepatitis B Virus

As a major contributor to and cause of HCC development in most developing regions in the world, this chapter contains a comprehensive review of HBV in an effort to explain its natural history and geographical distribution from a molecular perspective. This is to provide the reader with an understanding of the ways in which changes in the molecular biology may influence the natural history of viral pathogenesis, and thus lead to the development of LC.

2.1 Introduction

HBV is a known hepatotropic virus, and as such has an affinity for targeting the liver. The liver is a vital organ that carries out a wide variety of functions in the body including processing and storing nutrients, neutralizing toxins, maintaining blood homeostasis and immune response to pathogens or tissue damage (Robinson et al. 2016). In the non-diseased liver, tissue remodelling and regeneration require elements of inflammation (Tanaka & Miyajima 2016). The subsequent activation of resident hepatic macrophages, Kupffer cells, and other non-parenchymal cells plays a crucial role in resolving the inflammation and failure to do so may lead to chronic inflammation and disrupted tissue homeostasis. Consequently, the persistence of activated immune response contributes to liver fibrogenesis, which leads to the development of cirrhosis and HCC (Tanaka & Miyajima 2016). HBV has a tropism for hepatocytes, the liver parenchymal cells, which constitute about 60% of total liver cells (Tanaka & Miyajima 2016). The highly infectious nature of the virus and the presence of extensive reservoirs of extrahepatic virus means that as many as 20% of infected patients experience a spectrum of extrahepatic disorders (Rong et al. 2007). Following HBV entry into hepatocytes, the replication of the virus ensues and in so doing induces non-cytopathic effects that may interfere with the normal functioning of the cell (Zhang & Hu 2015). Although the replicative capability of HBV in extrahepatic tissues remains controversial, the highly infectious nature of the virus and the presence of extensive reservoirs poses a challenge for complete eradication of the virus (Rong et al. 2007).

2.2 Hepatitis B Virus Epidemiology

Worldwide, as measured by antibody against hepatitis B core antigen (anti-HBcAg) seropositivity, more than two billion people have been estimated to have been infected with HBV at some stage of their life (Shepard et al. 2006). In 2016, the estimated global prevalence of the HBV surface antigen (HBsAg) was 3.9% (3.4-4.6), corresponding to 292 million (95% CI: 251-341) chronically infected individuals (Polaris Observatory Collaborators 2018). HBV is the second most important human carcinogen following tobacco smoking (Zeng 2014) and was responsible for 887,000 deaths in 2015 (World Health Organization 2017c). The worldwide prevalence rates for HBV infection vary from 0.1% to more than 20% (Figure 2.1) (Lavanchy & Kane 2016). Globally, 78% of

HCC cases are attributable to hepatitis virus infection, with HBV infection more commonly associated with this malignancy than HCV (ranging from 75%-80% versus 10%-20%) (Perz et al. 2006). Accordingly, HBV is one of the most important aetiological risk factors for hepatocarcinogenesis (El-Serag 2012). Overall, approximately 45% of the world's population live in areas endemic for HBV (Papastergiou et al. 2015). In particular, HBV causes approximately two out of three cases of LC in less developed countries, with most cases aggregating in Eastern Asia and sub-Saharan Africa (Maucort-Boulch et al. 2018). Despite this, global LC deaths caused by HBV have been reported to have decreased from 1990 to 2013 (age-standardised mortality rate (ASMR) per 100,000 from 5.1 to 4.6, respectively; median change of -9.1%) (GBD 2013 Mortality and Causes of Death Collaborators).

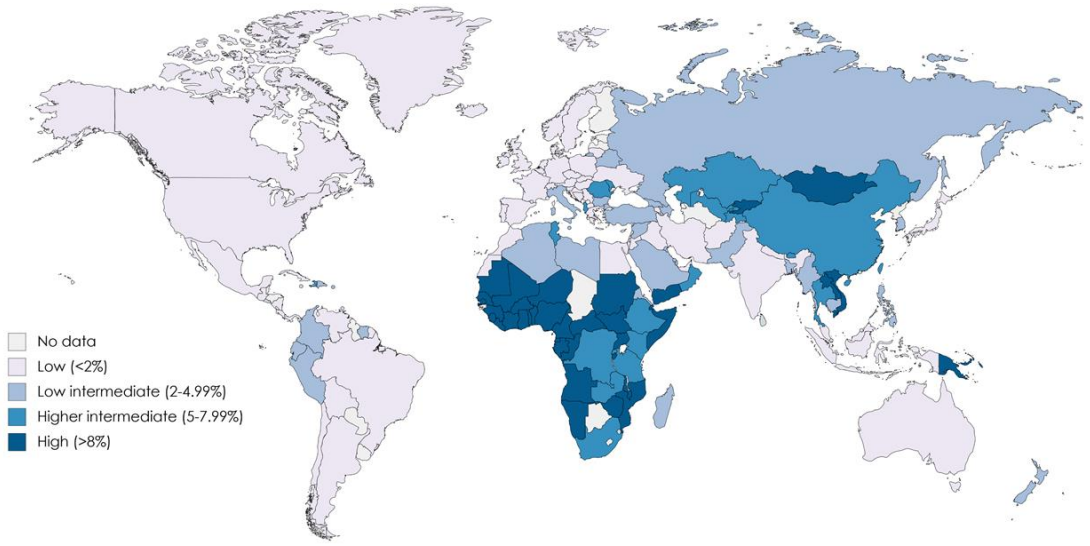

Figure 2.1 Global prevalence of Hepatitis B Virus infection, compiled using data from (Schweitzer et al. 2015). The map was created from https://mapchart.net.

Different regions have been categorised into four distinct groups based on the viral serological prevalence of the HBsAg (Schweitzer et al. 2015; Ott et al. 2012): high (≥8%), higher-intermediate (5%–7.99%), lower-intermediate (2–4.99%) and low (<2%). Except for Algeria, Eritrea, and the Seychelles, most countries in Africa were of higher-intermediate endemicity (SA [6.7%], Zambia [6.1%] and Kenya [5.2%]), or highly endemic for HBV (Swaziland [19%], Zimbabwe [14.4%], Cameroon [12.2%] and Mozambique [8.3%]). Countries in the Americas, such as Mexico [0.2%], Guatemala

[0.2%], USA [0.3%] and the Canada [0.8%] have mostly low endemicity levels, but high HBsAg prevalence was observed in Haiti [13.5%]. The Eastern Mediterranean region have a lower-intermediate endemicity, but Djibouti [10.4%], and Sudan [9.8%] showed a higher prevalence of HBsAg than other countries in the region such as Egypt [1.7%]. In the WHO Western Pacific Region, lower-intermediate to low-endemicity regions include the Philippines [4.6%], South Korea [4.4%], New Zealand [4.1%], Singapore [4.1%], Japan [1.0%] and Australia [0.4%]. Prevalence in China [5.5%] and Vietnam [10.8%] are amongst the highest in this region (Schweitzer et al. 2015).

Despite the low endemicity levels of HBV reflected in several western countries listed above, the underlying health risk from HBV-associated diseases has risen in some of these regions in the last half of the 20th century as a result of international population movements. Specifically, migrants may represent vectors for the introduction and transmission of viral hepatitis into host countries. In North America and Western Europe for example, migrants originate predominantly from continents and countries with high prevalence of viral hepatitis including sub-Saharan Africa, China and the Philippines (Nations and United Nations, 2018). Screening programs in the New York City (Centers for Disease Control and Prevention (CDC), 2006) and San Francisco (Gish and Cooper, 2011) for example have reported 2.4–15% prevalence of CHB among immigrant Asians. Accordingly, not only does this pose a unique challenge for the healthcare systems of the host nation, the burden of chronic liver disease and HCC continues to increase in these host countries (further discussed in Chapter 3). High HBV prevalence in the migrant population may be the result of a lack of universal standards for screening, vaccination and treatment of viral hepatitis in the country of origin.

2.2.1 Epidemiology of Hepatitis B Virus in Africa

With only 16% of the world's population, Africa carries 8.83% of the global burden of HBV (Schweitzer et al. 2015), of which an estimated 65 million are chronically infected (Kramvis & Kew 2007). In sub-Saharan Africa, 70% to 98% of adult black Africans show serological evidence of previous exposure to HBV (Kiire 1990), of which 8% to 20% are chronic carriers (Kramvis & Kew 2007). It is known that in this region HBV infection mainly occurs at a young age with most acquiring the HBV before the age of 30 years (81.5% and 71.4% in SA and Togo, respectively) (Kolou et al. 2017; Kew et

al. 2007), and thus are at a high risk of developing HCC (relative risk ranging from 9 to 23.3) (Kew 2010). The average ASIR of HCC in sub-Saharan Africa men and women is 18.9 and 8.0 per 100,000 persons/year, respectively (Kew 2013). Despite this, there is a general belief in sub-Saharan Africa that underreporting and inadequate collection of data contributes to the inaccurate reporting of the burden of HBV (Kolou et al. 2017; Kew 2013). Nevertheless, with 1.4 million annual deaths in 2013, HBV/HCV infection is of great public concern and threat to public health worldwide, and is higher in magnitude compared to HIV (1.3 million), malaria (0.9 million), and tuberculosis (1.3 million) (GBD 2013 Mortality and Causes of Death Collaborators).

The transmission of HBV occurs predominantly by percutaneous or mucosal exposure to infected blood and other body fluids of an infected person. Mother-to-child (perinatal/vertical) remains the most common route of viral transmission in areas of high prevalence such as Asia, whereas unprotected sexual contact or intravenous drug use are the main routes of infection in areas of low prevalence, such as Canada (Binka et al. 2018). Thus, the latter cases only acquire the infection later on in life, with majority (95%) experiencing spontaneous resolution of acute infection (Elgouhari et al. 2008). In contrast, in other regions of high HBV endemicity such as sub-Saharan Africa, most infections are acquired horizontally within the first 5 years of life (Edmunds et al. 1996; Burnett et al. 2012), presumably via contact with household members, unsafe injection practices and medical procedures (Simonsen et al. 1999), tribal tattooing, scarification and circumcision practices (Kew 2012). Unlike Eastern Asia, perinatal transmission of HBV in sub-Saharan Africa is less important partly as a consequence of the lower frequency of hepatitis B e antigen (HBeAg)-positivity, which is a major determinant of perinatal transmission (Thompson 2009; Edmunds et al. 1996; Kramvis 2016). Evidence from the pre- or early immunization period have reported very high infection rates in infants with HBsAg prevalence exceeding 10% in sub-Saharan Africa countries including Nigeria (Musa et al. 2015), Senegal (Coursaget et al. 1993) and SA (Mdlalose et al. 2016; Vardas et al. 1999). In these populations, fewer than 10% resolve the acute infection and progress onto various forms of sequelae related to chronic hepatitis B, including cirrhosis, liver decompensation or HCC (Elgouhari et al. 2008).

The transmission of HBV can effectively be prevented by vaccination, that is, the inclusion of the birth dose and subsequent doses prior to exposure to the virus. In 1992, the World Health Assembly formulated a resolution recommending the inclusion of hepatitis B vaccine in the national Expanded Programme on Immunization (EPI). In 2015, hepatitis B vaccine had been included in the EPI in 185 of 194 WHO Member countries (95%) (World Health Organization 2017a), with all of the countries in the WHO Africa region administering the vaccine (Breakwell et al. 2017).

The WHO recommends that all infants receive their first dose of HBV vaccine (HepB) immediately after birth to prevent mother-to-infant transmission and chronic infection with HBV, however this has proved challenging in sub-Saharan Africa. In 2015, the vaccine coverage of the HepB birth dose has increased to 39% globally (World Health Organization 2017a), but only fewer than 10 countries (Botswana, Djibouti, Mauritani, Namibia, Nigeria, the Gambia) in sub-Saharan Africa offered the birth dose vaccine (Tamandjou et al. 2017). This may be related to a higher frequency of home births, lack of services to reach infants born at home, inadequate and unreliable provision of the vaccine, inadequate documentation, and possible cultural factors (Breakwell et al. 2017). In SA, where no national HBV surveillance programme has been conducted, HepB is offered from 6, 10 and 14 weeks as a multivalent vaccine formulation since its introduction into the EPI in April 1995 (Burnett et al. 2012). A recent study reported that the HBsAg prevalence decreased favourably (<2%) during post-HepB compared to pre-HepB introduction in countries that do not offer a HepB birth dose including Cameroon, Ghana, Senegal, SA and Tanzania (Breakwell et al. 2017). In SA, the favourable childhood HBsAg carrier rate of less than 1% was also reported recently and the authors predicted that the population prevalence will reduce by approximately 20% by the year 2020 (McNaughton et al. 2017) in the absence of catch-up vaccination programmes for adolescents and school-based vaccination programmes (Burnett et al. 2012).

Comparison of HBV exposure and carrier rate between rural- and urban-born Blacks is important to the epidemiology of the virus, considering that a vast majority of the population resides in rural areas in SA. More specifically, rural-born and urban-born black Africans show variation in the burden of HBV-related HCC. In a study of 380

black Africans with HCC in SA, Kew *et al*. observed that those born and residing in a rural environment (45%) had a higher rate of HBsAg alone (anti-HCV negative) than rural-urban (37%) and urban-born (18%) black Africans (Kew, Houghton, et al. 1990). Moreover, rural-born and -residing HCC cases are diagnosed at a younger age than those who migrate to urban cities and their urban-born and -residing counterparts (34.7 vs 50.9 vs 46.7 years, respectively) (Kew 2008a).

2.3 Hepatitis B Virus - Virology

2.3.1 Taxonomy of hepatitis B Virus

HBV is a small enveloped DNA pararetrovirus with an RNA intermediate (Summers and Mason 1982) that causes B-type hepatitis and is a prototypic member of the family *Hepadnaviridae*. Hepadnaviruses are hepatotropic, yet non-cytopathic to infected hepatocytes (Seeger & Mason 2015; Bhat et al. 2011) and infect a narrow host range - which is divided into the mammalian *Orthohepadnavirus* and avian *Avihepadnavirus* genera (Fauquet 2005). HBV, in the former genus, is found only in humans, primates and in a special family of the sciuridae (Glebe 2007). Even so, each of these mammalian families may have their own species-specific HBV (Bonvicino et al. 2014).

2.3.2 Virion structure and subviral particles

HBV virions, also known as Dane particles (Dane et al. 1970), are comprised of a double shelled spherical structure with a diameter of 42 nm and is one of the smallest enveloped viruses infecting animals (Figure 2.2) (Liang 2009). The outer shell covered by a lipid bilayer membrane, densely packed with surface envelope proteins (small [SHBs], medium [MHBs] and large [LHBs]), that are acquired by budding at the endoplasmic reticulum. Enclosed by this outer lipid membrane, is the icosahedral capsid that has the intrinsic property to self-assemble from 180 or 240 units of the core protein (HBcAg). Within the capsid is a single copy of the HBV genome consisting of a 3.2 kb partially double stranded relaxed circular DNA molecule. The viral polymerase is covalently linked to the 5’ end of the complete (minus-sense) DNA strand, and serves as a protein primer. Besides virions, as many as 90% of secreted particles are actually subviral particles composed of empty HBsAg envelope proteins and host-derived lipids bound by covalently linked intermolecular disulphide bonds (Ning et al. 2011), with

long filamentous (17–22 nm diameter) and spherical (17–22 nm diameter) shapes (Dane et al. 1970). The non-infectious subviral particles released into the bloodstream are vastly more abundant than the mature circulating virions (100- to 10,000-fold) and can accumulate to concentrations of several hundred micrograms per milliliter in the blood of HBV-infected patients (Venkatakrishnan & Zlotnick 2016; Seeger & Mason 2015; Karayiannis 2017).

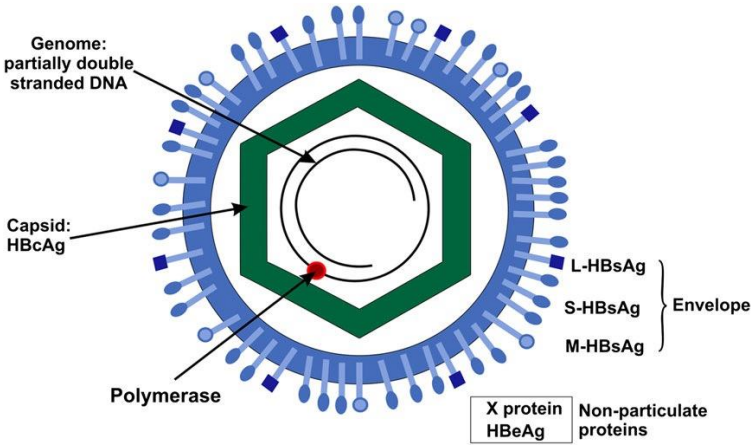

Figure 2.2 Schematic organization of the three hepatitis B virus surface proteins of a Dane and subviral particles (shown as spherical and filamentous form). The surface proteins are depicted as SHBs, MHBs (SHBs + preS2) and LHBs (SHBs + preS2 + preS1). Each core represents a dimer (120 dimers comprise a particle) and together the nucleocapsid harbours the partially double-stranded circular DNA (drawn as a single or double line) that is covalently linked to the viral polymerase. Reprinted with permission from (Kramvis 2016).

2.3.3 Genome organization and proteins

The HBV genome is partially double-stranded DNA with two asymmetric strands: The minus (long) DNA strand is complete except for a gap at a unique site, whereas the plus (short) strand is incomplete. Each of the strands incorporate a 11 nucleotide (nt) direct repeat sequences at their 5′ ends, namely DR1 and DR2, which is critical for priming virus replication (Seeger et al. 1986). The genome sequence has cohesive termini at the 5′ ends of the two strands, which overlap by 245 base pairs (bp) and maintain the circular configuration of the genome (Lien et al. 1987). This Watson-Crick pairing maintains the circular configuration of the DNA by bridging the gap in the minus

strand. Whereas the 5’ end of the plus strand has a capped oligoribonucleotide attached (Lien et al. 1986), a retrovirus-like reverse polymerase with transcriptase activity is covalently bound to the 5’ end of the minus strand (Summers and Mason 1982). The plus DNA strand acts as a template for the synthesis of the viral mRNA transcripts and ranges in length from 1700 to 2800 nucleotides (Ngui et al. 1999).

As a result of the highly compact nature of the HBV genome, every nucleotide is either utilised in one or the other of the coding regions or open reading frames (ORFs). The genome codes for seven proteins, in four partially overlapping, frame-shifted ORFs, as shown in Figure 2.3 and

Table 2.1. The ORFs are designated *S* (encodes the surface/envelope proteins), *C* (precore/core), *P* (polymerase) and the *X* (HBx protein). The S-ORF contains three in-frame initiation codons (PreS1, PreS2 and S) that code for three viral envelope proteins corresponding to HBsAg - termed the large (LHBs), middle (MHBs), and small (SHBs). These proteins can be distinguished by their different domains and glycosylation status (Glebe & Bremer 2013). The C-ORF is extended at the 5’ end by the PreC region, and consists of two in-frame codons encoding the nucleocapsid structural protein, HBcAg, and the extraparticulate HBeAg (Takahashi et al. 1983). The *X*-ORF encodes the multifunctional 16.5 kilodalton (kD) viral X regulatory protein (HBx) (Siddiqui et al. 1987). Finally, the *P*-ORF encodes for the large viral polymerase (about 800 amino acids).

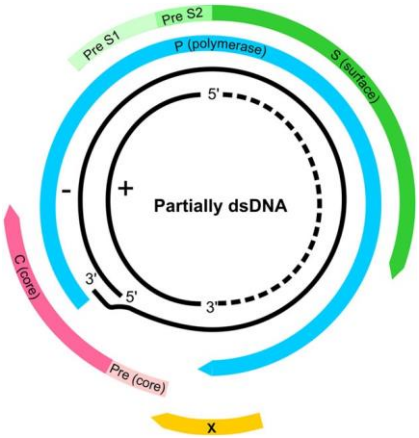

Figure 2.3 Schematic of the circular, partially double-stranded hepatitis B virus genome with the complete minus (-) and incomplete (+) strand. This includes four overlapping

reading frames that codes for seven proteins: *precore/core* (*preC/C*) that encodes the e antigen (HBeAg) and core protein (HBcAg); *P* for polymerase (reverse transcriptase), *PreS1/PreS2/S* for surface proteins (three forms of HBsAg: small (SHB), middle (MHB) and large (LHB)) and *X* for a transcriptional trans-activator protein. Reprinted with permission from (Kramvis 2016).

Table 2.1 HBV proteins and their respective coding regions in the viral genome. Positions are given with respect to reference strain AM282986.

Genomic Region	Protein	Reading Frame	Positions
PreS1	LHBs	Surface	2854-3210
PreS2	LHBs, MHBs	Surface	3211-154
HBsAg	LHBs, MHBs, SHBs	Surface	155-835
Precore (PC)	HBcAg, HBeAg	Core	1814-1900
Core	HBcAg	Core	1901-2458
X	X	X	1374-1900
Terminal Protein (TP)	Polymerase	Polymerase	2307-2843
Spacer (SP)	Polymerase	Polymerase	2844-129
Reverse Transcriptase (RH)	Polymerase	Polymerase	130-1161
RNaseH (RN)	Polymerase	Polymerase	1162-1623

The important regulatory elements are embedded in the protein-encoding genes and include the four promoters (PreC/C, S1, S2 and X), two enhancers (Enh I and Enh II), two direct repeat sequences (DR1 and DR2), a polyadenylation signal, encapsidation (ε), a glucocorticoid responsive element and a post-transcriptional regulatory element (Table 2.2) (Karayiannis 2017; Tur-Kaspa et al. 1986; Smith et al. 1998).

Table 2.2 HBV regulatory elements and their functional characteristics.

Element	Active on	Function/characteristics
Enhancer 1	cccDNA	Up-regulates transcription of the preC and HBx mRNAs and acts as binding sites for various transcription factors (Quarleri 2014).
Enhancer II	cccDNA	Up-regulates transcription of the preS1, preS2, and X promoters as well as the pg/preC mRNAs and acts as binding sites for various transcription factors (Quarleri 2014).
X promoter	cccDNA	Initiates transcription of X mRNA (Quarleri 2014).
preC/C promoter	cccDNA	Directs the transcription of both the precore mRNA and pgRNA and contains major nuclear binding sites (Quarleri 2014).
PreS1 promoter	cccDNA	Confers transcription of LHBs mRNA (Quarleri 2014).
PreS2 promoter	cccDNA	Confers liver-specific transcription of MHBs and SHBs mRNA (Quarleri 2014).
Glucocorticoid responsive element	cccDNA	Binds the glucocorticoid receptor and enhance the activity of enhancer I (Tur-Kaspa et al. 1986).
Negative regulatory element	cccDNA	Confers negative regulation of core/HBe mRNA transcription (Sun et al. 2001)
CCAAT box	cccDNA	Confers negative regulation of preS1 mRNA transcription; serves as activator of S mRNA transcript (Bock et al. 1999)
Polyadenylation signal	RNA	Termination of transcription (Hilger et al. 1991)
Post-transcriptional element	RNA	Activates the RNA export reporter system (Donello et al. 1996)
Encapsidation signal	Pregenomic RNA	Packaging of the RNA, initiation site for minus-strand DNA synthesis (Knaus & Nassal 1993; Beck & Nassal 1998)
Direct repeat 1	Pregenomic RNA	In priming the synthesis of minus-strand DNA (Rieger & Nassal 1996)
Direct repeat 2	Minus-DNA	Initiation site for plus-strand DNA synthesis (Habig & Loeb 2006)

2.3.3.1 Core Protein and HBeAg

The core promoter is responsible for the synthesis of mRNAs, which differ with respect to the start of their 5'end. The precore protein, which serves as a precursor for HBeAg, arises from the translation of the first start codon of a precore mRNA, which encodes a hydrophobic signal sequence the directs the protein to the ER. Here, 19 amino-acids of the protein are proteolytically cleaved at its N-terminus, and the subsequent removal of the arginine rich domain at its C-terminus occurs when it moves to the secretory pathway to generate the mature, secreted soluble HBeAg (Ito et al. 2009; Messageot et al. 2003; Wang et al. 1991; Schödel et al. 1993). The resulting 17 kDa HBeAg is a non-structural protein and is important for establishing persistent infection *in vivo*, elicits humoral and immune response, down-regulation of innate immune response as well as act as T cell tolerogen (reviewed in Revill et al. 2010). A previous study using cell receptor transgenic mice expressing receptors for the HBcAg or HBeAg suggest that a function of HBeAg is to suppress the immune response to the HBV core protein (Chen et al. 2005) and elicit T cell tolerance (Chen et al. 2005; Chen et al. 2004), thus predisposing to chronic infection in newborns. Thus, HBeAg detected in serum is an important diagnostic marker of HBV replication and the seroconversion is marked as the transition from immune-active phase to the inactive carrier state (described below).

The other transcript is the pregenomic RNA (pgRNA) and is initiated five nucleotides downstream from the precore initiation codon. The pgRNA acts as a bicistronic mRNA independently encoding for both the polymerase and the capsid core protein (ORFs overlap by an estimated 150 bp) (Wang et al. 2013; Radziwill et al. 1990). In addition, it also serves as a template for DNA synthesis by reverse transcription within the nascent nucleocapsids, as explained below. The 183 aa HBV core protein or capsid protein (HBcAg) is the organizing framework of the viral shell composed of two soluble icosahedral moieties, namely T = 3 (30 nm diameter, 180 core particles) or T = 4 (34 nm diameter, 240 core particles) (Crowther et al. 1994; Lamontagne et al. 2016). Translated from the pgRNA, the first 149 aa of the core protein form the assembly domain that is responsible for core protein dimer formation and self-assembly into capsid structures (Klumpp et al. 2015). The remaining 34-36 aa makes up the arginine-rich C-terminal domain acts as an essential auxiliary component in HBV replication that

is critical for capsid formation (Wynne et al. 1999), nuclear localization of core proteins (Liao & Ou 1995), pgRNA encapsidation, initiation of reverse transcription and formation of rcDNA (Melegari et al. 2005; Nassal 1992; Jung et al. 2014; Zlotnick et al. 2015). The diverse functions of core protein in the HBV life cycle including replication and pathogenesis, were recently reviewed (Diab et al. 2018).

2.3.3.2 Surface Proteins

All three of the HBV surface proteins (SHBs, MHBs and LHBs) that envelope the spherical structure of the HBV are encoded within a single S-ORF from in-frame AUG codons. They all share the same carboxy-terminus part but exhibit different amino-terminal extensions, that is, the carboxy-terminal S-domain (226 amino acids [aa] long) is present in all surface-proteins, preS1 (55 aa) only in LHBs, preS2 (108-119 aa depending on the genotype) in LHBs and MHBs (Glebe & Bremer 2013).

The preS1 and preS2 regions of the LHBs protein are essential for viral replication, which have been recently reviewed (Chen 2018). The functional sites can be divided into two sections of the preS1, where the N-terminal half (aa 1-57) contains the hepatocyte binding site (aa 2-48) necessary for the attachment of the virus to hepatocytes (Figure 2.4). The C-terminal half of preS1 (aa 58-119) contains a heat-shock protein 70 (Hsc70, aa 74-118) binding site and cytosolic anchorage determinant (CAD, aa81-105) essential for dual topology of the LHBs proteins; nucleocapsid binding site (NBS, aa103-127) for virion morphogenesis; S-promoter (nt 3045-3180) and CCAAT binding factor (CBF, nt 3137-3147) binding site necessary for S RNA transcription. The preS2 region contains NBS, viral secretion (VS, aa 1-5) and polymerized human serum albumin (pHSA, aa 3-16) binding sites. The HBV preS1 and preS2 regions play a crucial role in immune responses, as they are both carriers B-cell and T-cell epitopes.

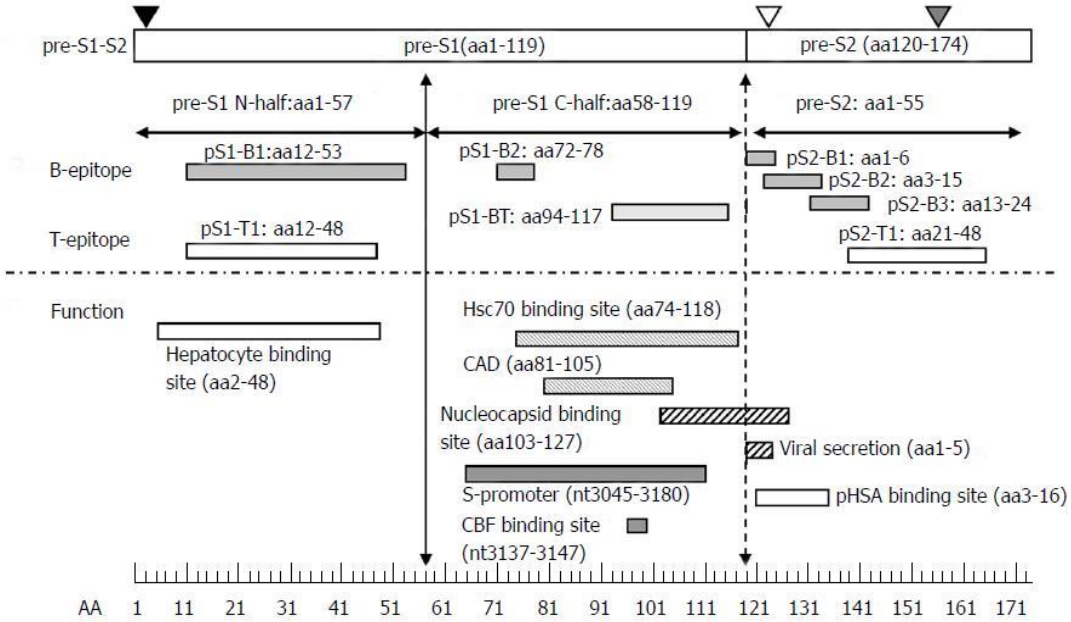

Figure 2.4 The HBV preS region contains immune epitopes and functional domains reprinted with permission from (Chen 2018): hepatocyte binding site (Hb), S promoter (Sp), CCAAT box (CT), topology domain (Topo) includes heat shock protein 70 binding site and cytosolic anchorage determinant (CAD), nucleocapsid binding site (NBS), viral secretion domain (Vs), polymerized human serum albumin binding site (pHSA).

Translation of the mRNAs coding for the three forms of surface proteins is driven by two independent promoter regions, thus allowing for regulation of variable protein expression. First, the preS1 promoter region, characterised by a canonical TATA box, contains binding sites for liver-specific transcription factors (e.g., HNF1) (Raney et al. 1991) and is located upstream of the first start codon of the LHBs and thus, responsible for 2.4 kb long mRNA that gives rise to LHBs (24 kD). The other is called preS/S promoter region, which is devoid of a TATA box and is located in the preS1 region. The preS/S promoter is responsible for the generation of two types of mRNAs that give rise to the MHBs (31 kD) and SHBs (39 kD) using the second or third start codon, respectively.

The virion envelope consists of the three proteins (SHBs, MHBs and LHBs) at a ratio of 4:1:1, respectively. Surface proteins synthesised in the ER can either be incorporated

into the virion envelope or when produced in excess, undergo multimerization together with the host lipids. The latter process occurs by budding from the ER/Golgi compartment for both non-infectious spherical or filamentous lipoprotein particles (Siegler & Bruss 2012). Such subviral particles (SVP) are typically secreted from infected hepatocytes in great excess over the virion, and may induce immune tolerance. Spherical SVPs of an estimated 100 protein molecules are produced by cells expressing the S protein only (without other HBV-encoded proteins) represent the antigenic component of HBV vaccines. Spherical SVPs are assembled and secreted by a biochemical pathway distinct from the production and release of Dane particles and filaments (Patient et al. 2007; Jiang et al. 2015).

2.3.3.3 Polymerase Protein

HBV polymerase has both DNA polymerase and reverse transcriptase activity (Pol/RT), and is encoded by the longest ORF *P* that spans almost 70% of the HBV genome (Glebe & König 2014). It overlaps the entire surface gene and contains between 834 and 845 codons depending on genotype. The hepadnaviral polymerases consist of four domains: N-terminal terminal protein (TP) domain followed by the spacer domain, reverse transcriptase (RT) domain and a C-terminal RNase H domain (Jones & Hu 2013). Located at the 5′ end of viral minus-strand DNA, the TP domain is responsible for binding to the encapsidation signal (epsilon (ε)) of the pgRNA, RNA packaging, and protein priming. Although the spacer domain has no recognised function, it may also play a role in RNA packaging as well as have a structural role in polymerase function (Jones & Hu 2013; Radziwill et al. 1990). On the other hand, the spacer domain may serve as an important variable site for maintaining heterogeneity for the adaptive evolution of HBV to escape from the host immune system, as well as providing flexibility for conformational changes and response to immune selective pressure without compromising the polymerase activity (Chen et al. 2013). The RT domain contains the tyrosine–methionine–aspartate–aspartate (YMDD) polymerase active site that is required for both protein priming and all subsequent DNA synthesis while the RNase H domain, located at the C-terminus, is responsible for the degradation of the viral pgRNA template during the minus-strand DNA synthesis (Jones & Hu 2013).

2.3.3.4 X ORF

The HBV X (HBx) protein is translated from a small subgenomic RNA controlled by the 465 nucleotides long HBx ORF (from nucleotides 1374-1900), and was originally termed as such because of the lack of homology with known sequences. The mechanism and function of HBx remains not completely understood, however, it is well established that HBx participates in generating an environment that is conducive to the replication of HBV and may contribute to the oncogenic potential of the virus (Slagle & Bouchard 2016). The non-structural HBx protein has both a N-terminal negative regulatory domain and a C-terminal transactivation/coactivation domain (Tang et al. 2005), which regulate transcription and host cell proliferation during the course of infection. HBx is also a multifunctional viral regulator that modulates gene transcription, signalling pathways, genotoxic stress responses, protein degradation, cell-cycle control, cell proliferation, and apoptosis (Murakami 2001; Tang et al. 2005; Lucifora & Protzer 2012; Bouchard & Schneider 2004). These activities may play an important role in viral replication and proliferation with relevance to HBV associated pathogenesis such as the development of HCC (Xie et al. 2018; Liao et al. 2017; Kew 2011).

2.4 Hepatitis B Virus Life Cycle and Replication

As shown in Figure 2.5, HBV replication is a complex process that have been extensively studied, and divided into several stages: binding and entry of the virion to the host hepatocyte, cytosolic transport of the core particles within the cell, conversion of relaxed circular DNA (rcDNA) to covalently closed circular DNA (cccDNA), viral transcription, expression of core and polymerase proteins, encapsidation, reverse transcription, synthesis of positive strand DNA, circularization, maturation and secretion of virions and subviral particles and formation of a closed circular DNA pool (Glebe & Bremer 2013).

The initial stage of HBV infection involves the attachment of virus to the membrane of hepatocyte. Evidence suggests low-specific binding to heparan sulfate proteoglycans (Schulze et al. 2007; Sureau & Salisse 2013) first takes place, followed by binding to high-specific receptors. The high specificity interaction occurs between the N-terminal 75 amino acids of the preS1 domain of the LHBs and sodium taurocholate

cotransporting polypeptide, a multiple transmembrane transporter predominantly expressed in the liver (Ni et al. 2014; Yan et al. 2012). Upon receptor-mediated entry of the virion by endocytosis (Cooper & Shaul 2006), the virus undergoes disassembly whereby the HBV nucleocapsid containing the rcDNA is released into the cellular cytoplasm and actively transported along microtubules and into the nucleus (Rabe et al. 2006; Blondot et al. 2016). Here, productive viral infection proceeds with the conversion of the liberated rcDNA into a nuclear, episomal cccDNA occurs, with the assistance of host enzymes that are components of the DNA repair machinery (Qi et al. 2016; Königer et al. 2014). This is achieved by covalently ligating both DNA strands after completion of the shorter (plus) DNA strand, as well as the removal of obstructive terminal modifications such as the 5' covalently-linked viral polymerase and the 3' linked RNA primer (Schädler & Hildt 2009). The resulting stable form of the viral genome (cccDNA) represents the hallmark of an established infection and serves as a template for transcription of viral genomic and subgenomic RNAs.

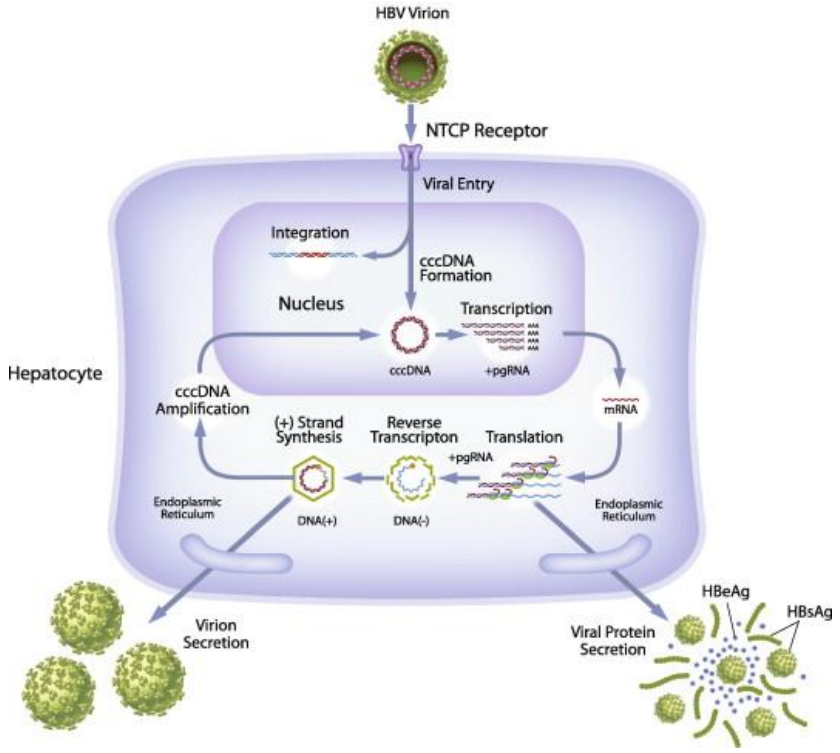

Figure 2.5 Schematic representation of various stages of the hepatitis B virus (HBV) life cycle. NTCP, sodium-taurocholate cotransporting polypeptide receptor; cccDNA,

covalently closed circular; pgRNA, pregenomic RNA; Hepatitis B e antigen (HBeAg) and surface antigen (HBsAg). Reprinted with permission from (Gish et al. 2015).

Unlike other retroviruses that exploit the cellular replication machinery for amplification by integrating their DNA into the host genetic material, this stage is not necessary for the replication of hepadnaviruses. Nevertheless HBV DNA integration throughout the host genome at double strand DNA breaks, does occur frequently, suggesting an evolutionary benefit for the virus. This integration seems to be a main driver for oncogenic transformation of infected hepatocytes, despite the exact mechanism remaining unclear and poorly characterised (Tu et al. 2015). The negative strand of the cccDNA in the nucleus serves as the a transcriptional template for the synthesis of the overlength pgRNA, serves as the template for reverse transcriptional synthesis of viral DNA and as a transcript for the translation of the for the polymerase (90 kDa), the core protein (21 kDa) and precursor early antigen (24kDa) (Schädler & Hildt 2009). An important *cis*-element for the initiation of the replication step is the highly conserved stem loop structure termed as *epsilon*, 'ε', the encaspdiation signal, found at the 5' and 3' ends of the pgRNA. However, only the 5′ ε is specifically recognised and bound by the viral polymerase (Rieger & Nassal 1996), which prompts the recruitment and packaging of the pgRNA-polymerase complex in core protein homodimers (Chiang et al. 1992; Bartenschlager et al. 1990). Besides initiation of pgRNA-polymerase encapsidation, the exclusive requirement of the 5' ε in the interaction with the polymerase ensures that only the pgRNA is packed and synthesised into rcDNA inside the capsid. This synthesis occurs by reverse transcription of the minus-DNA strand using the pgRNA as template, followed by the synthesis of the plus-DNA strand using the minus-DNA strand. Additionally, the core protein is crucial for reverse transcription, as defects in DNA synthesis have been shown to result from either deletion or modification of the C-terminus assembly region of the core protein (Nassal 1992; Kock et al. 2004; Diab et al. 2018). Thus, not surprisingly, replicative intermediates in the form of non-enveloped HBV DNA-containing nucleocapsids and enveloped pgRNA-containing nucleocapsids occur throughout the HBV morphogenesis and some are secreted (Beterams & Nassal 2001). For instance, minor forms of subviral particles characterised as genome-free (empty) enveloped virions, with or without capsid, are secreted and the biological function(s) remains unknown (Hu & Liu 2017).

On the other hand, the major form of HBV produced by infected hepatocytes are the nucleocapsids containing rcDNA. These can either be enveloped and secreted out as mature infectious HBV virions to continue with the viral life cycle (Patient et al. 2009; Watanabe et al. 2007), or recycled back into the nucleus, to add or replenish the intranuclear cccDNA pool (Zhang et al. 2003). For instance, when the secretion of the envelope protein is inhibited, LHBs is retained in the endoplasmic reticulum leading to LHBs overexpression relative to MHBs and SHBs. This phenomenon produces the characteristic ground glass appearance of hepatocytes and is recognised as a distinct histopathological hallmark that has been implicated in the pathogenesis of HCC (Mathai et al. 2013; Su et al. 2008).

2.5 Hepatitis B Virus Diversity

Historically, genetic variability within the HBV was defined by serotypes based on the antigenic determinant "*a*" (aa 121–149) that maps to the HBsAg (Cassidy et al. 2011) and four other serological subdeterminants of HBV that display aa polymorphisms at specific positions: "*d*" subtypes have a lysine whereas "*y*" subtypes have arginine at aa 122, "*r*" subtypes carry a arginine at aa 160 (Okamoto et al. 1988) where as "*w*" is determined by variation in up to four aa positions (aa 122, 127, 140, and 159) (Purdy et al. 2007). Based on the different combinations of these allelic variations, this give rise to an apparently complex classification of ten serological subtypes of HBV (*ayw*1, *ayw*2, *ayw*3, *ayw*4, *ayr*, *adw*2, *adw4*, *adwq*, *adr*, and *adrq*-) (Kramvis 2014). The relationship between serotypes and genotypes are not entirely clear with the possibility of the same serotype classified into different genotypes (Okamoto et al. 1988; Kramvis 2014).

More recently, based on intergroup nucleotide divergence of >7.5% of the HBV genome sequence instead of antigenic variations in the surface protein, nine major genotypes (labelled A-I) of HBV have been phylogenetically identified worldwide (Kramvis & Kew 2005; Kramvis 2016). As shown in Figure 2.6, these can have distinct geographical distribution (Norder et al. 1992; Kramvis 2014; Kramvis et al. 2005), while some, such as A and D are more widely spread and are also the predominant genotypes in North, East, and Southern Africa (Kramvis & Kew 2007; Yousif et al. 2013). The reasons of this non-uniform distribution are not completely clear, but human

population migration and increased risky behaviours, among other factors, may alter the prevalence of genotypes in a region (Kostaki et al. 2018). Currently, genotype B and C are confined to Asia and Oceania (Huy & Abe 2004; Tian & Jia 2016) while genotype E is restricted to Africa (Suzuki et al. 2003; Shimakawa et al. 2015). Genotype F and H on the other hand are frequently identified in the Americas (Kramvis 2014; Arauz-Ruiz et al. 2002). Genotype I is rare, and has been described in Laos, India, China (T. Shen et al. 2014) and Vietnam (Huy et al. 2008; Tian & Jia 2016). A putative tenth genotype, J has been isolated from a single individual from Japan who had resided in Borneo (Tatematsu et al. 2009), and is suggested to be a recombinant of genotype C and gibbon HBV in the S region (Locarnini et al. 2013).

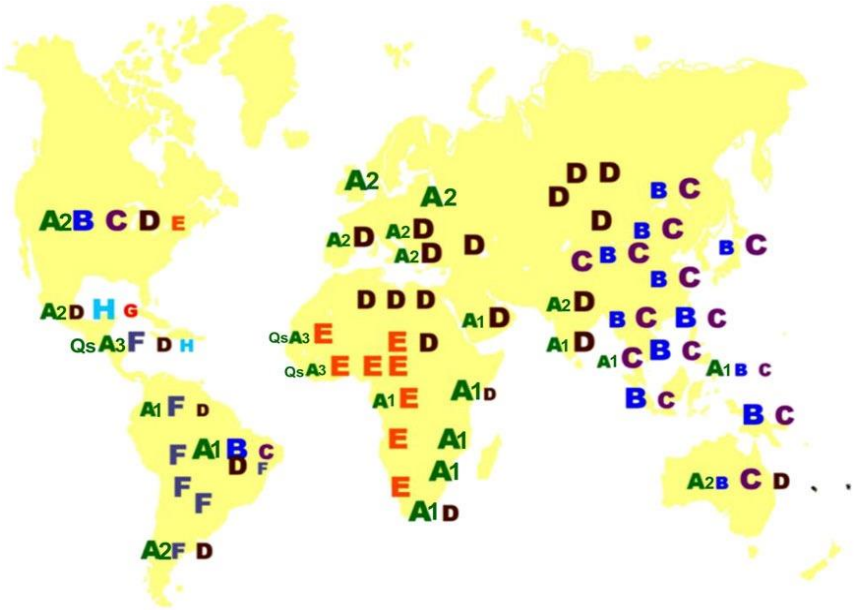

Figure 2.6 Geographic distribution of hepatitis B virus genotypes worldwide. Reprinted with permission from (Kramvis 2016).

Further diversity also exists within each of the HBV genotypes (A-D and F) and thus are further comprised of multiple subgenotypes, which are defined as sequences with a intergroup nucleotide divergence between ~4% and 8% (Kramvis et al. 2008; Kramvis 2016). For instance, sub-genotypes have been described for genotypes A (A1/Aa, A2/Ae and quasi-subgenotype A3), B (B1/Bj, B2/Ba and B3-B5), C (C1/Cs, C2/Ce and C3-C5), D (D1-D6) and F (F1a, F1b and F2-F4) (Schaefer 2007; Kay & Zoulim 2007; Yousif & Kramvis 2013 Kramvis 2016). The distribution of HBV genotypes and their respective subgenotypes varies geographically, and is well-established that disease

severity and progression, clinical manifestation of illness, host response to antiviral treatments and distribution of mutations can be influenced by certain HBV genotypes and in some cases subgenotypes (Kramvis & Kew 2005; Kramvis 2014; Mayerat et al. 1999; Q. Zhang et al. 2015; Kramvis 2016). For example, certain genotypes are associated with increased risk of HCC development (Genotype A1, C, B2-5 and F1) while others is associated with lower rates of disease-related complications (Genotype B1, B6, and A2) (McMahon 2009).

Regions where two HBV genotypes are common, such as Southeast Asia (genotype B and C) (Chan & L-Y Chan 2004; Orito et al. 2001) and India (genotype A and D) (Gopalakrishnan et al. 2013) have provided valuable information for researchers to evaluate the clinical important of these genotypes (Lin & Kao 2013). For example, most but not all retrospective or case-controls studies have reported genotype C is associated with a worse prognosis (including cirrhosis and HCC) and a lower response rate to alpha interferon therapy than is genotype B (Chan & L-Y Chan 2004; Orito et al. 2001; Kao et al. 2000). Additionally, genotype A is strongly associated with chronic infection when compared with genotype D (Mayerat et al. 1999), while southern African patients with subgenotype A1 have a 4.5-fold increased risk of developing HCC compared to those infected with non-A genotypes (Kew et al. 2005), but genotype D has been reported to be more severe as compared to A in some studies (Thakur et al. 2002; Toan et al. 2006). Furthermore, HBV DNA viral load levels are significantly lower in subgenotype A1 compared to subgenotype A2 and genotype D (Tanaka et al. 2004), and subgenotype A2 is more likely to become chronic compared to genotype B or C in Japanese population (Sugiyama et al. 2017). Patients with liver disease (Yousif et al. 2013) and blood donors (Mahgoub et al. 2011), had viral loads that were significantly higher in genotype E-infected individuals compared to genotype D-infected individuals and furthermore patients infected with with genotype E were more likely to be HBeAg positive. Finally, studies on recurrence of HBV after liver transplantation have also shown that genotype C infections have greater risk and severity of recurrence (Huang et al. 2008), while HBV genotype A and D did not have a significant impact on HBV reactivation and mortality after liver transplantation (Girlanda et al. 2004).

In regions where two or more HBV genotypes are prevalent, multiple exposures can lead to co-infection of multiple genotypes. In these populations, recombination in which the exchange or cross over of sections of a gene sequence may occur between the disparate strains of HBV and is common in many areas of the world. Recombination has been suggested as a potential mechanism for the development of divergent HBV strains (Morozov et al. 2000), and recombinant strains have been misclassified as new genotypes or sub-genotypes (Shi et al. 2013). Genotype A/D recombination has been found in 13% of asymptomatic Black SA adults (Owiredu et al. 2001a), with D/A (Bollyky et al. 1996; Bowyer & Sim 2000), A/E (Garmiri et al. 2009; Olinger 2006) and D/E (Mahgoub et al. 2011; Abdou Chekaraou et al. 2010) recombination also being reported in other sub-Saharan Africa countries. Others include H/B recombinant strains isolated in a Japanese patient (Uchida et al. 2013), and C/D in Tibet and China (Liu et al. 2017; L. Shen et al. 2014). In a previous study from West Africa, it has been suggested that the core gene is a preferred site for recombination between A and E genotypes (Garmiri et al. 2009), however, if whether other recombinant strains share similar preferential site for genetic rearrangement remains unclear.

2.6 Hepatitis B Virus gene specific mutants

Despite HBV being a DNA virus, it replicates with a mutation rate that is approximately 10 times higher than other DNA viruses (Du et al. 2017), with an estimated mutation rate of $\geq 2 \times 10^{4}$ base substitutions/site/year (Buti et al. 2005). The high mutation rate is associated with the viral polymerase that lacks proofreading capabilities and RNA as the intermediate (pgRNA) (Caligiuri et al. 2016). This replicative strategy introduces mutations throughout the HBV genome and are not restricted to any of the genes. However, because of the overlapping nature of the HBV genome, a mutation in one gene may inadvertently select for a mutation in another that is in direct overlap with it. Naturally occurring mutants may lead to different populations of viral variants called “quasi-species” that can coexist together and confer a pathogenic potential by modulating specific host immune responses (Cao et al. 2014). However, a prevalent population may arise during the course of a patient’s infection, at least in part, through antiviral pressure of the host immune system or specific therapies. These naturally occurring mutants selected by “viral fitness” may display modified epitopes that render

them unrecognizable by the host immune system, have enhanced viral replicative ability and/or resistant to antiviral therapies (Caligiuri et al. 2016). Similar to their wild type strains, these mutants are potentially transmissible and thus have implications in regards to public health (Chen et al. 2000; Thakur et al. 2005).

2.6.1 Basic Core Promoter (BCP), Precore (preC) and core (C) gene mutants

Mutations in the preC and core promoter (CP) have been described. The core promoter (CP, nt1591-1822) consists of the basic core promoter (BCP, nt1742-1849) and the upper regulatory region (URR, nts 1613-1742) (Kramvis & Kew 1999). The BCP region is involved in both HBV replication and morphogenesis, and initiates the transcription of precore mRNA and pgRNA *in vivo*. The BCP also contains DR1, which is necessary for reverse transcription. Mutations occurring in this region may influence both viral replication and HBeAg expression at three different stages of protein synthesis (i.e. transcription, translation and post-translational modification), thus HBeAg expression may be down-regulated or even abolished entirely (Kramvis & Kew 2007). BCP and preC mutations suppress the synthesis of HBeAg, one of the hallmarks of chronic HBeAg-negative and anti-HBe-positive HBV infection. BCP mutations contribute to reduced synthesis of HBeAg by suppressing the production of the preC mRNA at the transcriptional level, whereas the preC mutations may inhibit the translation of the HBeAg protein as a result of the introduction of frameshift or nonsense mutations (Kramvis & Kew 2007).

A1762T/G1764A. The major double core promoter mutation (A to T at nt 1762 and G to A at nt 1764; A1762T/G1764A) is a well-known mutation that interferes with HBeAg expression. This occurs at the viral transcription level by selectively reducing the affinity of liver-enriched transcription factors to this region, while high viral load levels may be associated with increasing pgRNA packaging and viral progeny production (Buckwold et al. 1997; Buckwold et al. 1996; Gunther et al. 1998; Ghabeshi et al. 2013). Additionally, the removal of a nuclear receptor binding site and subsequent creation of hepatocyte nuclear factor 1 sites (Li et al. 1999) have also been reported to be responsible for the abolished preC RNA synthesis, thereby diminishing production of HBeAg whereas the pgRNA synthesis remains unaffected (Buckwold et al. 1996; Gunther et al. 1998; Moriyama et al. 1996). The BCP double mutation is suspected to

be intermediate in the development of the double mutant, with A1762T being the favoured site for the initial mutation (Wang et al. 2015). Earlier transfection studies investigating revertants with either one or the other BCP mutation demonstrated that compared to the A1762T-mutant alone, G1764A-mutant alone conferred a lower viral replication levels, which was similar to that of the wild type HBV (Moriyama et al. 1996).

The BCP double mutation has been described in patients with fibrosis (Yan et al. 2017), fulminant hepatitis, including children with liver cirrhosis (Zhong et al. 2016), those with HBeAg- and anti-HBe-positive chronic hepatitis (Ledesma et al. 2011), cirrhosis (Tseng et al. 2015) and in liver tumour tissues and sera of patients with HCC (Chachá et al. 2017), but less frequently in asymptomatic chronic carriers (Baptista et al. 1999). It has been reported that the BCP double mutant is associated with the progression of liver disease, with a 4-fold increased risk of developing HCC (95% CI: 2.77–5.65) (Wei et al. 2017). The mechanism by which the BCP double mutant induces hepatocarcinogenesis is not completely understood, but it has been suggested to confer a selective advantage for variants that do not express/secrete HBeAg during an immune response in patients with chronic infection (Frelin et al. 2009). This is because, hepatocytes containing HBV expressing HBcAg and HBeAg are more susceptible to immune-mediated clearance than hepatocytes expressing either of the seromarker alone (Liu et al. 2009; Kawai et al. 2003) which may result in necroinflammation of hepatocytes. In addition, double mutants develop before or during HBeAg seroconversion, which predisposes to immune-mediated liver damage in response to increased levels of HBV viremia with little or no HBeAg (Ferns et al. 2007), and thus leads to more rapid cellular turnover and consequently fibrosis (Tong et al. 2005).

Core promoter mutations other than the double mutation may play a role on HBV replication and HBeAg expression: the A1762T/G1764A mutation in addition to substitution at position T1753C and C1766T in combination conferred higher viral replicability and lower levels of HBeAg (Parekh et al. 2003). In addition, studies from patients with chronic infections have further detailed the double A1762T/G1764A mutation in relation to the different genotypes. In comparison with genotypes A and B, patients infected with genotypes C and D experience lower rates and delayed onset of

spontaneous HBeAg seroconversion. In particular, this mutation is more prevalent in genotype C and correlates with high levels of HBV replication than genotype B (Liu & Kao 2013; Constantinescu et al. 2014). Similarly, the frequency of the double mutation have been reported to be higher in genotype D compared to genotype A (Liu & Kao 2013), but was similar in other studies (Chachá et al. 2017). Enhanced pgRNA and lowered preC mRNA transcription have been reported in genotype A with C1766T and Y1768A mutation in fulminant hepatitis patient (Nishizawa et al. 2016).

T1753V. The A1762T and G1764A, the T1753V (V represent C/A/G) mutations lead to amino acid changes K130M, V131I and I127L,T,N,S in the overlapping HBx protein, respectively (Kim, Lee & Kim 2016). The loci have been frequently identified as mutational hot spots associated with HBeAg-negativity (Cho et al. 2011), liver disease progression (Chen et al. 2005; Cho et al. 2011) and HCC (Yang et al. 2015; Jang et al. 2012; Zhu et al. 2010). In HBeAg-negative patients, the T1753V mutation has been suggested as a predictive marker for hepatocarcinogenesis (Chen et al. 2005). A multivariate survival analysis showed that the T1753V mutation is an independent predictor of HCC survival (Xie et al. 2014). Takahashi et al. reported that C1753 is a common mutation in the HCC (51%) and CH group (50%) compared with blood donors (13%) among HBeAg-positive patients. In contrast, this occurred more frequently in HCC group (45%) than in either CH (9%) and blood donors (8%) group with HBeAg-seronegativity, which suggests the tight association with advancement of liver disease in the presence of HBeAg (Takahashi et al. 1999). Meta-analysis showed that the T1753V was associated with a 2-fold increased risk of HCC (95% CI: 1.92–4.01), which significantly correlated with progressive liver disease (Liao et al. 2012; Yang et al. 2015). The T1753V mutation is more prevalent in chronic patients infected with HBV genotype C compared to other HBV genotypes (Castelain et al. 2017), and in those with severe liver disease (HCC or cirrhosis) than milder types of diseases (Kim et al. 2008). These findings suggest that T1753V mutation may play a critical role in liver disease progression. A recent study consisting of 93 HBeAg-negative French patients chronically infected with HBV showed that the T1753V mutation was associated with the presence of A1762T/G1764A double mutation, and the combination of which correlated with HBV viral load >20,000 IU/ml (Castelain et al. 2017). Similar findings

were also observed in an *in vitro* study, with only minor reduction in HBeAg expression (Parekh et al. 2003).

While the exact mechanism underlying the role of T1753V mutation in hepatocarcinogenesis remains to be elucidated, some explanations have been proposed. This mutation together with the A1762T/G1764A double mutation may influence the binding of transregulating nuclear factors to HBV, including transcription factor Sp1, CCAAT/enhancer-binding protein and HNF4 may lead to hepatocarcinogenesis (Yang et al. 2015).

With the HBV core promoter partially overlapping the HBx coding sequence, nucleotide 1762 and 1764 mutations induce HBx protein 130 and 131 substitutions, increasing the risk for HCC by 3.75-fold (95% CI: 1.101-12.768, P = 0.033) in patients with chronic HBV infection (Lee et al. 2011). The HBx protein is potentially oncogenic via multistep carcinogenesis through the proliferation of hepatocytes (Madden & Slagle 2001), enhancement of cell invasion and metastasis (He et al. 2017), HBV replication (Xu et al. 2002) and integration of the viral genetic material into the human host genome (Bonilla Guerrero & Roberts 2005), thereby all contributing to hepatocarcinogenesis.

1809–1812. Another specific and frequently detected substitution is the double or triple mutations at nucleotides 1809-1812 of the Kozak sequence (Kozak 1986) preceding the precore/core initiation codon. The mutation of ^{1809}GCAC^{1812} to ^{1809}TCAT^{1812} modifies that Kozak region from an optimal to suboptimal translational context (Kimbi et al. 2004), thereby resulting in the impairment of HBeAg expression by a leaky scanning mechanism (Ahn et al. 2003) and subsequently resulting in a more vigorous host immune response. The negative effect of these mutations on the expression of the HBeAg protein may further be enhanced in an additive manner when combined with the presence of the 1762T/1764A mutations (Kramvis & Kew 2007). The precore start codon is located within the BCP region in all genotypes, at co-ordinate 1814, while position 1813 is conserved as a C. A mutation of “T” at 1809 and at 1812 results in amino acid changes from an alanine-to-serine and proline-to-serine substitutes hydrophobic amino acids to that of smaller polar ones. In SSA, the 1809-1812 mutations are observed in patients with acute hepatitis, children, ASC, CH and HIV-

coinfected (Belyhun et al. 2018; Ahn et al. 2003). Tanaka *et al*. have reported a higher prevalence of the 1809-1812 mutations in genotype A than genotype D (Tanaka et al. 2004). More specifically, Kimbi *et al*. (Kimbi et al. 2004) found that these variations are exclusive to subgenotype A1 only, and not in A2 or other genotypes/subgenotypes. Earlier studies that identified 80% HBV strains circulating in SA have a double or triple mutation at 1809, 1811 and/or 1812 (Ahn et al. 2003; Baptista et al. 1999), thus further suggesting that these mutations might represent a natural variation in subgenotype A1 rather than an adaptive mutation (Kramvis 2008). Site-directed mutagenesis and transfection experiments have demonstrated that the expression of HBeAg protein is adversely affected by the $1809^{T}/1811^{T}/1812^{T}$ and $1809^{T}/1811^{C}/1812^{T}$ triple mutations in comparison to the $1809^{T}/1812^{T}$ and $1809^{A}/1812^{T}$ double mutations (Ahn et al. 2003).

G1862T. A transversion from G to T at 1862 that affects codon 17 of the precore protein is common in HCC patients infected with HBV genotype A, particularly subgenotype A1, than in other genotypes (Chachá et al. 2017; Kramvis 2008; Kramvis and Paraskevis 2013). Despite the G1862T mutation is located at the bulge of ε encapsidation signal (which overlaps the *preC* region) (Kramvis et al. 1997), it does not appear to destabilise this stem-loop structure and may instead be a replication competent variant that may survive independent of the wild type strain (Hou et al. 2002). This mutation leads to a substitution of valine for phenylalanine missense mutation, which affects the signal peptide cleavage site (between residue 19 and 20) and results in the retention of the first 19 amino acids of the precore/core protein that otherwise would have been removed during its processing into HBeAg protein in the ER (Hou et al. 2002; Kramvis et al. 1997). By site-directed mutagenesis and transfection experiments, showed inhibited but not abolished HBeAg secretion by introducing valine to phenylalanine missense mutation at residue 17 of the preC region (Hou et al. 2002), which leads to intracellular retention in the ER-Golgi intermediate compartment (Chen et al. 2008; Bhoola and Kramvis 2016; Bhoola and Kramvis 2017). This defect may therefore be responsible for the HBeAg-negative serostatus and lower viremia titers in patients infected with HBV subgenotype A1, in which this mutation occurs most frequently. The prevalence of this mutation varies and may range from 17.5% of HBsAg–positive southern African black carriers (Kramvis et al. 1997) to 82% of subgenotype A1 Brazilian patients who were chronically infected with HBV (Mello et

al. 2014). Moreover, 83% of HBV subgenotype A1 isolates from Eastern India harbored the G1862T mutation irrespective of HBeAg status (Chandra et al. 2007; Gopalakrishnan et al. 2013), further supporting these mutations as characteristic traits of subgenotype A1 (Kramvis 2018).

G1896A. Among the mutations that have been reported, the preC mutation that is frequently associated with inhibited expression of HBeAg at a translational level is the G to A transition at nucleotide 1896 (G1896A), that results in a premature stop codon (TGG→TAG) at the 28th codon (Carman et al. 1989). By virtue of the location of nucleotide 1896 in the stem loop of ε, genotypes harbouring a T nucleotide (genotype B, D, E and part genotype C and F) at the opposite position 1858 foster a stronger Watson-Crick base-pair critical for viral replication (Lok et al. 1994). Thus the more stable G1896A/C1858T double nucleotide mutation has been determined as an independent factor associated with high viral replication (Castelain et al. 2017). On the other hand, the formation of G1896A mutation is precluded in genotype A, which has a C nucleotide as reported in infected patients from SA (Lok et al. 1994; Li et al. 1993; Kramvis et al. 2008), Brazil (Conde et al. 2004) and Spain (Rodriguez-Frias et al. 2006), thus the high prevalence of HBeAg seronegativity associated with genotype A is therefore unlikely attributed to this variation. In general, the consequential reduction in HBeAg level may play a crucial role in HBV replication, and thereby affecting progression of liver disease, particularly in severe fulminant hepatitis (Hayashi et al. 2008) and acute exacerbation of chronic hepatitis B (Suppiah et al. 2015). A recent meta-analysis reported that the G1896A mutation significantly increased risk of developing HCC (summary OR of 2.04, 95% CI: 1.41-2.95) (Wei et al. 2017), with many other studies reporting a similar correlation to HCC disease and HBeAg-negativity in CH patients (Liu, B.-F. Chen, et al. 2006; Zheng et al. 2011; Kim et al. 2012; Jang et al. 2012; Kim, Lee, Do, et al. 2016), but not in acute and self-limited hepatitis B (Omata et al. 1991). In contrast, other studies showed that the G1896A mutation has no appreciable effect on HBV replication capacity (Jammeh et al. 2008) or disease severity (Yoo et al. 2003). A longitudinal study reported higher HBV DNA levels in patients infected with the mutant after seven years of follow-up in comparison to those infected with the wild type variant, but with no consequential liver damage (Besharat et al. 2015). These conflicting results between the association of G1896A

mutation and HCC development may likely be attributed to confounding factors. For instance, in a large prospective cohort study consisting of 89,293 men and women (Yang et al. 2008), Yang *et al*. reported that the G1896A mutation was associated with a lower risk of HCC when compared with the wild type variant, and this negative association was observed when adjusting for confounding factors including sex, age, cigarette smoking status, alcohol consumption status, serum ALT level, and cirrhosis at study entry, as well as HBV genotype, viral load, and BCP mutant, thus suggesting that the mutation may not be directly associated hepatocarcinogenesis. The lack of association of G1896A/C1858T mutation and HCC was also confirmed in a meta-analysis consisting of 11,582 HBV-infected participants, of whom 2801 had HCC (S. Liu et al. 2009).

2.6.2 PreSurface (preS) and surface (S) gene mutants

The detection of naturally occurring or vaccine/antiviral therapy induced mutants with one or more in frame deletions at the *preS/S* region of the HBV genome is not an uncommon event in CH patients. In a recent review by Chen (Chen 2018; Caligiuri et al. 2016), the authors describe the *preS/S* gene the HBV genome as having high heterogeneity, with variants categorised into five groups: 1) preS deletion; 2) preS point mutation; 3) preS1 splice variant; 4) C-terminus S point mutation; and 5) preS/S nonsense mutation. These mutations are invariably associated with changes in the highly variable spacer domain in the P gene that completely overlaps the *preS* region (preS1 and preS2) (Chen et al. 2013). Several of these variants may have important pathobiological and clinical implications, which are increasingly recognised, particularly in the context of HCC development (Hunt et al. 2000; Zhang 2016; Pollicino et al. 2014; Su et al. 2008; Günther et al. 1999; Cao et al. 2014; Chen 2018; Sugauchi et al. 2003; Zhong et al. 1999; Kao et al. 2012; Yeung et al. 2011). In fact, evidence from a previous study shows that preS mutations consecutively increase during the progression of liver disease from the asymptomatic HBV carrier state (10%) to CH patients (20%), patients with liver cirrhosis (35%) or HCC (50%) (S. Liu et al. 2009). Specifically, the preS2 start codon mutations and/or deletions in the 5′-terminal half of the preS2 and the 3′-terminal half of the preS1 region have been commonly implicated in hepatocarcinogenesis (Raimondo et al. 2004; Chen 2018; Gopalakrishnan

et al. 2013; Sugauchi et al. 2003; Chen 2016). The different types of preS mutations in relation to the HBV genotype was investigated in a previous study, and the authors reported that preS1 deletion mutants were common in HBV/D, preS2 start codon mutation and preS2 deletions in HBV/A in HBV/C, respectively (Biswas et al. 2012). The deleted sequences often correspond to viral regions consisting of B or T cells epitopes (Chen 2016), therefore resulting in the production of an antigenically modified surface protein, which compromises the host ability to eliminate the acute infection. These immune escape mutants may confer a selective advantage, which favours the clonal proliferation of hepatocytes with the preS mutants against wild type HBsAg (Kreutz 2002), while also being potentially infectious to immune-protected patients (Perazzo et al. 2015). Despite the decreasing prevalence of HBsAg in children in HBV endemic areas that have implemented the universal hepatitis B vaccination, a study reported that S gene mutations were prominent among vaccinated Taiwanese children with breakthrough HBV infection (Chang 2010). In addition, these variants with preS deletions may be undetectable by the commercially available HBsAg assays (Hossain & Ueda 2017) and they may account for cases of occult HBV infection that is reported around the world.

The proposed mechanism of preS deletion mutants in inducing hepatocarcinogenesis is the imbalance in the synthesis of surface proteins, with the overproduction and intracellular accumulation of the LHBs protein, leading to the activation of ER stress pathways and the consequential oxidative DNA damage, genomic instability, and thus liver disease as shown in Figure 2.7 (Su et al. 2008; Wang et al. 2005; Chen 2018; Li 2016). In this context, the observation that intracellular accumulation of surface protein in the hepatocytes may have a potential growth advantage (Wang et al. 2005) and a direct and deleterious cytopathic effect favouring the progression of the liver disease toward cirrhosis as demonstrated in transgenic mice (Chisari et al. 1987) and *in vitro* experiments (Wang et al. 2012; Bock et al. 1997). The typical histological feature of ground glass hepatocytes (designated types I & II) have been implicated to be a precursor lesion of HCC (Wang et al. 2003; Su et al. 2008; Wang et al. 2012). In addition, truncated preS2 proteins act as transcriptional activators that may exert a tumour promoter-like function, thus enhancing hepatocyte proliferation and tumour development (Hildt 2002).

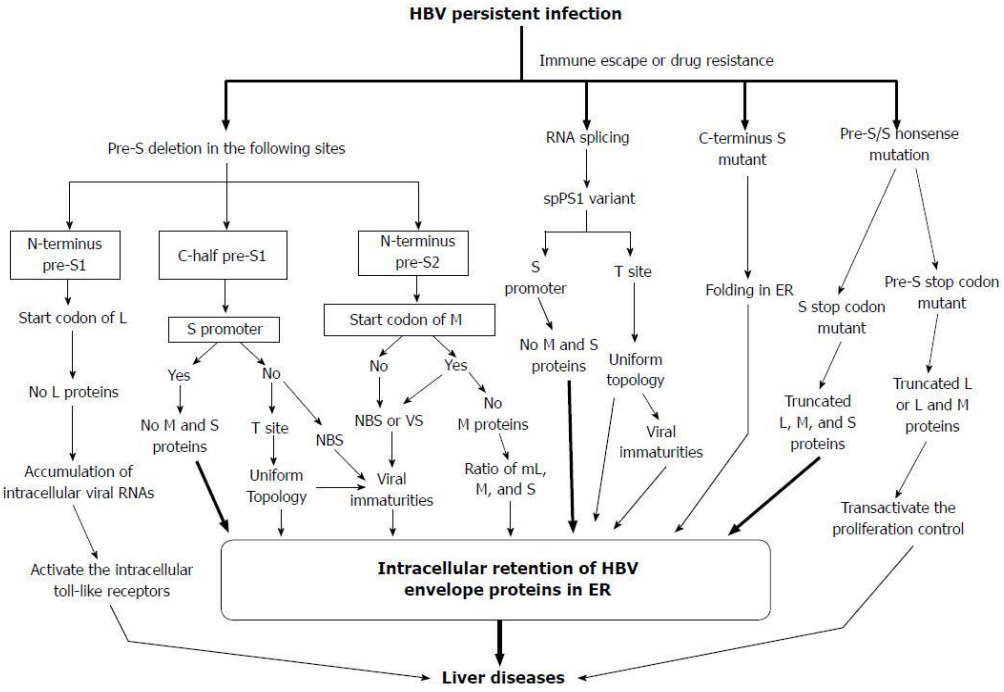

Figure 2.7 Proposed model for the generation of preS/S variants and their possible roles in liver damage and carcinogenesis. HBV: Hepatitis B virus; ER: Endoplasmic reticulum; NBS: Nucleocapsid binding site; VS: Viral secretion. Reprinted with permission from (Chen 2018).

2.7 Pathogenesis of HBV infection

Because HBV replicates profusely and produces high titres in the blood (10^8 to 10^{10} virions/mL) (Elgouhari et al. 2008), HBV spreads from infected individuals to non-infected via various bodily fluids that harbour virus (such as blood, saliva, semen, vaginal secretions, breast milk, sweat, tears and urine) (Kramer et al. 2006; Lavanchy 2004). HBV is reported to be capable of surviving outside the body for at least one week (Kramer et al. 2006), and is estimated that the risk of transmission of this virus from a contaminated needlestick is 1% to 6% if the blood is HBsAg-positive but HBeAg-negative, and much higher, 22% to 40% if positive for both antigens (Elgouhari et al. 2008). HBV can be transmitted either vertically either *in utero* or perinatally from mother-to-infant at the time of birth (Li et al. 2015), or horizontally by non-sexual close bodily contact for children (Heiberg et al. 2010), blood transfusion, body piercing, unsafe injection and sexual contact (Yang et al. 2015; Kramvis 2016). HBV infection is associated with a diverse spectrum of clinical manifestations that can result in both adult

and childhood developing hepatitis B infection that is acute, thus attaining complete immune clearance of virus yielding lifelong immunity. Alternatively, they can progress to chronic hepatitis B, liver cirrhosis and HCC. The outcomes of the viral infection are indicated in Figure 2.8. The natural history of HBV infection can be broadly divided into acute hepatitis B that rarely results in fulminant liver failure, and chronic hepatitis B with HBV persisting for longer than 6 months (Rajoriya et al. 2017).

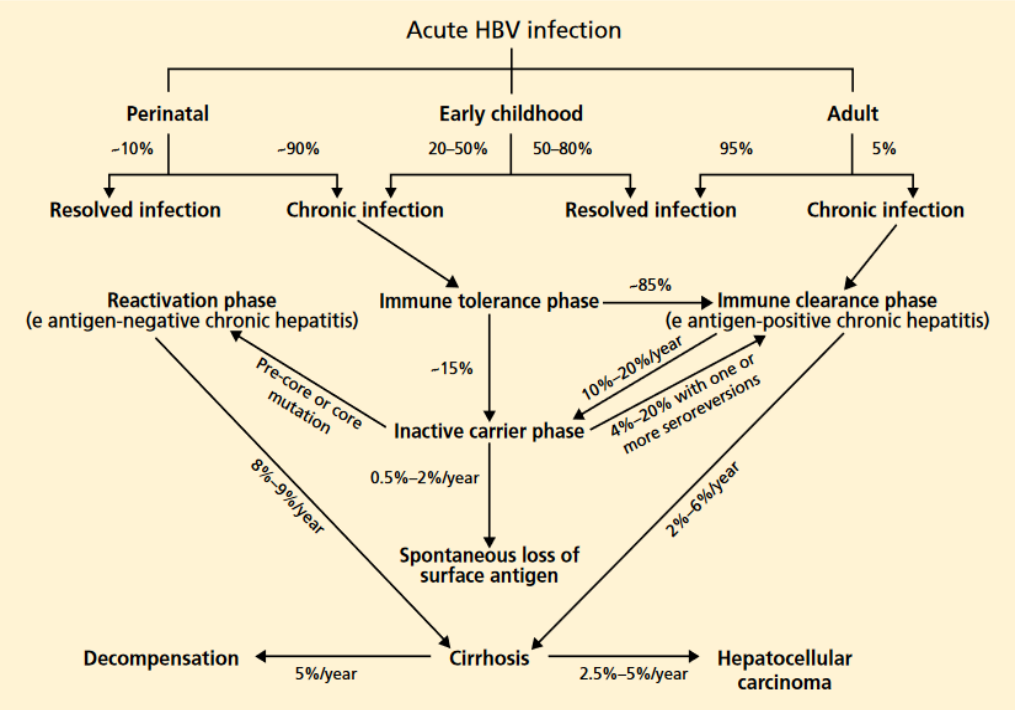

Figure 2.8 Natural history of acute hepatitis B virus infection and the transition to chronic hepatitis and sequelae related to this disease. Reprinted with permission from (Elgouhari et al. 2008).

Exposure to HBV infection sets off a cascade of complex interactions between the host immune system and the virus, leading to various clinical manifestations including asymptomatic carrier state, acute hepatitis, fulminant hepatitis and ultimately chronic disease with progression to cirrhosis and/or HCC. Additionally, confounding factors such as host factors (age, gender, genetics and immune status), viral factors (the size of the inoculum, the virulence of the HBV strain causing the infection and co-infection with other pathogens [HIV/HCV]) as well as environmental factors (alcohol use and exposure to dietary toxins) all influence the natural history of HBV infection (Fattovich et al. 2008).

2.7.1 Acute HBV Infection

Acute hepatitis B infection is generally self-limiting where complete recovery from this infection is almost always certain. However, others can clinically manifest as anicteric (subclinical) hepatitis, icteric hepatitis, or, rarely complete liver failure, termed acute fulminant hepatitis (0.1%-0.5% of patients) (Elgouhari et al. 2008; Kim & Ray Kim 2007). Serum HBsAg becomes detectable after an incubation period of 4 to 10 weeks, but can be as short as six days (Figure 2.9) (Krugman et al. 1979). This is followed by the appearance of anti-HBc, which is predominantly of the immunoglobulin M (IgM) class in the early phase, but declines in titre as levels of IgG anti-HBc rise. However, during the so called window phase laboratory testing may not detect HBsAg because of early clearance and IgM anti-HBc is the only detectable antibody, while HBV DNA may be low or undetected (Lok et al. 2007). A delayed increase in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) occurs because of the time required for specific cytotoxic T lymphocyte responses to develop against the infected hepatocytes (Pungpapong et al. 2007). HBV DNA levels typically reach very high levels (from 200 million IU/mL to 200 billion IU/mL or 10^{9}–10^{12} copies/mL) (Ribeiro et al. 2002), which is accompanied by a high HBsAg titre, and clinical symptoms of hepatitis such as jaundice may appear. This high level of HBV replication and infectivity subsequently leads to the presence of a third serological marker, HBeAg, which is detectable in most patients with acute HBV infection (Liang et al. 2002).

In self-limited cases, HBeAg levels is usually cleared early at the peak of clinical illness, after the characteristic peak in serum ALT activity. This coincides with the presence of anti-HBe and a period where HBsAg and HBV DNA persist in the serum for the duration of clinical symptoms (Yotsuyanagi et al. 1998), but cleared with recovery (Krugman et al. 1979). Thus, the loss of HBeAg and appearance of anti-HBe is a favourable event during acute infection as this indicates recovery. Antibody to HBsAg (Anti-HBs) arises late during the course of infection (approximately 32 weeks), usually during recovery or convalescence after HBsAg clearance, and may remain detectable after recovery for a decade as the protective antibody against HBV (Krugman et al. 1979). However, an estimated 10%-15% of recovered patients may only reveal anti-HBc-positivity alone, suggesting past infection. In contrast, approximately 5%–10%

experience ongoing infection of the virus and thus progress to a chronic carrier state (Liang 2009).

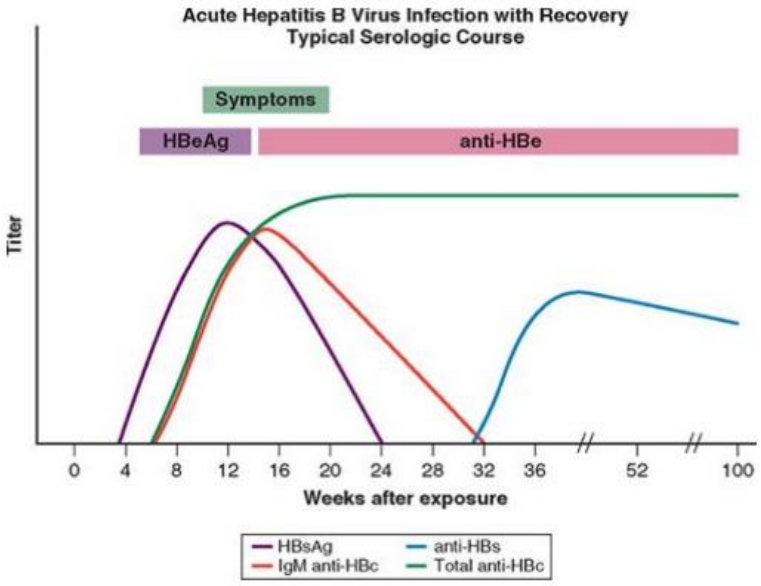

Figure 2.9 Graphical representation of the typical serologic course during an acute HBV infection to chronic hepatitis B (http://virology-online.com/viruses/HepatitisB.htm, accessed on 11 April, 2018).

2.7.2 Chronic HBV Infection

Chronic HBV infection is defined as the persistence of HBsAg in the sera for longer than six months (Lok et al. 2007), which follows after the failure of the host immune system to effectively clear an acute HBV infection. Unlike acute infection, chronic hepatitis has a variable and dynamic course, so much so that studies over the last few decades have tried to classify the phases into three (Raimondo et al. 2003) and four phases (McMahon 2004; Azmi 2014). In a report by Gish *et al* (Gish et al. 2015), the natural course of chronic hepatitis B was classified into five different phases: 1) an initial high replicative, low inflammatory state (HBeAg-positive, high HBV DNA, low to normal aminotransferases and minimal necro-inflammatory activity); 2) immune clearance phase (fluctuating HBV DNA and aminotransferases levels, and inflammation); 3) HBeAg negative chronic hepatitis phase (moderate to high HBV DNA levels and persistent necroinflammation leading to progressive liver disease); 4)

non-replicative phase (low or undetectable serum HBV DNA, HBeAg negativity, and normal aminotransferases levels); 5) HBsAg loss/occult phase (HBsAg -negative but intrahepatic persistence of entire, episomalv cccDNA chromatinized episomes, HBV DNA detectable in the liver and phases of undetectable and very low levels in serum) (Gish et al. 2015). The outcome of chronic infection varies, with the progression through these phases involving sequelae of the liver that can be fatal including hepatic fibrosis, cirrhosis, fulminant hepatitis, and HCC (Chen 1993).

Generally, the chronicity rate is highest in individuals infected at birth (90%), but falls to less than 5% in individuals infected at adulthood (Elgouhari et al. 2008). Immunocompromised populations, such as those infected with HIV, have a higher risk of developing chronic HBV. Initially, these individuals display similar serological patterns as acute hepatitis (HBV DNA, HBsAg, HBeAg, and anti-HBc in Figure 2.10) but at higher titres, with evidence of mild to moderate elevations in serum aminotransferase levels (Liang 2009; Yotsuyanagi et al. 1998). HBeAg persistence for more than three months is an indication that the infection is likely to become chronic (Pollicino et al. 1996), and may last for an extensive period of time (years) before seroconversion to anti-HBe occurs - which is linked to diminishing viral replication and improved liver disease (Liaw 2009). The damage done to the liver from ongoing inflammatory responses may vary between individuals depending on both host-specific (age, gender, genetic, immune status) and viral factors (HBV DNA levels, HBV genotype, HBV mutations), with a third of chronically infected individuals developing sequelae such as cirrhosis, end-stage liver disease, or HCC (Liang 2009).

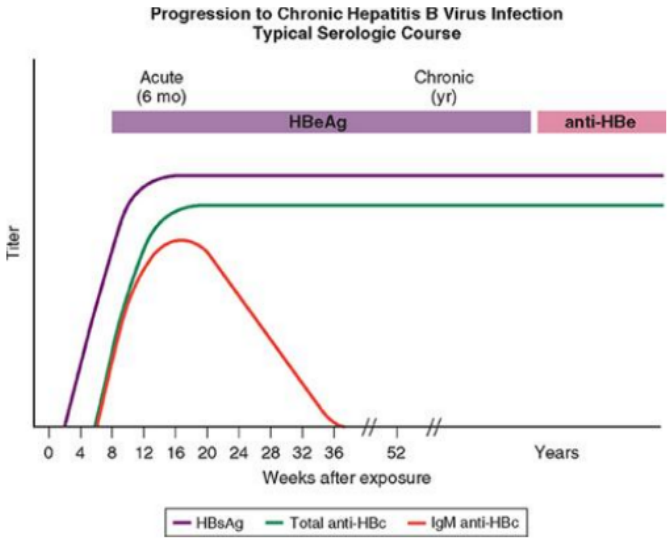

Figure 2.10 Graphical representation of the typical serologic course during the progression from acute HBV infection to chronic hepatitis B (http://virology-online.com/viruses/HepatitisB.htm, accessed on 11 April, 2018).

2.8 Occult HBV Infection

The detection of HBV is routinely carried out using the relatively inexpensive and easy to implement enzyme-linked immunosorbent assay (ELISA) test for HBsAg. The consequential reduction in the spread of the infection among blood donors is one of the many beneficial impacts of these tests when carried out nationwide, however, ELISA fails to prevent HBV transmission via blood components negative for HBsAg (Liu, Chen, et al. 2006). In the absence of HBsAg, low HBV DNA concentrations of less than 200 IU/mL have reportedly been detected in the serum and liver tissue biopsies by real-time polymerase chain reaction (PCR) and this variant form of HBV infection is termed occult HBV infection (OBI) (De Mitri et al. 2010). One of the explanations for the low or undetectable levels of serum HBV DNA in OBI include the strong suppression of viral replication and gene expression by antiviral cytotoxic T cells (Bes et al. 2012). Others have proposed the persistence of competent wild type HBV with low level replication rate may be possible (without any mutations in the precore/core promoter regions) (la Fuente et al. 2011).

On the other hand, mutant HBV variants characterised by mutations in the S gene may either impair the synthesis of viral proteins or express a modified HBsAg proteins that are no longer recognised by the commercially available ELISA assays (Makvandi 2016). It is also suggested that co-infection with other pathogens such as HCV may assist in down-regulation of viral replication, therefore resulting in OBI (Rodríguez-Iñigo et al. 2005). OBI is a complex variant of HBV infection in that it is comprised of many clinical conditions and different situations (Raimondo et al. 2013).

The persistence of coinfection of OBI with either HCV or HIV may favour or accelerate the progression towards fulminant hepatitis and HCC (Mphahlele et al. 2006; Branco et al. 2007). Evidence from a meta-analysis showed increased risk of chronic liver disease was 4-fold (95% CI: 1.7–9.0) (Covolo et al. 2013), while another showed that the risk of HCC increased by 3-fold (95% CI: 1.6–4.1) (Branco et al. 2007; Shi et al. 2012). It is generally believed that OBI maintains most of the pro-oncogenic properties that subtend HCC development as in HBsAg-positive (overt) patients (Raimondo et al. 2013). The residual risk of HBV transmission is mainly related to orthotopic liver transplant or blood product transfusion from donors negative for HBsAg, but with low level HBV DNA viremia. Additionally, OBI may also carry the potential risk of HBV transmission in individuals with impaired host defence systems including haemodialysis, cancer chemotherapy, HIV infection, hematological malignancies and treatment with potent immunosuppressive drugs. In these individuals, the virus, which persists by maintaining the cccDNA reservoir intracellularly, can evade the host immunity and can reactivate at a later stage (Kwak & Lim 2017; C.-J. Liu et al. 2013).

2.9 Aims and Objectives

It should be noted that a different study was conducted with an overall aim of:

- Retrospectively describing the occurrence of viral (HBV, HCV and/or HCV infection) and non-viral (socio-demographics and lifestyle behaviours) risk factors that are associated with HCC, in treatment-naïve black African patients attending public hospitals in Johannesburg, SA.

Furthermore, the main objectives of this study was to:

- Identify the socio-demographic lifestyle-related risk factors that are associated with HCC development in black African patients attending public hospitals in Johannesburg, SA.
- Determine the sero-prevalence of HBV/HCV/HIV mono- and co-infection with regards to HCC aetiology.
- Test whether there is an additive or multiplicative association with HIV infection and the presence or absence of HBV/ HCV co-infection in relation to the risk of HCC.
- Determine the risk of HBV, HCV and/or HIV for HCC development after controlling for other known risk factors such as age, gender, alcohol consumption and tobacco smoking.
- Determine the HBV genotypes and mutations in patients with HBV-related HCC.

This study was divided in Chapter 5 and Chapter 6 using data and serum samples collected from the Johannesburg Cancer Case Control Study (JCS) in which patients newly diagnosed with neoplasms were recruited prior to treatment from the oncology units in the greater Johannesburg public referral hospitals that offer cancer treatments to cancer patients, in SA.

— CHAPTER 3 —

Trends in Incidence Rates of Liver Cancer in South Africa

Given the continued growth in the number of persons with cancer in the world, the primary prevention of this disease remains an urgent public health priority. In particular, as the field of LC prevention continues to mature and scientific knowledge evolves, it is becoming more imperative to evaluate changes in the population demographics in order to define prevention strategies accurately. For instance, advancing age is one of the most important risk factor for LC overall. However, equally important is that many people commonly underestimate or overlook age as a risk for LC, and thus, are less likely to be aware of symptoms and are more likely to be diagnosed in the advanced stages. Unlike other southern African countries, SA is unique in that it is a low- and middle-income country with the highest population of people living with HIV/AIDS in the world. While LC is considered a non-AIDS defining cancer. the consequences of HIV infection are both dire and complex in that it intersects and interacts with various social and behaviours changes (Kabudula et al. 2017), ultimately exerting an invisible force that drives a change in the population demographics. As such, studying LC among a population largely influenced by HIV infection offers an opportunity to understand the extent to which this malignancy may differ to that observed in other nations with lower to negligible HIV prevalence. Furthermore, this chapter provides for a comprehensive assessment of trends in LC diagnosed in SA over a period of two decades that overlaps the period of pre- and post- antiretroviral therapy (ART) introduction, and how these patterns can be viewed from the perspective of a dynamic population demographics.

3.1 Abstract

Background: Exposure to risk factors for LC are specific to subgroups within a population, and thus studies these subgroups in SA may provide valuable insight for prevention and treatment strategies in a region where LC is documented to be of lower prevalence. Furthermore, LC incidence trends from 1993-2012 were determined.

Methods: LC incidence data were obtained from the SA NCR database. The ASIR for LC were reported in the present study, and the trends and age-period-cohort effects in the ASIR for LC were evaluated using regression analysis methods.

Results: Over the 20-year period, there were 7148 confirmed LC cases, with the tumour presenting approximately 2.4 times more frequently among men than in women (5021 *versus* 2127, respectively). The highest proportion of cases were reported in the black African population (75%), who were approximately 10 years younger than the white population, who accounted for 16.8% of reported cases. HCC was the most frequently diagnosed LC histological subtype (59%). Overall LC incidence rates decreased significantly between 1997-2003/4 (Annual percentage change (APC) -15.2% and -12.9% in men and women, respectively, $p<0.05$). However, despite the observed stable trends in the most recent years in black Africans, non-significant increasing rates were found among older black men (70-79 years) and middle-age black women (40-59 years). Age-period-cohort analysis indicated a population-group disparity, with younger black African men at increased risk of LC (30-34 years). Using the specific results of Wald tests, we found statistically significant period effects for both sex and population groups.

Conclusion: Decreasing LC incidence rates among black African and white population groups, and the non-significant increase in LC incidence rates in recent years may suggest that the nadir of the disease may be near or may have passed. Findings of age and population group variations in LC burden can inform control efforts, while more extensive investigations of the underlying aetiological risk factors are needed to provide targeted interventions for the different sex- and population-subgroups.

3.2 Introduction

Globally, primary LC is a major public health concern in developed and developing nations as both share similar fatality rate ratios (incidence-to-mortality) in both men (1.21 vs. 1.05) and women (1.08 vs. 1.03) (Wong et al. 2017). Despite this, the epidemiology of this disease is constantly in flux and the degree at which this occurs may vary considerably between different geographical regions. Worldwide LC incident cases have increased by 75% from 1990 to 2015 (Global Burden of Disease Liver Cancer Collaboration 2017). While the reasons are manifold, changes in the prevalence of risk factors and population demographics play a major role in the disease pattern.

In the United States, the noteworthy increase in the rate of LC is more rapid in African Americans (Average Annual Percentage Change [AAPC] of 5.8 and 2.6 in men and women, respectively) than in White Americans (3.8 and 1.4 respectively) (Wong et al. 2017). Previous studies comparing racial differences between survival and treatment choices among individuals with LC found, apart from the frequent presentation of elevated AFP and cirrhosis in white patients, black patients had earlier age of onset, present with late stage/with metastatic disease, larger tumour size, are less likely to be screened and to receive treatment for LC (Xu et al. 2016; Stewart et al. 2016; Davila et al. 2010). In turn, perhaps not surprisingly, these differences in late stage disease presentation mean that many patients with LC are no longer amenable to curative treatment. Even if treatment was available, the overall 5-year HCC survival rate is lowest among African Americans (21.3%, 95% CI: 19.5%-23.1%) (Ha et al. 2016). These findings together with the more rapid increase in incidence seen in African Americans (Wang et al. 2016) translates into a widening disparity in LC disease burden between African and White Americans.

Although both sub-Saharan Africa and East Asia are the most affected regions of the world owing to the high prevalence of risk factors for LC in these continents, data on this disease is by far more published and available in the latter region than the former (Sankaranarayanan & Swaminathan 2011). Moreover, from the available data, it is evident that the survival rate of people with LC in sub-Saharan Africa regions is generally poor, and much lower than that in East Asia and even lower than in developed countries (Xu et al. 2016; Allemani et al. 2018; Sankaranarayanan & Swaminathan

2011). The median survival rate of LC patients in North Africa is significantly higher than in sub-Saharan Africa, being 10.9 (95% CI: 9.6–12.0] months in Egypt compared to 2.5 (95% CI: 2.0–3.1) months in other African countries ($p<0.0001$) (Yang et al. 2017). The 5-year survival rate of patients with LC in Uganda and Gambia is 3.2% (Gondos et al. 2005) and 3% (Bah et al. 2011) respectively, much lower than 9% in Denmark (Montomoli et al. 2011) and 14.8% (whites: 14.3% and blacks: 11.3%) in the United States (Momin et al. 2017). In SA, the mean survival period of rural blacks is only 6 weeks from diagnosis and 11.2 weeks from the onset of symptoms (Kew 1981). Furthermore, since most cases of LC are only presented during the advanced stages of the disease, resectability is only at 1%-2% and remission or prolongation of survival are seldom achieved (Kew 1981). In addition to the above, inefficiencies in prevention and public health intervention, diagnostic challenges and poor treatment of HCC in West Africa (Ladep et al. 2014) and sub-Saharan Africa (Kew 2012), all suggest that LC in sub-Saharan Africa is of deserving of an in depth understanding.

Despite SA being ranked as an upper-middle income nation that has one of the largest economies and the highest total health expenditure per capita in sub-Saharan Africa (Dieleman et al. 2016), many black South Africans are subject to economic disparity compared to other population groups (Berg & Louw 2005). Furthermore, the demographic is shifting as many of these individuals are aging in significantly high proportions in comparison to the past (Lehohla 2014).

In addition, with 7.03 million individuals infected with HIV in 2016, the total HIV prevalence of 12.7% in SA is amongst the highest in the world. Moreover, the impact of this infection is felt predominantly by the black African population group who represent more than 80% of total population (StatsSA 2017). Despite this, it is positively noted that HIV-related diseases have decreased following the massive roll-out of ARTs nationwide between 2004 and 2005 (Reniers et al. 2017). Even though similar ASIRs for LC in developed regions have been reported to those of developed regions (6.5 and 3.5 per 100,000 person-years in men and women respectively) (Ferlay et al. 2015), it is believed that incidence cases in the former region are grossly underestimated (Kew 2011). More specifically, this underestimation of LC incidence rates primarily stems from a high degree of under diagnosis of cases due to the inaccessibility to and

unavailability of healthcare services and diagnostic facilities. While cancer registration of the population remains uncommon in sub-Saharan Africa (Gakunga et al. 2015), SA has the benefit of reporting high-quality incidence data due to an existing pathology-based cancer registry at the national level, as well as a reasonably well-developed program for the collection of vital statistics (StatsSA).

Thus. the present study examines the variation in LC incidence by age and population group in SA over a period of two decades from 1993 to 2012. There are several reasons for investigating this issue. Firstly, there are known differences between population groups in SA in terms of patterns of exposure to LC-related risk factors and detection, and although these differences are anecdotally recognised, no study has yet shown this in SA. Moreover, demographic patterns and the epidemiology of risk factors are changing over time and they do so differentially between socioeconomic and ethnic groups. We would therefore expect that population group patterns in the LC burden may also be changing over time. These differences are likely to vary depending on the age, as they do between population groups in other countries. In addition, patterns of LC incidence between population groups based on risk factor distribution may not always be clear, and thus suggest that there may be an alternative aetiological explanation for the differences observed. There is value for data collection and analyses in this region because public health programs and policies are not as advanced and adequately implemented as those seen in developed countries. Moreover, information in sub-Saharan Africa can also be useful for healthcare clinicians to understand community needs for care and aid in identifying LC hot spots that need more investigation to understand the root causes of disease.

At present, no studies in sub-Saharan Africa have examined recent trends using age-period-cohort analysis, which has previously been reported to be more useful than conventional cross-sectional analysis in evaluating trends (Rosenberg & Anderson 2011). The present analysis allows for the investigation of the effects of three factors, namely age, period and cohort. The age effects represent the differing risks associated with each age bracket; the period effects are usually generated by factors that affect all ages concurrently, such as improvements in diagnostic practice and treatment; the cohort effects are attributed to risk factors that vary in prevalence across groups of

individuals with the same birth years—that is, for successive age groups in successive time periods, such as the inclusion of the HBV vaccine in the EPI in 1995 in SA (Burnett et al. 2012).

The current analysis and the research results not only lay the foundation for discussing the relationship between LC burden between the different population groups in SA, but also can assist in the evaluation of the specific risk factors that may influence the development of this cancer. Furthermore, the research results can provide a scientific reference in the drawing up of national cancer prevention strategies.

Thus, the aims of this study are:

1. To describe the LC incidence trends in the different SA population groups by sex;
2. To examine cohort and period effects by sex and population group using age-period-cohort modelling.
3. To compare the LC incidence with the national HIV prevalence rates in the black African population.

3.3 Methods and Materials

3.3.1 Population

Following the first comprehensive national census conducted in 1996 after the fall of apartheid, the declared total number of resident citizens in SA was 40.5 million (Lestrade-Jefferis et al. 2000). The 2011 Census was carried out in October 2011 and showed that the population had climbed to over 50.5 million people (StatsSA 2012) (Figure 3.1). That represents a rise of nearly ten million people over the course of the sixteen years, with notable increases occurring in age groups below 40 years and a decrease in those aged 80 years and older (Figure 3.1). According to the census, five separate population groups were defined: Black African, White, Coloured (mixed ancestry) and Indian/Asian, each of which accounted for 79.2%, 8.9%, 8.9% and 2.5% of the total SA population, respectively (StatsSA 2012). In SA where the median age is intermediate (25 years), the age structure largely differs between population groups: 24

years for the black African population, 27 years for the coloured population, 32 years for the Indian/Asian population, and 39 years for the white population (Lehohla 2015). In SA, there was a dramatic decrease in life expectancy at birth in SA from 57 years in 1996 (StatsSA 2001) to 53.5 years in 2005 and 2006 (StatsSA 2017). By 2012, the life expectancy was at 61,1 years.

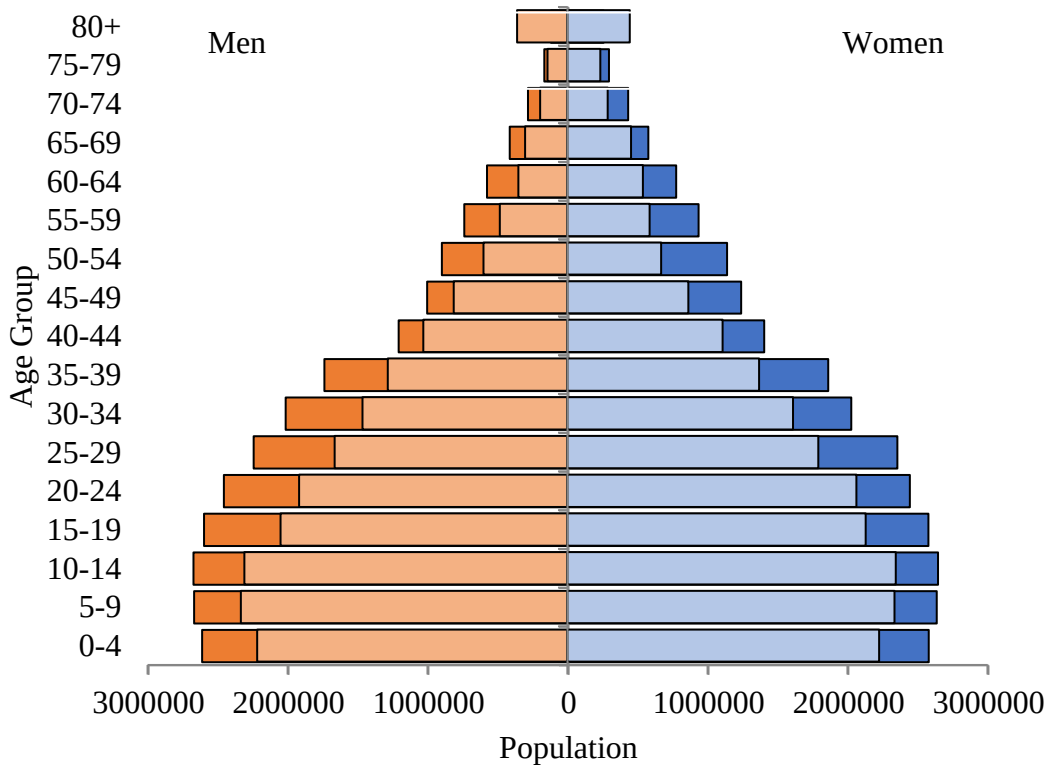

Figure 3.1 South African population for 2011 (Outer, darker shade) and 1996 (Inner, lighter shade) compiled using data from (StatsSA 2012).

3.3.1 Data sources and selection criteria

Cancer incidence data during 1993–2012 were obtained from the SA NCR, which is a pathology-based cancer surveillance system that passively records and collects pathology reports verified through laboratory tests (microscopy, haematology, histology, and cytology) from a total of 84 private and public laboratories throughout the country. Briefly, the NCR is a division of the National Health Laboratory Service and is the primary cancer surveillance system with the most extensive repository of cancer data in SA. In 1986, the NCR was established as a voluntary, pathology-based

cancer surveillance system. Moreover, the NCR database contains over 1,2 million cancer records with approximately 80,000 new cases reported annually. The passing of the SA legislation (National Health Act-Regulations relating to cancer registration, 2012 Act No. 61 of 2006 No. R. 380) enables the NCR to extend their registry to include a population-based system (Kielkowski et al. 2012). Of note, the NCR reports covering 1993 to 1995 include summarised data for the 3-year period, however, the number of observed cases were given per year. Although, the NCR reports covering 1996 and 1997 has the coloured and white population groups pooled together, the NCR was able to provide the number of cases reported for each group separately (Appendix A).

LC incidence was defined by International Classification of Diseases for Oncology (ICD-O), Third Edition using topography and morphology codes listed in these reports consists of laboratory results and interviews in a representative cross-section of the population, including all ages, sex, population groups, locality types and provinces. Furthermore, it covers the epidemiology of HIV from socio-behavioural and structural aspects that contribute to the spread of this virus in the population as well as providing statistical data on the ART exposure.

Table 3.1. Additional details sourced directly from the NCR database include the age of diagnosis, sex and population group. Given the period of political, economic and social challenges experienced in SA under the Apartheid regime that ended in 1994, the healthcare delivery system was adversely affected with most government central referral hospitals operating below capacity, and key information of the general population was not optimally gathered. For this reason the present study analysed all new cases with LC reported to SA NCR from 1993 until at the time of writing the latest publication which was for the year 2012 (up to 2014 is now available).

The South African population demographics were provided by StatsSA. Briefly, StatsSA is the national statistical service of SA, that publishes approximately 300 different statistical releases each year covering various national economic, population and social statistics. StatsSA captures, processes, assesses, analyses and disseminates the most comprehensive statistical reports and datasets on mortality and causes of death in SA, based on administrative records from death notification forms collected biweekly from the South African Department of Home Affairs. The completeness level of

reporting total adult deaths (15 years and older) is estimated at more than 95% for the 2011–2016 intercensal/survey period.

Information about the HIV prevalence were derived from the SA National HIV Prevalence data that is available for 2008 (Shisana et al. 2009) and 2012 (Shisana et al. 2014), by sex. These reports consists on laboratory results and interviews in a representative cross-section of the population, including all ages, sex, population groups, locality types and provinces. Furthermore, it covers the epidemiology of HIV from socio-behavioural and structural aspects that contribute to the spread of this virus in the population as well as providing statistical data on the ART exposure.

Table 3.1 ICD codes used to identify and group liver, gallbladder and biliary tract cancers.

Definition	ICD10	Abbreviation
Subsites		
Liver, gallbladder and biliary tract	C22-C24	Total
Liver cell cancer	C22.0, C22.2-C22.7	LCC
Intrahepatic bile ducts	C22.1	IHBD
Gallbladder	C23	GB
Extrahepatic bile ducts	C24.0	EHBD
Other parts of the biliary tract	C24.1-C24.9	OPBT
Definition	ICD-O	Abbreviation
Histological types		
Hepatocellular carcinoma	M817	HCC
Adenocarcinoma	M814	
Neoplasm NOS+others	M800 and other codes not specified	
Cholangiocarcinoma	M816	CCA
Carcinoma NOS	M801	

ICD, International Classification of Diseases; NOS, not otherwise specified.

3.3.2 Statistical Analysis

3.3.2.1 Demographics

Continuous variable such as the age at which LC was diagnosed was summarised as means ± standard deviation (SD). Furthermore, the age-specific LC incidence rates (ASpIR) was used to provide a general overview of the number LC diagnosed in each age group (0-4, 5-9, 10-14, 15-19, … 75-79, 85+) and sex in SA,

3.3.2.2 Joinpoint Analysis

Joinpoint regression was used to evaluate the trends of LC ASIR by population group and sex using the Joinpoint regression Software (Version 4.5.0.1) (Statistical Research & Applications Branch, National Cancer Institute, Bethesda, MD, USA). This was expressed as the annual percentage changes (APCs) and their 95% CIs for each period. Average annual percentage change (AAPC) was computed as a geometric weighted average of APCs to summarise the trend over the entire observation. The detection of patterns (i.e trends) in these ASIR permits the identification of the direction in which LC is developing or changing.

Since the occurrence of LC in black Africans below 15 years old and whites below 30 years old is very rare, and evaluations on individuals over 80 involve deaths from other competing causes, only rates for those between 15 and 79 years old were considered in this study. Additionally, the ASIR data for years 1993-1995 and 1996-1997 was pooled together and cannot be reported separately, they were therefore combined and reflect the average ASIR for these periods. The years in which the coloured and white populations were also pooled together were omitted from the joinpoint analysis.

3.3.2.3 Age-period-cohort analysis

Age-period-cohort regression for each sex and population group was done to better understand the effects of age, period and cohort on the observed temporal trends by year of diagnosis, using the online Age Period Cohort Analysis Web Tool developed by the US NCI (Rosenberg et al. 2014). The web tool provides useful estimable parameters including longitudinal age curves from the observed cohort-specific age-specific rates and cross-sectional age trend (age trend − period trend). In addition “local drifts” can be

generated from log-linear regressions. The central age group, period, and birth cohort are often defined as the reference, respectively. Since only 5-year age groups are available, the same period interval was used to group calendar periods, birth cohorts and population data. Thus, for these models, LC incidence count was extracted from the SA NCR and the population data from the StatsSA database, respectively. The input data were classified into 23 five-year age groups from 15-79 years, four quinquennial time calendar periods (1993-1997, 1998-2002, 2003-2007 and 2008-2012), and 16 consecutive cohorts including those born in 1918–1922 and aged 75–79 years in 1993; to those born in 1997–2001 and aged 15–19 in 2012.

The age-specific incidence models (Joinpoint, age-period-cohort) did not include 0-4, 5-9, 10-14 age groups, as well as Indian/Asian and Coloured populations, owing to the low number of cases in these groups. By removing these groups where the risk of being diagnosed with LC is very low, this reduced the negative impact on model fitting. Data from those aged 80 years and older were also not included as this was an open-ended age group and incidence data were not available within five-year age brackets. This study was performed with the approval from the Medical Ethics Committee of University of Witwatersrand and in compliance with the Helsinki Declaration. No information that identifies individual subjects was included in the study.

3.4 Results

Over the 20-year time period (1993 to 2012), a total of 9685 pathologically identified primary liver and biliary tract cancers were reported by the SA NCR. Of this total, 7148 (72.5%) were confirmed LCs, with the tumour occurring approximately 2.4 times more frequently among men (5021) than women (2127). In 1993, 741 new cases of LC were reported and two decades later, there were 302 new cases (2012).

In SA from 1993 to 2012, the mean age and SD of all men and women at diagnosis is 50.4 (18.2) and 51.7 (18.9) years, respectively (Figure 3.2). The mean age was highest in the white population group (men: 59 [17.6]; women: 57.5 [19.4]) and the lowest in the black African population group (men: 48.5 [17.5]; women: 50.2 [18.8]). Notably, only men from the white population group had higher mean age compared to women. In contrast, men from other population groups all had lower mean age compared to women

in the same population group (Figure 3.2), although these differences were not significant. The highest proportion of cases were reported in the black African population (5363, 75%), who were approximately 10 years younger than their white counterparts (Figure 3.2), which accounted for 16.8% of the reported cases (1204) (Figure 3.3). Both the coloured (428; 6%) and Indian/Asian (153; 2.1%) populations each had fewer than a hundred cases reported each year (Figure 3.3).

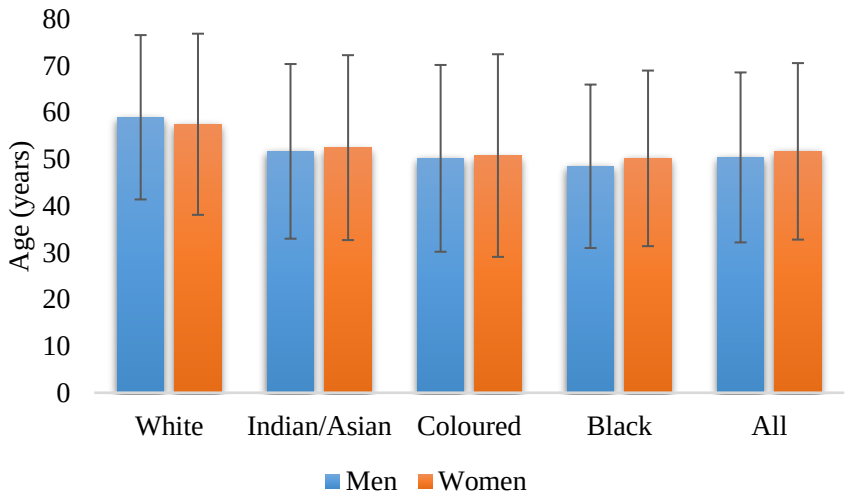

Figure 3.2 Mean age of cancer cases reported to the South African pathology-based National Cancer Registry (NCR) from 1993 to 2012 in each population group, by sex.

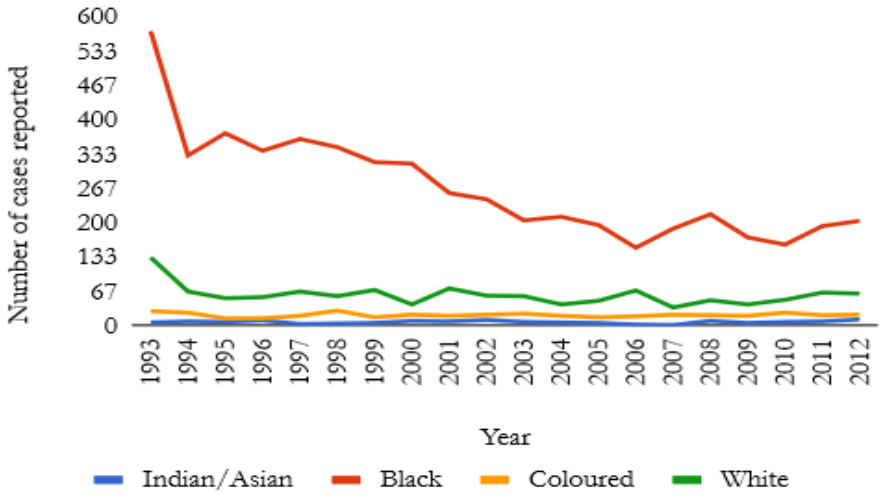

Figure 3.3 Number of liver cancer cases reported to the South African pathology based National Cancer Registry (NCR) from 1993 to 2012 by the population groups; white, black, coloured, and Asian/Indian. Years 1996-1997 were pooled together for coloured

and whites in the published report but NCR provided the number of cases per year (Appendix Table A).

Table 3.2 and Table 3.3 provides for the summary statistics and overall LC ASIR per 100,000 for men and women by population groups, respectively. The overall ASIR for LC in men decreased from 3.86 to 1.7 and in women from 1.81 to 0.9 between 1993-1995 and 2012. In the most recent year (2012), the highest incidence rates from LC were from men and women from the white population group(2.4 and 1.4, respectively). Since 1998 and 2001, the ASIR in the white population have consistently been the highest compared to other populations in both sexes.

Table 3.2 South African National Cancer Registry summary statistics for liver cancer in men from 1993-2012, by population group.

	Lifetime risk (0-74)(LR)					Age standardised rates (ASR)					Percentage of all cancers (%)				
Year	All	Black	White	Coloured	Asian	All	Black	White	Coloured	Asian	All	Black	White	Coloured	Asian
*1993-95	217	227	496	476	323	3.86	3.88	4.03	1.91	2.53	NR	NR	NR	NR	NR
1996	223	205	NR	NR	179	3.77	4.34	2.37†		4.21	1.29	4.11	NR	NR	2.56
1997	282	239	417	417	345	3.18	3.68	2.22†		2.2	0.92	3.54	NR	NR	1.12
1998	286	271	385	435	435	2.8	3.11	1.93	2.03	1.5	0.84	3.04	0.59	0.42	1.04
1999	369	345	455	770	371	2.34	2.57	1.91	1.06	1.46	0.71	2.84	0.36	0.45	0.92
2000	375	360	465	371	396	2.39	2.55	1.74	2.26	2.4	0.73	2.99	0.39	0.55	1.77
2001	430	477	324	495	403	2.03	1.88	2.62	1.98	1.6	0.6	2.14	0.3	0.3	0.98
2002	464	502	396	612	273	1.85	1.79	2.02	1.43	2.92	0.6	2.13	0.38	0.48	2.21
2003	559	627	422	560	651	1.5	1.37	1.99	1.49	1.31	0.53	1.9	0.29	0.46	1.22
2004	611	579	737	538	2128	1.4	1.52	0.98	1.57	0.54	0.54	2.04	0.39	0.48	0.52
2005	584	601	548	523	536	1.43	1.4	1.64	1.35	1.39	0.59	1.83	0.33	0.41	1.3
2006	685	798	482	584	743	1.32	1.19	1.85	1.39	1.07	0.53	1.55	0.34	0.56	0.79
2007	623	631	567	663	878	1.33	1.35	1.41	1.08	0.64	0.62	1.72	0.41	0.63	0.48
2008	528	523	525	588	1091	1.57	1.64	1.43	1.34	1.04	0.59	1.9	0.37	0.37	0.9
2009	701	809	522	536	1135	1.31	1.27	1.75	1.31	0.65	0.59	1.57	0.32	0.59	0.55
2010	638	655	575	767	590	1.43	1.35	1.59	1.42	1.87	0.99	1.72	0.42	0.77	1.32
2011	593	714	427	540	626	1.56	1.29	2.13	1.71	1.4	0.82	1.59	0.41	0.65	1.25
2012	561	600	434	729	504	1.7	1.5	2.35	1.25	1.92	0.8	1.58	0.4	0.46	1.26

*Summary statistics for the years 1993–1995 (3 years) together

†1996-1997: White and coloured population groups were pooled together

Asian is the Asian/Indian population group

NR: not reported

Table 3.3. South African National Cancer Registry summary statistics for liver cancer in women from 1993-2012, by population group.

	Lifetime risk (0-74)(LR)					Age standardised rates (ASR)					Proportion (%)				
Year	All	Black	White	Coloured	Asian	All	Black	White	Coloured	Asian	All	Black	White	Coloured	Asian
*1993-95	455	313	333	1000	476	1.81	2.56	2.5	0.85	1.56	NR	NR	NR	NR	NR
1996	435	401	NR	NR	152	2.07	2.24	1.33†		6.11	1.95	2.04	NR	NR	3.33
1997	503	477	NR	NR	910	1.69	1.8	1.44†		1.42	1.55	1.54	NR	NR	0.64
1998	508	527	401	770	1251	1.53	1.43	2.1	0.85	0.87	1.4	1.16	0.35	0.96	0.48
1999	695	667	667	1429	5001	1.23	1.26	1.33	0.85	0.61	1.22	1.01	0.33	0.57	0.4
2000	821	915	635	862	535	1.1	1.01	1.35	1.08	1.07	1.3	1.13	0.34	0.88	0.7
2001	912	939	800	1937	433	0.96	0.91	1.14	0.67	1.75	1.03	0.95	0.44	0.81	1.06
2002	1062	1304	623	1251	1205	0.89	0.75	1.3	0.92	0.84	1.01	0.84	0.39	0.56	0.52
2003	1211	1499	771	1076	1193	0.74	0.64	1.02	0.85	0.81	0.87	0.8	0.36	0.58	0.68
2004	1016	1289	660	703	822	0.83	0.65	1.38	1.09	1.01	0.82	0.68	0.19	0.59	0.7
2005	1199	1354	825	1199	1577	0.64	0.71	1.01	0.78	0.71	0.9	0.85	0.35	0.6	0.56
2006	1211	1239	933	1503	1311	0.74	0.66	1.12	0.79	0.79	0.86	0.69	0.42	0.62	0.61
2007	1232	1300	992	983	1461	0.75	0.68	1.05	0.98	0.72	0.92	0.77	0.39	0.57	0.54
2008	1102	1185	873	1203	820	0.75	0.73	0.97	0.59	0.8	1.04	0.77	0.39	0.64	0.6
2009	1155	1285	1045	734	773	0.75	0.7	0.87	0.94	0.9	0.89	0.75	0.39	0.69	0.55
2010	1274	1356	1006	1134	1397	0.69	0.67	1	0.59	0.42	0.56	0.75	0.34	0.37	0.33
2011	1125	1308	689	1785	1988	0.8	0.73	1.16	0.46	0.8	0.55	0.82	0.36	0.26	0.4
2012	997	1139	716	844	882	0.92	0.75	1.42	1.07	0.99	0.58	0.76	0.39	0.56	0.68

∗Summary statistics for the years 1993–1995 (3 years) together

†1996-1997: White and coloured population groups were pooled together

Asian is the Asian/Indian population group

NR: not reported

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As shown in Figure 3.4, HCC was the most frequently diagnosed LC histological subtype, accounting for approximately 59% of all LC incident cases (67.2% and 44% in men and women, respectively). Cholangiocarcinoma, the 3rd most common subtype, accounted for 4.7% (4% and 5.9% in men and women, respectively).

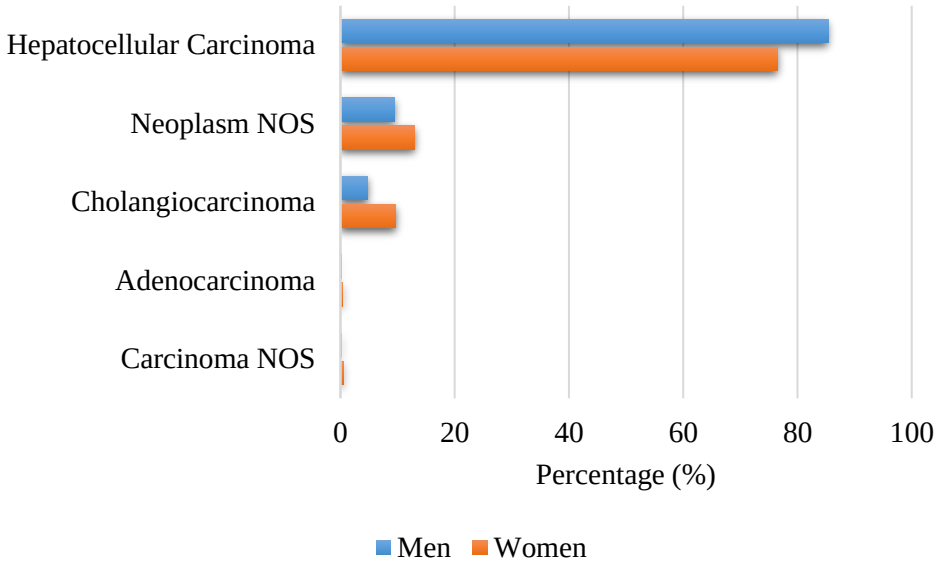

Figure 3.4 Proportion of the top 5 morphological subtypes of liver cancer from South African pathology based National Cancer Registry, 1993-1995 to 2012.

Figure 3.5 compares the ASpIR per 100,000 in both sexes over the study period separated into approximately four 5-year periods. LC AspIR was relatively low in men and women under the age of 29, but increased thereafter with increasing age. The peak ASpIR appeared in age groups 70–74 and 80-84 years for men (during 1998-2002) and 80-84 years for women (during 2003-07), which then descended rapidly. In the most recent period (2008-2012), ASpIR was higher than the previous years (2003-07) in men but lower in women. Of note, because the source of the incidence data is pathology reports, the numbers of reported incidence cases may be incomplete as a consequence of late diagnosis due to delayed or limited access to all tiers of healthcare facilities and services (Babb et al. 2014). Nevertheless, despite the possibility that the true incidence of LC is higher than that reported here, it does not suggest that the conclusion of the study should be different, as the factors that are involved in the detection of LC in our study should have played a similar role across time.

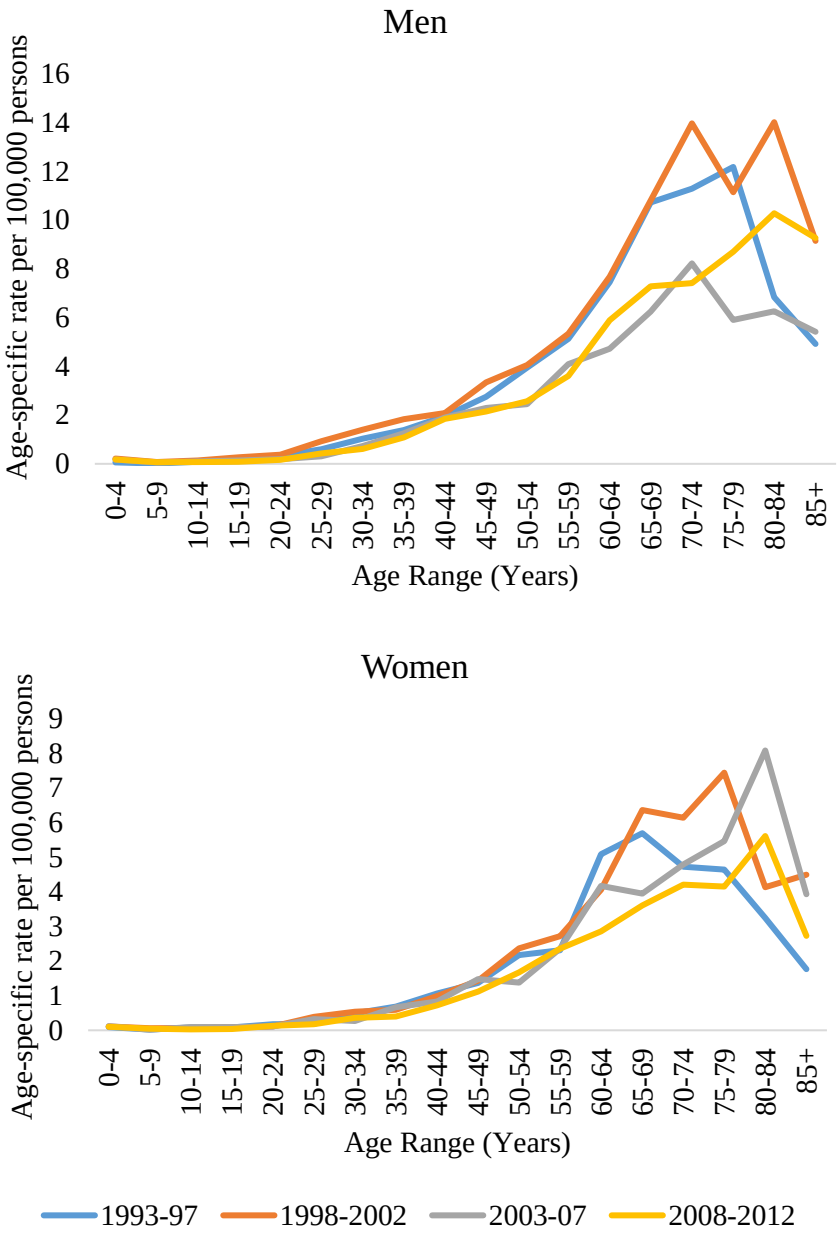

Figure 3.5 Age-specific incidence rates (ASpIR) for liver cancer from South African pathology based National Cancer Registry over the period of 1993-1995 to 2012, by sex.

3.4.1 Joinpoint analysis

The APC's for specific period segments and AAPCs of ASIR for LC are presented in Table 3.4 and Figure 3.6. The joinpoint analysis showed that, between 1993-1995 and 2012, overall ASIR trend relatively remained stable in both sexes. The best fitting

model for the overall LC incidence trends had three segments in that they were identical in both men and women: the first segment from 1993-1995 to 1997 showed a rapid increase (likely a result of improved access to healthcare and improved diagnosis during the transition to a democratic government); the second segment from 1997-2003/4 showed a dramatic fall in ASIR (APC of -12.9% and -15.2% in men and women respectively, $p<0.05$); the third segment fitting LC incidence trends from 2003/4-2012, showed a non-significant increase. The AAPC differed by population group, either showing a decrease in men and women from the Coloured and Indian/Asian population group and in women only in the white population group; or a stable trend in men and women from the black African population group. In the black African population, the substantial change in LC incidence was the observed fall beginning 1996 until 2003 (decreased by 13.4% and 14.4% in men and women, respectively), which was then followed by a more stable APC trend in men (-0.8%, $p>0.05$) but a significant increase in women (1.3%, $p<0.05$).

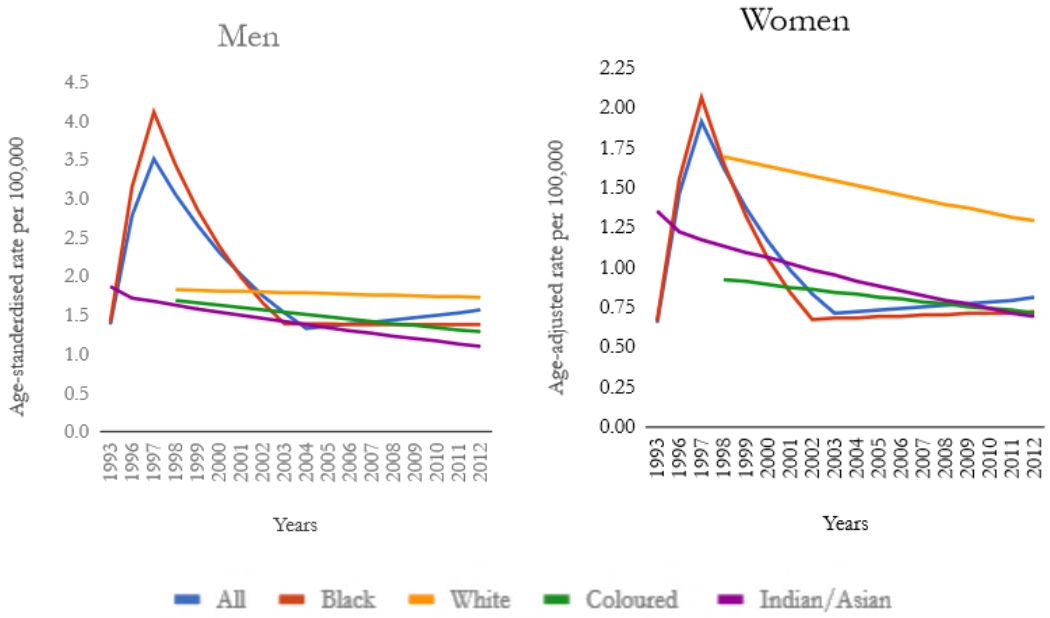

Figure 3.6 Joinpoint analysis of age-standardised incidence rates trends for liver cancer from 1993-1995 to 2012 in South Africa, by sex.

Table 3.4 Joinpoint analysis of the age-standardised incidence rates for liver cancer over the period of 1993–1995 to 2012 in South Africa, by sex and population group.

		Trend 1		Trend 2		Trend 3	
Population group	AAPC	Range of Years	APC	Range of Years	APC	Range of Years	APC
Men							
All	0.7	1993-1997	26.2*	1997-2004	-12.9*	2004-2012	2.1
Black African	-0.1	1993-1997	30.7*	1997-2003	-16.6*	2003-2012	-0.1
Coloured	-1.9	1998-2012	-1.9				
Indian/Asian	-2.8	1993-2012	-2.8				
White	-0.4	1998-2012	-0.4				
Women							
All	1.1	1993-1997	30.6*	1997-2003	-15.2*	2003-2012	1.5
Black African	0.5	1993-1997	33.0*	1997-2003	-20.1*	2003-2012	0.7
Coloured	-1.8	1998-2012	-1.8				
Indian/Asian	-3.5	1993-2012	-3.5				
White	-1.9	1998-2012	-1.9				

*The annual percent change (APC) is significantly different from 0 (two-sided $p < 0.05$).
AAPC = average annual percentage change

3.4.2 Joinpoint: Incidence Patterns by Age and Race

The APC of LC ASIR by sex, population group and age group are shown in Table 3.5 and Table 3.6. For the black African population, our results showed that that AAPCs significantly decreased in men and women of all ages over the entire period (20-29, 30-39, 40-49, 50–59, 60–69 and 70–79 years). In contrast, significant declines in ASIR over the full period were only found in white men aged 50–59 years and older white women aged 60–79 years. More specifically, the best fitting model of two segments found in the black African population showed that the decreasing ASIR occurred from 1996 until the mid-2000's. In particular, it is noticed that the APCs of middle-aged black Africans aged 40-49 and 50-59 years stabilized in men but non-significantly increased in women.

Table 3.5 Joinpoint analysis of the age-standardised incidence rates for liver cancer over the period of 1996 to 2012 in South Africa, by sex, population group and age group.

		Trend 1		Trend 2	
Population group	AAPC	Range of Years	APC	Range of Years	APC
All[a]					
Age (Years)					
20-29	-9.0*	1996-2012	-9.0*		
30-39	-6.0*	1996-2012	-6.0*		
40-49	-3.9*	1996-2004	-8.1*	2004-2012	0.4
50–59	-5.5*	1996-2004	-10.3*	2004-2012	-0.4
60–69	-4.2*	1996-2006	-10.5*	2006-2012	7.3
70–79	-5.6*	1996-2004	-12.6*		
Black African[a]					
Age (Years)					
20-29	-9.2*	1996-2012	-9.2*		
30-39	-6.8**	1996-2012	-6.8*		
40-49	-4.6*	1996-2004	-8.8*	2004-2012	-0.3
50–59	-6.0*	1996-2004	-12.4*	2004-2012	0.9
60–69	-7.6*	1996-2003	-16.4*	2003-2012	-0.2
70–79	-6.7*	1996-2006	-14.9*	2006-2012	9
White[b]					
Age (Years)					
40-49	11.1	1998-2012	11.1		
50–59	-3.4*	1998-2012	-3.4*		
60–69	-2.6	1998-2012	-2.6		
70–79	-0.1	1998-2012	-0.1		

[a] Incidence data are from SA National Cancer Registry from 1996 to 2012

[b] Incidence data are from SA National Cancer Registry from 1998 to 2012

* The APC is statistically significantly different from 0 ($p<0.05$).

AAPC = average annual percentage change

APC = annual percentage change

Table 3.6 Joinpoint analysis of the age-standardised incidence rates for liver cancer over the period of 1996 to 2012 in South Africa, by sex, population group and age group.

		Trend 1		Trend 2	
Population group	AAPC	Range of Years	APC	Range of Years	APC
All[a]					
Age (Years)					
20-29	-4.7*	1999-2015	-4.7*		
30-39	-5.4*	1999-2015	-5.4*		
40-49	-4.5*	1999-2015	-4.5*		
50–59	-3.6*	1999-2012	-3.6*		
60–69	-7.5*	1999-2009	-20.3*	2000-2012	-2.7
70–79	-4.5*	1999-2004	-4.5*		
Black African[a]					
Age (Years)					
20-29	-5.0*	1996-2012	-5.0*		
30-39	-8.3*	1996-1999	-30.3	1999-2012	-2.3
40-49	-5.2*	1996-2004	-13.6*	2004-2012	4.1
50–59	-3.1	1996-2005	-10.6*	2005-2012	7.5
60–69	-8.7*	1996-2002	-21.7*	2002-2012	0.2
70–79	-5.1*	1996-2012	-5.10*		
White[b]					
Age (Years)					
40-49	-3.5	1998-2012	-3.5		
50–59	-4.6	1998-2012	-4.6		
60–69	-4.2*	1998-2012	-4.2*		
70–79	-4.2*	1998-2012	-4.2*		

[a] Incidence data are from SA National Cancer Registry from 1996 to 2012

[b] Incidence data are from SA National Cancer Registry from 1998 to 2012

* The APC is statistically significantly different from 0 ($p<0.05$).

AAPC = average annual percentage change

APC = annual percentage change

3.4.3 Age-period-cohort Analysis 1993-2012

The longitudinal age curves of LC in SA black and white population groups showed considerable variation as illustrated in Figure 3.7 (Appendix B). After controlling for period effects, the age-period-cohort analysis (Longitudinal age curve) showed that unlike the white population that showed similar rates that decreased with increasing age for both sexes, the rates in black African men were significantly higher at younger ages compared to women, and the rates converged at older age groups (>75 years). Moreover, the results indicated that younger age black Africans were predominantly susceptible to LC: risk peaked in early 30s in men and late 20s to early 30s in women. These patterns were very different from the age patterns seen in the white population that had a higher risk at ages 20-24 years of age but declined with increasing age. Furthermore, findings showed that a distinction in age curves of LC rate patterns exists between population groups of the same sex: In men, the risk was substantially higher in black Africans than in whites from 20 to 54 years of age, which converged thereafter with increasing age. In contrast, the risk was higher in white women from ages 20 to 49 years of age, which converged thereafter with increasing age.

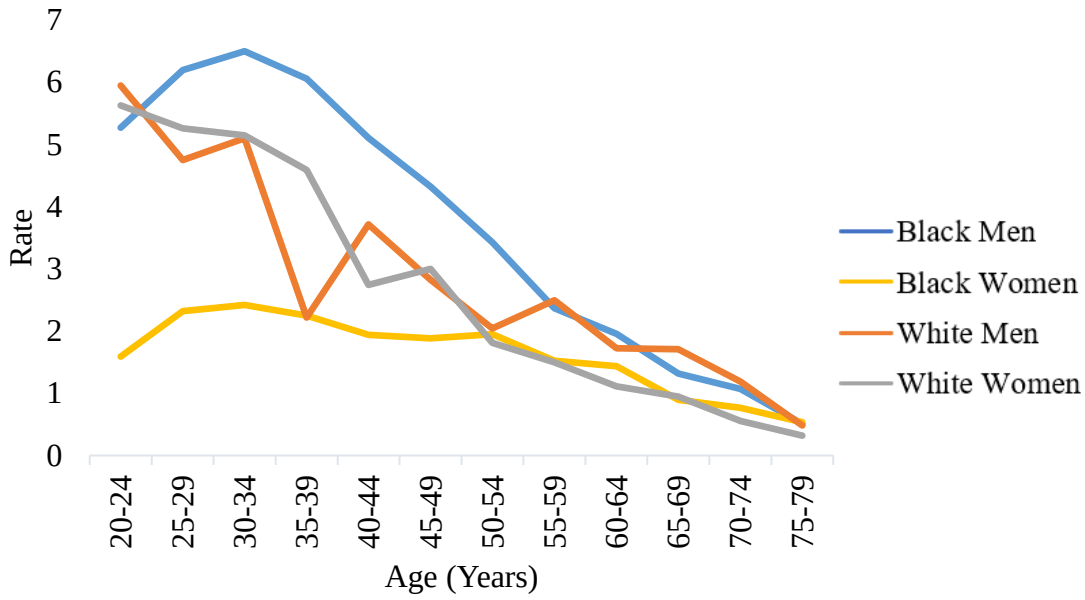

Figure 3.7 Longitudinal age curves of the liver cancer incidence rates per 100,000 person-years by sex and population group in South Africa.

The local drift values by sex and population group for specific age groups (Appendix C), which indicate the age-period-cohort in the LC rates during the study period are displayed in Figure 3.8. The overall net drift was similar in both men and woman from the black African (-9.675 [95% CI: -10.759, -8.578] and -8.052 [95% CI: -9.527, -6.553], respectively) and the white population groups (-10.588 [95% CI: -13.394, -7.692] and -10.525 [95% CI: -13.541, -7.402], respectively). With regards to age groups in black Africans, all had local drift values below 0, which were higher at ages 35-49 years and ≥70 years in men, and ≥70 years in women. Similarly, for the white population, we observed local drift values under 0 in all age groups, which were higher at younger ages of 30-44 years in men and 20-34 years in women.

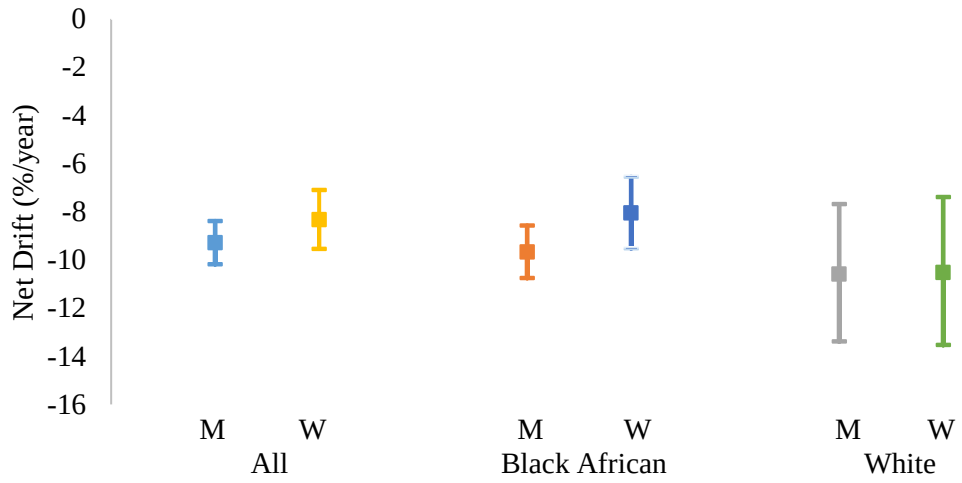

Figure 3.8 Incidence net drift in liver cancer incidence rate per 100,000 person-years and the corresponding 95% confidence intervals for men (M) and women (W) by population group.

The estimated period and cohort effects by sex and population group are displayed in Appendix D and E. We observed similar period effects for both the black African and white population groups, which showed downward trends since the 1993-1997, before reaching a stable trend in the early 2000s. For both the black African and white population, the cohort effects in both sexes showed a general downward trend. Using the specific results of Wald tests (Table 3.7), we found statistically significant period effects for both sexes and population groups.

Table 3.7 Wald $\chi 2$ tests for estimable functions in the age-period-cohort model for liver cancer in South Africa by sex and population group.

	All		Black African		White	
Null Hypothesis	Chi-Square	P-value	Chi-Square	P-value	Chi-Square	P-value
Men						
NetDrift = 0	373.59	0.00	272.83	0.00	47.35	0.00
All Age Deviations = 0	16.50	0.09	18.31	0.05	11.43	0.33
All Period Deviations = 0	64.52	0.00	31.65	0.00	46.61	0.00
All Cohort Deviations = 0	6.53	0.93	7.33	0.88	3.92	0.99
All Period RR = 1	543.56	0.00	373.63	0.00	118.88	0.00
All Cohort RR = 1	401.62	0.00	282.11	0.00	123.08	0.00
All Local Drifts = Net Drift	5.70	0.93	7.33	0.84	3.22	0.99
Women						
NetDrift = 0	162.78	0.00	103.46	0.00	40.38	0.00
All Age Deviations = 0	9.11	0.52	10.02	0.44	3.66	0.96
All Period Deviations = 0	44.69	0.00	26.87	0.00	21.51	0.00
All Cohort Deviations = 0	7.90	0.85	9.09	0.77	6.30	0.93
All Period RR = 1	247.56	0.00	153.85	0.00	78.99	0.00
All Cohort RR = 1	194.83	0.00	118.06	0.00	91.22	0.00
All Local Drifts = Net Drift	7.84	0.80	9.08	0.70	6.30	0.90

Figure 3.9 shows a comparison between the HIV prevalence (years 2008 and 2012) and the LC ASIR in the same years for the black African population, by sex and age group. It should be noted that in contrast to LC, which is often found to predominate in men, the HIV prevalence is observed to be higher in women. Whereas the HIV prevalence rose sharply and peaking in the late 20's and early 30's age groups, the LC ASIR's increase is gradual at younger age groups, but becomes more rapid as the HIV prevalence decreased below 5%. More interestingly, the intersection between the HIV prevalence and LC ASIR was found in the 50-59 year age group, for both sexes and for both years 2008 and 2012. Compared to the year 2008, the 2012 peak HIV prevalence shifted from 25-29 to 30-34 in women and 30-34 to 35-39 in men. In contrast, LC ASIR peaked at 70-74 years in both 2008 and 2012 regardless of sex.

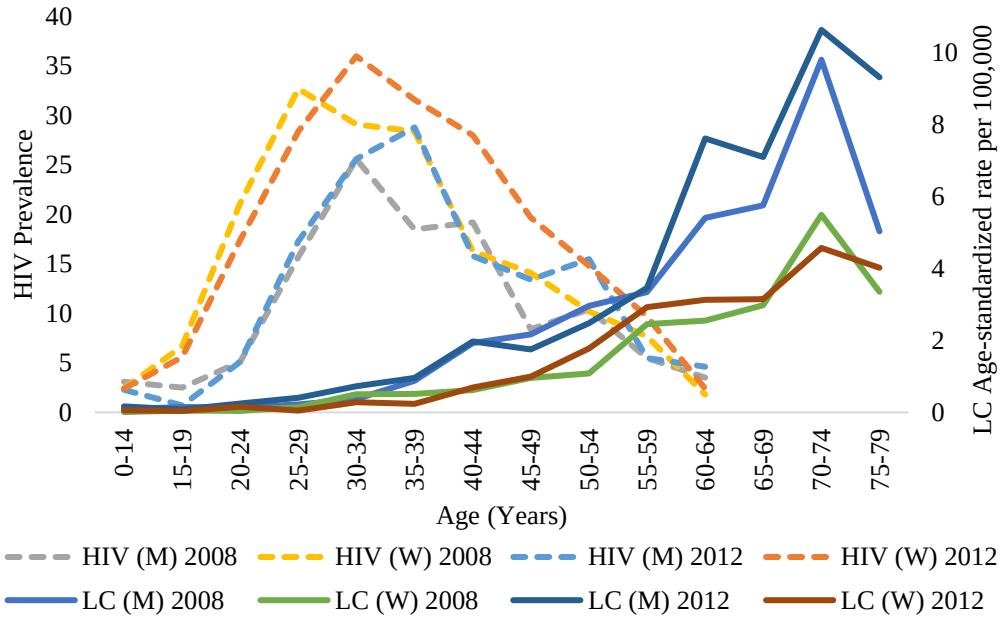

Figure 3.9 Liver cancer incidence rate per 100,000 person per year (solid lines) and human immunodeficiency virus (HIV) prevalence (doted lines) in South African men (M) and women (W), in years 2008 and 2012.

3.5 Discussion

From 1993-1995 to 2012, the cancer burden in SA increased by 33% (from 49,939 cases to 74,519 cases) (SA NCR 2012; SA NCR 1998), while the population increased by an estimated 27.7% (from 37.8 million to 52.3 million) according to the mid-year population estimates by StatsSA (StatsSA 4 October, 2013; StatsSA 2000). During this period, analysis of the SA NCR data showed an overall decrease in LC incidence, which varied between sex, age and population group. To our knowledge, this is the first study in SA to define the burden of LC at the national level using current SA NCR data. In doing so, the current study demonstrated that, despite the positive decreasing trend in LC incidence found in the black African population during 1997-2003, more recent patterns indicate that this is no longer the case. More specifically, the incidence rates have remained stable since 2003, which may have been offset by the increasing trend found in 70-79 year old men, and middle-aged women (40-59 years). Age-period-cohort analysis further showed that LC incident trends are likely to be associated with period effects, that is, a change/s that occurs in a particular time, which affect/s all age groups and cohorts uniformly in the observed population. In particular, the present study

showed that younger age is directly associated with a higher prevalence of HIV infection, whereas older age is directly associated with increased LC diagnosis. Thus, by looking at long-term trends of a large nationally representative sample, our study allowed for a broad examination of the national LC epidemiology from 1993-2012, and measured the differential burden of this disease by the different sex, age and population groups.

Figure 3.10 shows a comparison of ASIR for LC between the SA NCR and other regions of the world. It is clear that, the ASIR observed in SA black African men and women is significantly lower than that observed in men (9 to 11 times) and women (11 to 16 times) from Mozambique and Zimbabwe. This difference is less pronounced when compared to black African Americans (7 and 2 times in men and women, respectively), and less so when compared to a South African rural/regional population-based cancer registry based in Eastern Cape Province (3 and 2 times in men and women, respectively). In contrast, the LC ASIR in SA black African men is 3-fold higher than that reported in Calabar, Nigeria.

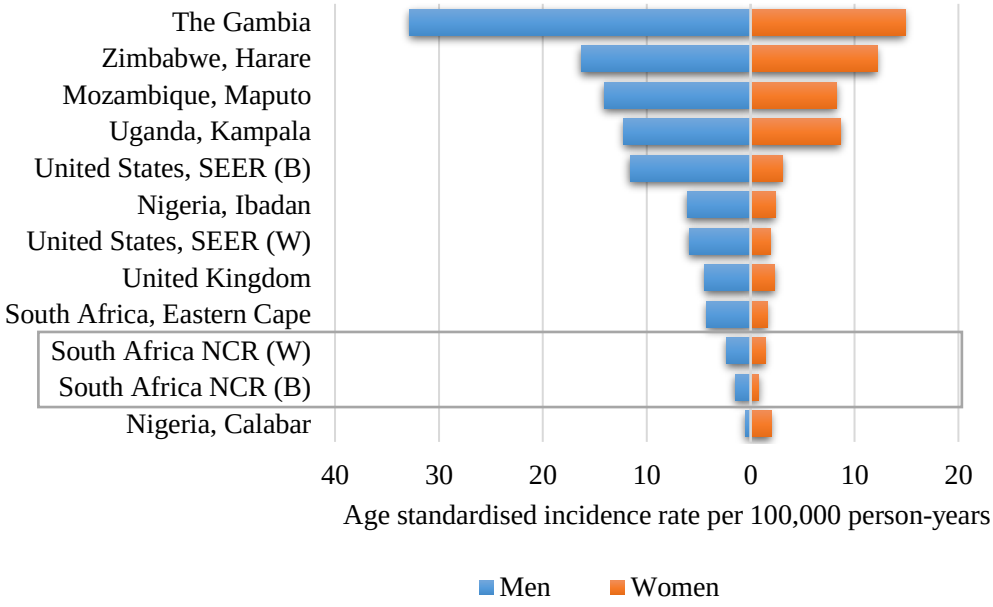

Figure 3.10 Comparison of liver cancer age-standardised incidence rates per 100,000 person-years from Sub-Saharan African countries including: Zimbabwe, Harare (1991-2010) (Chokunonga et al. 2013); Mozambique, Maputo (1997-2008) (Lorenzoni et al. 2015); Uganda, Kampala (1991-2010) (Wabinga et al. 2014); The Gambia (1988-2006)

(Sighoko et al. 2011); United States, Seer (1973-2007) (Y. Zhang et al. 2015); South Africa, Eastern Cape Province (1998-2012) (Somdyala et al. 2015); Nigeria, Calabar (2009–2013) and Ibadan (2006-2009) (Ekanem & Parkin 2016a); and the South African pathology-based National Cancer Registry (1993-2012) (in box). The graph is ordered by descending LC incidence rates in men; B: Black African and W: White population groups.

In the SA NCR, evidence showed that the ASIR in the black African population group decreased from 3.1 to 1.5 in men, and 1.4 to 0.8 in women over the period of 1998 and 2012 (Table 3.2 and Table 3.3). This however differed to a rural/regional population-based cancer-registry based in Eastern Cape Province reported that reported an increase in LC ASIR in women (from 0.9 to 1.6), but a decrease in men (from 4.4 to 2.8) over the same period (Somdyala et al. 2015). These differences may likely be attributed to the difference in the sample population.

Whereas the SA NCR represents the total population living in SA, Eastern Cape Province is largely a rural area that only represents 12.7% of the total South African population (6.56 million of 51.8 million) and 13.8% of the total land area of SA (168,966 of 1,220,813 square kilometers) (StatsSA 2012). Furthermore, it was reported that during the 10-year period between the 2001 and 2011 Censuses (StatsSA 2012), Eastern Cape Province experienced the highest negative net migration of people (-278,261), meaning that more people have migrated out of this province than have moved in over time. Historically a large proportion of migrants are working-age men who migrated to urban cities to seek better opportunities for work (Clark et al. 2007). However, this trend has since gradually changed as women, particularly those of working-age, are increasingly participating in the rural-to-urban migration. Thus, the observed LC ASIR decrease in men may suggest that the outward migration may play a role as more men leave their home village in the Eastern Cape Province. In contrast, the increase in LC ASIR for women living in Eastern Cape Province may not be as clear, that is the increasing trend in LC incidence may be a reflection of either more older aged women who generally remain in their rural home village, or possibly more younger age women returning home when ill. Evidence from an earlier study in a rural area of northeastern SA suggests that many of these people who participate in the

circular rural-to-urban migration and become seriously ill while living away from home, would return to their rural homes where their families live and where social networks and support systems may be stronger, to convalesce and possibly to die (Clark et al. 2007).

Comparisons LC incidence trends as indicated by the AAPC between SA and other regions of the world are shown in Figure 3.11. The trend of LC incidence rates in the SA black population group was significantly lower and more stable (AAPC: 0.1% and 0.5% in men and women, respectively; Table 3.5 and Table 3.6) when compared to its neighbouring southern African nations. For instance, a population/regional-based cancer registry based in Harare, Zimbabwe, reported a decrease in LC ASIR per 100,000 from 33.5 to 16.3 in men (AAPC: -5.4% [95% CI: -7.3%-3.5%]), and 14.4 to 12.2 in women (0.6% [95% CI: -3.5%-2.4%]) over the period of 1991 to 2010 (Chokunonga et al. 2013). In another population/regional-based cancer registry based in Maputo, Mozambique, a decrease in LC incidence rates was observed in men (18.9 to 14.1; AAPC: -1.6% [95% CI: -4.0-0.7]), but increased in women (7.3 to 8.3; AAPC: +2.4% [95% CI: -1.1-5.8]) (1997-2008) (Lorenzoni et al. 2015). In Kampala, Uganda, rates increased in both men (9 to 12.2; AAPC: +2.8% [95% CI: -1.0-6.8]) and women (5.2 to 8.7; AAPC: +4.0% [95% CI: 0.5-8.4]) (1991-2010) (Wabinga et al. 2014). Similarly, rates increased in Gambian women (11.71 to 14.9; AAPC: +3.01% [95% CI: 0.3-5.8]), but not men (38.36 to 32.84; AAPC: +0.02% [95% CI: 21.8-1.9], p=0.98) (188-2006) (Sighoko et al. 2011).

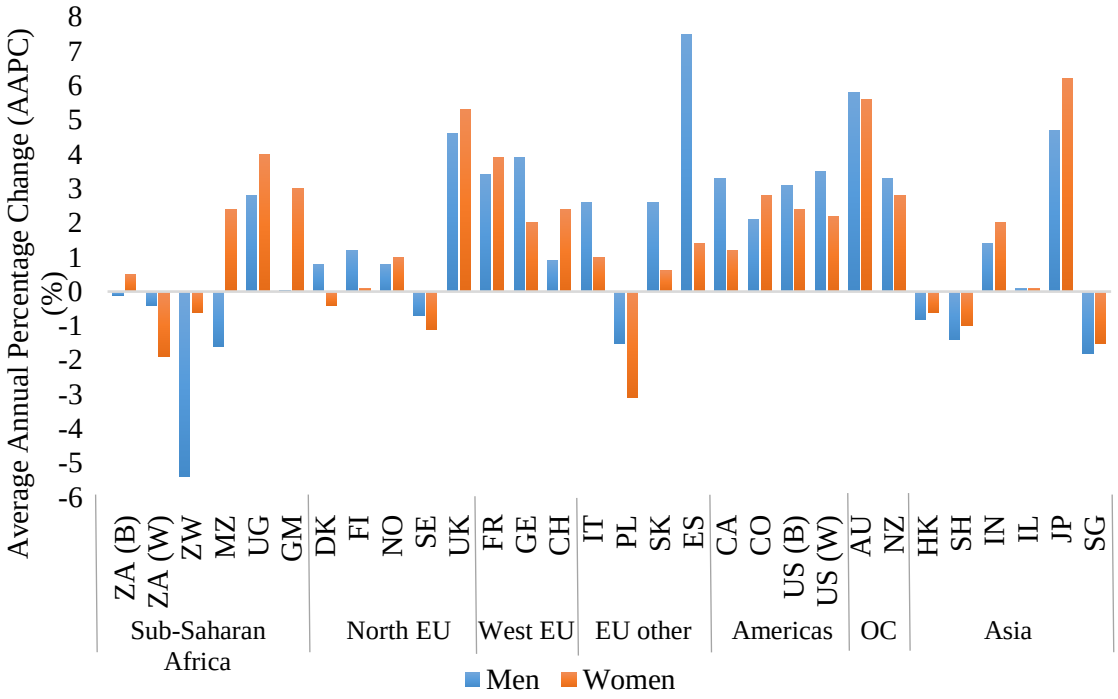

Figure 3.11 Liver cancer incidence average annual percentage change (AAPC) (%) for men and women from different regions in the world (Sub-Saharan Africa: South Africa Black [1994-2012], ZA (B); South Africa White [1994-2012], ZA (W); Zimbabwe Harare [1991-2010] (Chokunonga et al. 2013), ZW; Mozambique Maputo [1991-2008] (Lorenzoni et al. 2015), MZ; Uganda Kampala [1991-2010] (Wabinga et al. 2014), UG; The Gambia [1988-2006] (Sighoko et al. 2011), GM; Northern Europe, North EU: Denmark, DK; Finland, FI; Norway, NO; Sweden, SE; England, UK; Western Europe, West EU: France, Bas-Rhin, FR; Germany, Saarland GE; Switzerland Geneva, CH; Europe Other, EU other: Italy Varese Province, IT; Poland Krakow, PL; Slovakia, SK; Spain Navarra, ES; Americas: Canada British Columbia, CA; Colombia Cali, CO; United States SEER Black, US (B); United States SEER White, US (W); Oceania, OC: Australia New South Wales, AU; New Zealand, NZ; Asia: China Hong Kong, HK; China Shanghai, SH; India Mumbai, IN; Israel Jews, IL; Japan Osaka Prefecture, JP; Singapore Chinese, SG) [1973-2007] (Y. Zhang et al. 2015).

Furthermore, in the South African white men and women, the ASIR (2.5 and 1.3) as reported are relatively lower in comparison to rates from other developed countries including United Kingdom (4.4 and 2.3, respectively) and the United States (5.9 and 1.9, respectively) (Zhang et al. 2015). Based on these comparisons between the different

population groups and other regions in the world, it is likely that there may be an underestimation of LC incidence in the SA NCR data. On the other hand, differences in incidence rates may also likely reflect the different characteristics between the SA NCR and cancer registries from other regions including the: type of registry (national vs regional), source of information (pathology reports vs population-based) and the mode of data collection (passive vs active). In SA where the NCR is a passive, pathology-based cancer surveillance, LC cases diagnosed without biopsy and histological examination of the liver tissue would therefore not be captured in the registry.

Additionally, in the absence of screening programs for LC in the general SA population, it is acknowledged that the inclusion criteria for LC cases diagnosed histologically may be problematic particularly in low-resource regions. This not only suggests that there may be an over-reliance on pathology laboratories in diagnosing this tumour, but also the inability to locate cases diagnosed by other methods. Consistently, concerns have also been raised in the population-based Calabar Cancer registry in Nigeria, whereby similarly low incidence rates were also likely a consequence of diagnosing cases based only on biopsy and histopathology (Ekanem & Parkin 2016b), hence the similar low ASIR between the SA NCR and this cancer registry as shown in Figure 3.10.

In a study consisting of 894,449 LC patients (of a total 28.7 million cancer patients) from 279 cancer registries that represent 67 countries in the world (ten registries from nine African countries) (Allemani et al. 2015), the authors reported that morphological confirmation varied widely from 72% in the Americas to 32.5% in Asia (Figure 3.12). Interestingly, Africa reported more than half (53.9%) of the LC cases with morphological verification (Allemani et al. 2015). However, this only consisted of 445 diagnosed LC patients from 10 cancer registries: Algeria (MV: 74.6%), Libya (MV: 24.6%), Mauritius (MV: 100%), SA (Eastern Cape Province; MV: 69.4%), The Gambia (MV: 1.2%) and Tunisia (MV: 100%).

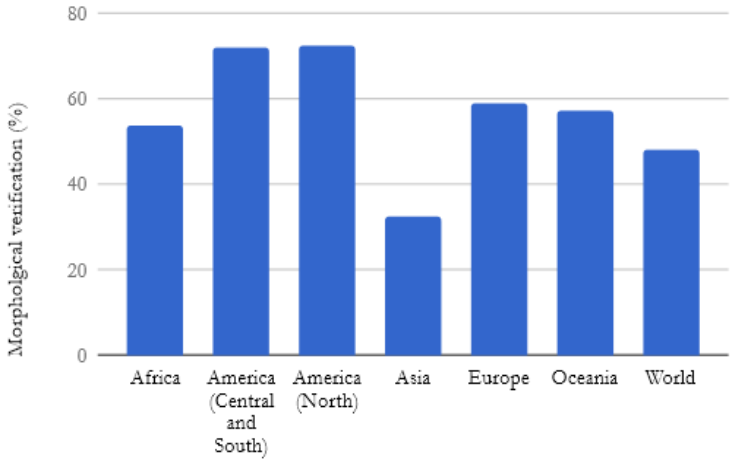

Figure 3.12 Data quality indicator as the percentage of diagnosed cancers with morphological verification, by region from 1995-2009 with permission to reprint from (Allemani et al. 2015).

3.5.1 Population group disparities in LC incidence trends

Distinctive features of our study includes a focus on population group disparities in LC burden. The current study demonstrates that the disease burden is lower in the black Africans than their white counterparts (Table 3.2 and Table 3.3), and is consistent with previous SA studies investigating tumours of the breast (Vorobiof et al. 2001) and the prostate (Babb et al. 2014). A possible reason for this population group disparity in health outcome is borne by the difficulties of detecting LC in its earlier stages, possibly as a consequence of socioeconomic disparities and the gap between private and public healthcare system. While, LC screening and early detection has been shown to improve the survival outcomes of patients with LC (Singal et al. 2017; Izzo et al. 2013), the detection of potentially curable LC are potentially cost prohibitive (Cadier et al. 2017).

Furthermore, this may also require trained medical practitioners/technicians and specialized diagnostic laboratories, which are often unavailable in resource-limited countries or settings. Even if these are available, there is often an imbalance in the geographic distribution of these facilities between the public and private healthcare sectors, while competition for limited personnel resources further aggravates the quality gap between the public and private services (Cadier et al. 2017; van Rensburg 2014). This is evident in SA where the private healthcare sector employs more than 70% of the

country's doctors (Cadier et al. 2017; van Rensburg 2014; Pillay-van Wyk et al. 2016), but only serve less than 20% (17.9%) of the SA population that have health insurance: this included 75.1% of the white population, 41.7% of the Indian/Asian population, 20.9% of the coloured population and only 10.4% of the black African population (Lehohla 2013) (Figure 3.13). The remaining, yet significant proportion (84%) of the population without private health insurance, can be said to be entirely dependent on the public sector for their healthcare services, and many of whom face challenges in accessing and receiving quality care. Studies in the United States have reported that uninsured adults with LC are much less likely than insured adults to have received routine health check-ups and less likely to receive basic preventive services related to cancer screening and/or treatment (Wang et al. 2016). This has also further been reported that even with the equalization of access to health care, health disparities may persist (Guy & Yee 2009; Martin et al. 2007). Further investigation of population group disparities in the development of LC should also consider other factors such as genetic makeup, virological, behavioural, cultural, environmental, and social determinants which are said to play a role either individually or in combination (Vorobiof et al. 2001).

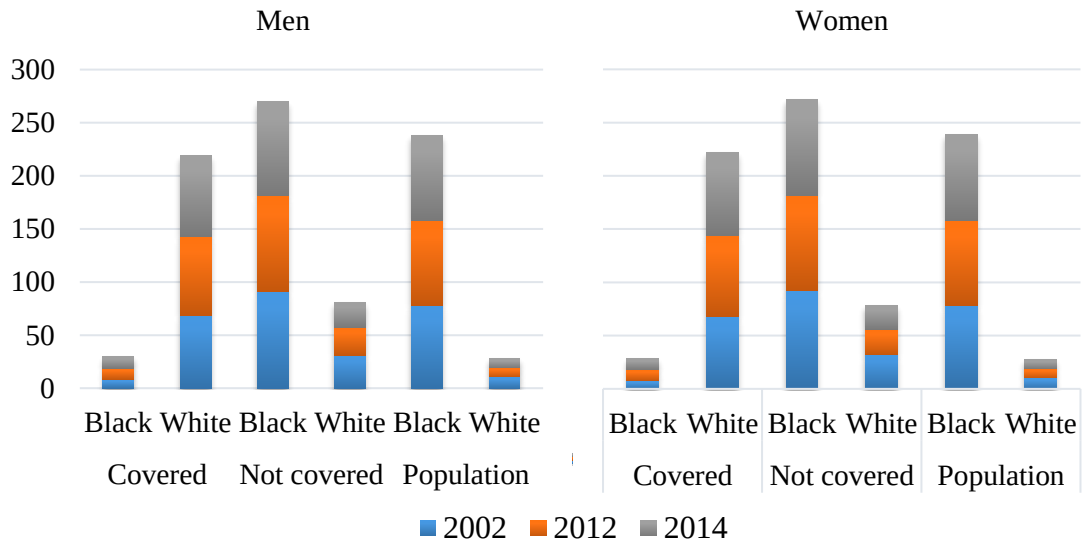

Figure 3.13 Percentage of individuals covered/not covered by private health insurance and the population size in South Africa, by population group. Compiled using data from (Lehohla 2013).

While previous studies on LC incidence are based on analysis of age-standardised rates, trends outlined in these studies may be misleading particularly when time trends differ between age groups as shown in this present study. Thus, the present study demonstrated that, although the AAPC was stable in black African population, significant decreases in rates from 1997 until mid-2000's were seen in all age groups, and non-significant increases in older men (60-69 years) and middle-aged women (40-59 years) occurred in the most recent years. This sex and population group disparity in LC disease epidemiology is an important observation and while the underlying aetiology of this phenomenon may not be immediately clear, several explanations may be proposed. Firstly, increasing trends in LC in sub-Saharan African women may be attributed to systematic under-detection or underestimation during earlier cancer registration periods, and that increasing rates in the recent period may primarily reflect improved registration of this malignancy. Additionally, rates may also possibly be attributed to increased life expectancy of this group.

While the stage of tumour at presentation is unknown, and thus the length of time between detection and its clinical manifestation and diagnosis could not be established for any particular population group, the average ages at LC diagnosis may reflect the age structure of the different SA population groups. For example, the age at LC diagnosis in the white population, was approximately a decade older than their black African counterparts, and this is similarly observed in economically developed countries (Ryder 2003; Davis et al. 2008) where the proportion of older age groups are substantially higher (He et al. 2016). As LC incidence rates are sensitive to the population size and the age structure, the age distribution and the number of competing causes of mortality in the various SA population groups must also be taken into consideration.

For example, LC risk factors such as HBV/HCV and metabolic diseases (obesity and diabetes type 2) differ in their contribution to LC burden in each group (gender, age and population), and changes in the prevalence of these factors may influence LC incidence trends. For example, higher rates of LC among black Africans (range of 5%-16% in men and 4%-12% in women) compared to whites (0.2%) are thought to reflect the higher prevalence of HBV infection (Kew 2008b). Public preventive interventions such

as universal infant HBV immunization programs initiated in 1995, are important, and have contributed to a decline in HBV prevalence, and ultimately HCC, among vaccinated cohorts of children and adolescents (Moore et al. 2008). However, the youngest cohort of LC cases that were included in the current study were born prior to the vaccine introduction in 1995, and therefore our observations are unlikely to reflect possible influences of HBV vaccination. Thus, in the immediate term, HBV infection prevalence remains high among unvaccinated adults, and may still represent an ongoing risk of LC.

On the other hand, the seroprevalence of chronic HCV infection is much lower in all population groups (0.75% and 0.16% in black Africans and whites, respectively) (Soni et al. 1993), and thus, is responsible for far fewer HCC cases (20%) who may also be relatively older (57.3 vs 37.5 years in those infected with HBV) (Kew et al. 1997). As a consequence, HCV is unlikely to play a major role in the observed LC trends in black African women.

Additionally, the stage at diagnosis and the lower compliance or acceptance of preferred treatment options have been also suggested to be a possible explanation for observed disparities among population groups (Stewart et al. 2016), while other factors including low socioeconomic status (Harrison 2004), lack of knowledge of the disease and risk factors (Fitzgerald et al. 2016), limited access to health care facilities and services (Davila & El-Serag 2006), and lack of health insurance (Yu et al. 2010) may all play a role in the difference in LC detection as observed between population groups.

Other factors that may account for the observed increase in LC incidence is the rising prevalence of metabolic disorders associated with obesity and diabetes. In SA, the high prevalence of obesity is largely attributed to women, which increased from 29.7% to 37.9% during 2008-2012 (Sartorius et al. 2015). In comparison, this only increased from 11.1% to 13.3% in men. A previous SA study showed that the factors associated with increased odds ratio of obesity included white ethnicity (OR 2.26, 95% CI: 1.44–3.54) among men and black African ethnicity in women, while urban residence and higher socio-economic status, lack of exercise were other factors that effected both sexes (Sartorius et al. 2015). Although data are limited, differences in overweight and obesity by gender, age and population group have also been observed to play a role in

SA. For instance, among individuals aged 35-64 years (the age group corresponding with higher incident rates of LC as observed in the present study), women were more likely to be overweight or obese when compared to men in all population groups, as were black African women (ranged from 75%-85%) and white men (ranged from 60%-70%) when compared to other population groups in the same sex- and age- group (Benade. et al. 1996). Moreover, more women than men tended to become obese as they grew older (Benade. et al. 1996). The prevalence of diabetes amongst adults in SA also has significantly increased from 3.5% (95% CI: 3.0–4.1%) in 2008 to 5.0% (95% CI: 4.3–5.7%) in 2012, with a higher prevalence among women (37.9%) than men (13.3%) (Sartorius et al. 2015). Although the prevalence rates for diabetes were higher in urban black Africans when compared to their rural counterparts, they were generally similar to those in whites for all age groups (except for 45-59 years) which were higher in urban black Africans (13% vs 8.7% in women and 9.2% vs 7.3% in men) (Bradshaw et al. 2007). In view of these findings, it appears that obesity and diabetes increased in the early part of the 21st century in black Africans, which may be a consequence of changes in lifestyle behaviours when living in urban cities.

In SA, there is currently no available data on the relationship between metabolic disease and the risk of developing LC. A recent nested case-control study conducted in the United States showed an association between excess bodyweight and increased risk of LC (Petrick et al. 2016). In this study, compared with persons of normal weight, the odds ratio of LC was 2.38 (95% CI: 1.68-3.36) for those who were obese, while diabetes conferred a modest increased risk of 1.28-fold (95% CI: 0.65-2.54) (Petrick et al. 2016). Among black Africans and women, obesity and diabetes were associated with at least a 2-fold increased risk of LC (OR 2.29, 95% CI: 1.22-4.28 and OR 2.00, 95% CI: 1.14-3.52, respectively) (Setiawan et al. 2014; Wang et al. 2012; Petrick et al. 2016). Despite the lack of data in SA, it is plausible that with the rapidly increasing prevalence of obesity and diabetes in the SA population, extrapolating the evidence of LC risk associated with these metabolic diseases from developed countries may suggest that they may play a more prominent role in the LC burden in the near future.

This is the first study to show that the age at which LC ASIR intersects with the HIV prevalence is at 55-59 years for both men and women (Figure 3.9). This age

corresponds to 25 and 20 year lag time from the peak HIV prevalence (in 2012) respectively. This may suggest that LC may require two to three decades to develop from the peak HIV prevalence age. In SA where the health of black African women is disproportionately and adversely impacted by the HIV epidemic (prevalence as high as 27%) (Welz et al. 2007), the massively scaled up roll out of highly active ART in 2004 has seen close to 40% of all HIV-infected women aged 25-49 years having access to this therapy (Zaidi et al. 2013). Consistent with the scientific literature (Silverberg et al. 2015), our results showed an increase in LC incidence in the period of ART availability (post-2004) and is specific to middle-aged black African women (40-59 years). Based on a meta-analysis of the epidemiology from Western countries (Cobucci et al. 2015), the overall increase in health and survival of ART-treated HIV/AIDS-infected individuals means that they now age with the disease, rather than die from it shortly after diagnosis. As a consequence, the authors reported that the risk of developing LC increased by 4-fold in people taking ARTs. In SA, the average life expectancy of women starting ART ranged between 36.8 years (95% CI: 34.0–39.7) at age 20 years, and 14.4 years (95% CI: 13.3–15.3) at age 60 years, and is substantially higher than that observed in men of the same age (27.6 years [95% CI: 25.2–30.2] and 10.1 years [95% CI: 9.3–10.8], respectively) (Johnson et al. 2013). In view of these findings, they may lend further support to the interpretation that while the ART scale-up improves the life expectancy of people living with HIV/AIDS, this may however loosen the constraint exerted on LC disease and ultimately result in an upswing in incidence, however, with a lag time of two to three decades as observed in our study.

Although it is difficult to establish whether this is caused by the drugs or by HIV itself, what is now known is that managing liver related complications is becoming an increasingly important component to the care of HIV-positive individuals with HBV and/or HCV co-infection (Park et al. 2016), alcohol-related disease (Rentsch et al. 2016), and non-alcoholic fatty liver disease (NAFLD) (Vodkin et al. 2015). Although these associations need to be further investigated in case-control and cohort studies, they may also suggest a need for focused LC control efforts and further investigations in ART-treated HIV-infected cohorts that are now surviving longer, in order to determine the underlying aetiological risk factors in this group.

In particular, amongst the SA population between the ages of 70-74 years, ASIRs exceeding 9 and 5 per 100,000 were seen in the black African and white population groups, respectively. One of the most pressing issues throughout Africa, is the growing recognition of population ageing, especially in SA where the proportions of older population is highest in Africa (National Research Council et al. 2006). As more persons live to older-ages with the overall increase in life expectancy, the growth rate of the total 60-and-over population (3.3 to 4.1 million from 2001-2011, +26%) (Lehohla 2014) has come to exceed that of the total population (1.3% from 2002-2013) (StatsSA 2013), with the gap expected to widen in the future. Although the increase in the percentages of 60 and older population is higher in the white (+35%) than the black African population (+20%), the absolute numbers are more important in terms of national population policy and program development, as the rapidly growing number of older persons and the service requirements generated by such growth in the black Africans (452,333) are approximately twice that of the white population (241,361) from 2001-2011 (Lehohla 2014). Given the anticipated growth and aging of the population over the ensuing decades, these results reinforce the need to prioritize LC related to ageing in national cancer control plans.

3.5.2 Limitations

This study has several strengths and limitations that warrant consideration. First, the current study includes the use of a large, comprehensive pathology-based cancer registry in SA, consisting of registered cases of LC based on pathological verification of the diagnosis, and a nationwide comparison of LC incidence and mortality trends in a southern African region. Despite this, obtaining comprehensive and reliable cancer registry data remains a daunting undertaking. Pathology-based incidence derived from cancer registries for fatal cancers, such as LC, may represent significant underestimates, as a proportion of cases may not be diagnosed particularly in resource-limited settings. Nevertheless, we believe that such under-diagnoses would be consistent over the years studied and unlikely to bias incidence trends, which were the main outcome of our study.

While the liver-biopsy diagnosed cases identified in this study may not be as ideal as those diagnosed using radiological modalities, it is an outcome of interest because it

reflects what is happening in the "real world" – in which routine screening of the population does not occur – and it captures a proportion of cases with access to healthcare, as well as highlighting the potential cases that are without access. With around 10 laboratories per million people on this subcontinent, SA has the highest density of pathologists compared to other sub-Saharan African countries, which is close to that seen in some European countries (Schroeder & Amukele 2014). Thus, this improves our ability to draw conclusions regarding the epidemiology of LC nationwide. Such studies are of great interest to health care policy makers focused on understanding the gaps in healthcare services in SA, especially in regards to population group disparities of diseases with very poor prognosis such as LC.

Finally, trend analyses on LC rates in the current study were descriptive analyses conducted at population levels and without inference at individual levels, and thus interpretations from these results do not necessarily hold for individuals. For these reasons, our study is unlike previous hospital-based reports for HCC in SA (Kew et al. 1986; Song et al. 1984), as it included essentially all the recorded cases in the country, and therefore, the case data that we analysed can be considered valid for the populations at risk and the results generalizable to South Africans. Therefore, all hypotheses raised in this study still need further confirmation in large epidemiological studies. It should be noted that with the implementation of a SA population-based cancer registry in the Ekurhuleni district (established in 2012), there may be a foreseeable rise in the documentation of the number of LC cases that may correspond with the increase in active case ascertainment of reportable neoplasms that are otherwise missed by the lack of biopsy (Elvira Singh et al. 2015).

3.5.3 Conclusion

In summary, this is the first study to estimate the total burden of the LC disease in SA, and the results of the present study show an upward trend in incidence rates in the black African population, in recent years. The reasons for this are likely many fold, but may be a consequence of improved life expectancy in the general population, who are reaching the age of increased risk of developing LC. In a region characterised by the lack of surveillance and suboptimal data collection, this analysis, albeit with the limitations discussed, provides a primary analysis and exposes key knowledge gaps

with regards to the burden of LC. The NCR data provides insight into the changing LC epidemiology in SA as they give minimum incidence rates of this disease, while continued surveillance will be vital in monitoring future trends. Moreover, while additional research is necessary to continue improving incidence measures and validating them, this study demonstrates the value of understanding LC as it allows more tailored interventions and services to meet the needs of their populations, as well as to ensure the efficient use of limited resources.

Our findings demonstrate that the LC incidence trends have stabilized after a rapid decline during the period when no ART was available, and may be largely attributed to the dramatic improvements in survival over time for HIV patients on ART. Additionally, the recognition of increasing burden of LC in older populations, especially black Africans, is a critical first step toward characterising the magnitude of this problem – given the that SA has one of the largest older aged populations in sub-Saharan Africa (Pillay & Maharaj 2012), and providing potential candidates for screening. In particular, LC risk may increase in those people living with HIV/AIDS in their late 20s and early 30s, albeit with a lag time of twenty years.

Research into the aetiologic factors contributing to this disease pattern in SA are sorely needed, especially in black African men who are most affected by the disease. Our findings of incidence patterns that differ among the black and white population groups, should encourage efforts to diminish disparities between these groups by focusing on increasing knowledge regarding healthy lifestyle and behavioural risk factors and also diminishing socioeconomic differences that impact on differences in access to medical care. In addition, the characterisation of these differing disease patterns is particularly essential in guiding the decentralised health and social systems on the effective targeting of limited resources.

— CHAPTER 4 —

Liver cancer mortality trends in South Africa: 1999-2015

Daniel Mak, Mazvita Sengayi, Wenlong C. Chen, Chantal Babb de Villiers, Elvira Singh, Anna Kramvis.

BMC Cancer Journal https://doi.org/10.1186/s12885-018-4695-9

References for literature cited in the following paper appear within the references section of the paper itself, and not necessarily in the references section of this thesis.

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In this chapter, population group variations in the mortality of LC were analysed using data for the whole population of SA during 1999 to 2015. This work is relevant because unlike incidence data, mortality data is not influenced by the availability of diagnostic. Important also is the fact that population-based relative ethnic distribution of LC mortality in SA has not previously been reported. This chapter of LC mortality trends will not only updates previously documented trends, but may provide insights into the impact of diagnostic and management of the LC disease burden during the last decade.

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Liver cancer mortality trends in South Africa: 1999–2015

Daniel Mak[1], Mazvita Sengayi[2,3], Wenlong C. Chen[2,5], Chantal Babb de Villiers[4], Elvira Singh[2,3*] and Anna Kramvis[1*]

Abstract

Background: In South Africa (SA), liver cancer (LC) is a public health problem and information is limited.

Methods: Joinpoint regression analysis was computed for the most recent LC mortality data from Statistics South Africa (StatsSA), by age group, sex and population group. The mortality-to-incidence ratios (MIRs) were calculated as the age-adjusted mortality rate divided by the age-adjusted incidence rate.

Results: From 1999 to 2015, the overall LC mortality significantly decreased in men (− 4.9%) and women (− 2.7%). Overall a significant decrease was noted in black African men aged 20–29 and 40–49 years, and white women older than 60 years but mortality rates increased among 50–59 and 60–69 year old black African men (from 2010/2009–2015) and women (from 2004/2009–2015). The mortality rates increased with age, and were higher among blacks Africans compared to whites in all age groups - with a peak black African-to-white mortality rate ratio of six in men and three in women at ages 30–39 years. The average MIR for black African men and women was 4 and 3.3 respectively, and 2.2 and 1.8 in their white counterparts. Moreover, decreasing LC mortality rates among younger and the increase in rates in older black Africans suggest that the nadir of the disease may be near or may have passed.

Conclusions: Findings of population-age subgroup variations in LC mortality and the number of underdiagnosed cases can inform surveillance efforts, while more extensive investigations of the aetiological risk factors are needed. Impact: There was a large race, sex and age differences in trends of LC mortality in SA. These findings should inform more extensive evaluation of the aetiology and risk factors of LC in the country in order to guide control efforts.

Keywords: Liver cancer, Trends, Mortality, Incidence, South Africa, Sub-Saharan Africa

Background

Liver cancer (LC) is the second largest contributor to cancer mortality worldwide [1], with 810,000 LC deaths recorded in 2015 [2]. The most common type of LC globally is hepatocellular carcinoma (HCC), accounting for 75 to 90% of primary LCs, followed by cholangiocarcinoma [3]. In 2015, close to 20.5 million years of healthy life [disability-adjusted life-years (DALYs)] were lost as a result of this cancer and thus it remains an important public health issue worldwide [2].

In the United States, population group disparities in LC mortality have been reported, with higher rates among African American young adults (35–49 years) and older ages (50–64 years) (2.0 and 18.6/100,000, respectively) compared to Whites (0.9 and 7.7/100,000, respectively) [4]. Invariably, African-to-white American mortality rate ratios are >1 in both men (1.7) and women (1.4) [5]. Declining LC mortality rates have been reported in young African and white Americans (5.3 and 2.8% annually, respectively) while those of older ages are increasing (by 6.2 and 6.3% annually, respectively). In America, Africans are more frequently diagnosed with LC at a younger age than Whites [6]. The former have larger tumour size, more advanced tumour stage/with metastatic disease, lower levels of alpha-fetoprotein and are least likely to present with cirrhosis [7, 8]. Reasons for these disparities have been attributed to differences

* Correspondence: Elvira.Singh@nhls.ac.za; Anna.Kramvis@wits.ac.za
[2]National Cancer Registry, National Health Laboratory Service, Johannesburg, South Africa
[1]Hepatitis Virus Diversity Research Unit (HVDRU), Department of Internal Medicine, School of Clinical Medicine, Faculty of Health Sciences, University of Witwatersrand, Johannesburg, South Africa
Full list of author information is available at the end of the article

in the prevalence of major risk factors including hepatitis B and/or C virus (HBV/HCV), obesity and diabetes [9], and to some degree, because of barriers to accessing high-quality care [10].

In contrast, data on LC mortality in South Africa (SA), and most regions of sub-Saharan Africa, are limited. The International Agency for Research on Cancer GLOBOCAN estimated 37,353 LC deaths occurred in this subcontinent in 2012 and predicted this number to increase to 64,525 by 2030 [1]. Thus, the present study aims to delineate the patterns and temporal trends of LC mortality in SA, based on currently available data from the national death registry from 1999 to 2015 by sex, age and population group.

Methods

Data sources and selection criteria

Cause of death was based on death certificate information reported to Statistics South Africa (StatsSA), a national statistical service that compiles routine mortality statistics based on medical certification of the cause of death registered with the Department of Home Affairs (DHA). LC (C22.0), as the first or underlying cause of death was selected according the ICD-10 for the period of 1999 to 2015. Analyses were restricted to four large population groups that were defined according to the SA National Census as Black Africans, White, Coloured (mixed ancestry) and Indian/Asian [11]. In 2015, each population group accounted for 80.5, 8.3, 8.8 and 2.5% of the SA population, respectively [12]. Since the majority of cases reported each year for the Coloured and Indian/Asian populations were fewer than 100, both these groups were excluded from further analysis.

Statistical analysis

Age-standardized incidence rates (ASIRs) and mortality rates (ASMRs) per 100,000 persons were calculated using mid-year population estimates provided by StatsSA and by direct standardization with the Segi's World Standard Population (1960) [13], by population group, sex and age group (summarized into 10-year age groups: 20–29, 30–39, 40–49, 50–59, 60–69 and 70+ years). Joinpoint regression trends were examined by population group, sex and age groups, based on a two joinpoint segment model [14]. Based on the criteria used in the National Cancer Institute's Cancer Trends Progress Report [15] and modified in a published report by Altekruse et al. [4], the trends were described as annual percentage change (APC) and categorized into five groups: 1: Significant decrease (APC < 0%, $p < 0.05$); 2: Non-significant decrease (APC < − 0.5%, $p > 0.05$); 3: Stable (Absolute value of rate change less than or equal to 0.5% per year, $p > 0.05$); 4: Non-significant increase (APC > + 0.5% per year, $p > 0.05$); and 5: Significant increase (APC > 0%, $p < 0.05$). The APC and average annual percentage change (AAPC) were calculated using Joinpoint software (Version 4.5.0.1) from the Surveillance Research Program of the US National Cancer Institute [16]. Statistical significance was taken at $p \leq 0.05$. The mortality-to-incidence ratios (MIRs) were calculated as the age-adjusted mortality rate divided by the age-adjusted incidence rate for LC from 1999 to 2012, and was used to compare population group and sex disparities. Age-Adjusted incidence rates for MI ratios were derived from the South African National Cancer Registry's pathology-based cancer surveillance system.

Results

Figure 1 shows the sex and population group-specific overall ASMR for LC in SA. The results of the joinpoint regression analysis, the APCs for each trend, and the AAPCs in both genders are depicted in Table 1. During the 17-year mortality study period, a total of 27,791 deaths from LC were reported. Of the total cases of known population group (22,435, 81%), majority were men (62.1%) and black African (73.2% vs 15.4% whites). Women died of LC at significantly older ages than men, with 83.5% versus 79.1% being older than 40 years, and 59.6% versus 50.1% being older than 60 years, respectively. LC ASMRs in men and women (overall) did not significantly change throughout the study period (5.3 and 2.1/100,000 in 1999 to 5.4 and 2.5/100,000 in 2015), with an AAPC of 0.3 and 0.8% ($p > 0.05$) respectively. The segmented joinpoint analysis identified a period of decrease in mortality rates (men: 2004–2009, APC of − 4.9%; women: 2002–2009, APC of − 2.7%), after which the rates began to increase (2009–2015, APCs: 3.4 and 2.6% respectively). In men, age-group analysis revealed that, during the entire study period, the mortality rates decreased significantly for all age groups >20 years old; whereas in women this only included 40–49 and >60 year olds. However, segmented joinpoint analysis by age groups distinguished between two different time periods, i.e. an initial period characterized by a significant decrease in 50–59 year old men (1999–2010) and 60–69 year old women (1999–2009), and a second period with an increase or levelling-off in rates until 2015.

Over the study period, population group analysis showed that black African men (6.2/100,000) had the highest LC ASMR, followed by white men (4.5/100,000), black African women (3.1/100,000) and white women (1.8/100,000) (Fig. 1). The corresponding black African-to-white LC mortality rate ratio increased and peaked at ages 30–39 years (6-fold in men and 3-fold in women), but decreased thereafter to around 1.0 in those >70 years old in both sexes (Fig. 2). Segmented joinpoint analyses of population-age subgroups by sex are shown in Table 2. In black Africans, a decreasing or stable trend in ASMRs was observed among

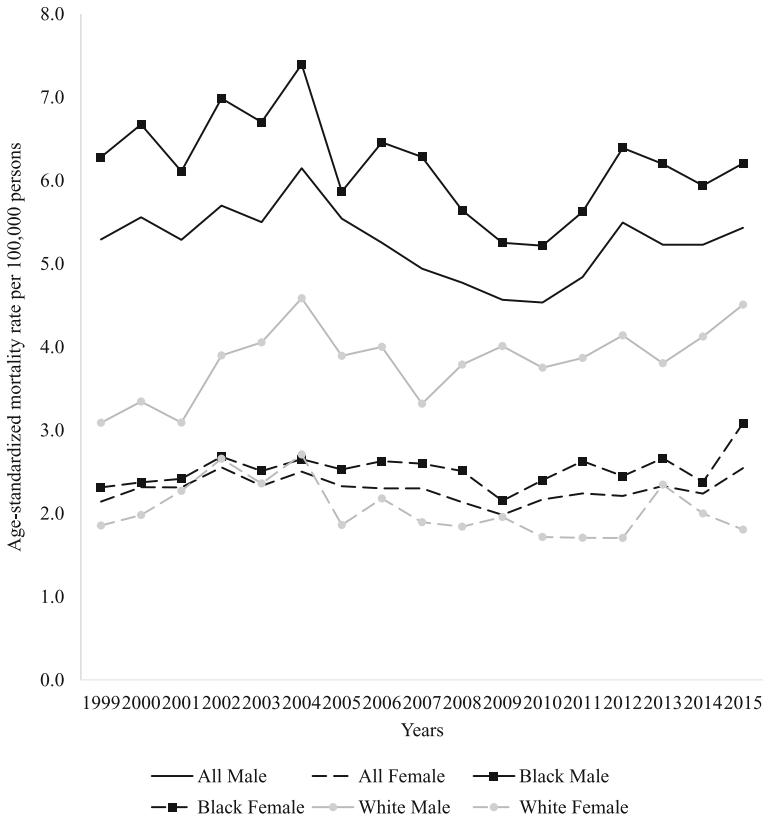

Fig. 1 Liver cancer age-standardized mortality rates in South Africa by population group and sex, 1999–2015

Table 1 Joinpoint analysis of age-standardised liver cancer mortality rates by sex and age group in South Africa; 1999–2015

All		Trend 1		Trend 2		Trend 3	
Age (Years)	AAPC (95% CI)	Range of Years	APC	Range of Years	APC	Range of Years	APC
Men							
Overall	0.3 (− 1.2, 1.8)	1999–2004	2.1 (− 0.7, 5)	2004–2009	−4.9* (− 8.6, − 1.1)	2009–2015	3.4* (1.2, 5.6)
20–29	−3.2* (− 4.9, − 1.4)	1999–2015	−3.2* (− 4.9, − 1.4)				
30–39	−1.8* (− 3, − 0.6)	1999–2015	−1.8* (− 3, − 0.6)				
40–49	−2.1* (− 3.2, − 1.1)	1999–2015	−2.1* (− 3.2, − 1.1)				
50–59	−1.8* (− 3.3, − 0.3)	1999–2010	− 3.7* (− 5, − 2.4)	2010–2015	2.6 (− 2, 7.3)		
60–69	−3.4* (− 4.4, − 2.4)	1999–2015	−3.4* (− 4.4, − 2.4)				
70+	− 2.3* (− 3.3, − 1.3)	1999–2015	−2.3* (− 3.3, − 1.3)				
Women							
Overall	0.8 (−0.6, 2.2)	1999–2002	5.6 (−0.6, 12.2)	2002–2009	−2.7* (− 4.7, − 0.7)	2009–2015	2.6* (0.6, 4.8)
20–29	−1.5 (− 3.1, 0.1)	1999–2015	−1.5 (− 3.1, 0.1)				
30–39	−1.4 (−3, 0.3)	1999–2015	− 1.4 (− 3, 0.3)				
40–49	−1.7* (− 2.4, − 1)	1999–2015	−1.7* (− 2.4, − 1)				
50–59	−0.9 (−1.9, 0)	1999–2015	− 0.9 (− 1.9, 0)				
60–69	−2.6* (− 3.9, − 1.3)	1999–2009	− 4.4* (− 5.8, − 3)	2009–2015	0.5 (− 2.7, 3.7)		
70+	− 2.3* (− 3, − 1.5)	1999–2015	−2.3* (− 3, − 1.5)				

*The average annual percentage change (AAPC) and/or annual percent change (APC) is statistically significant ($p < 0.05$)

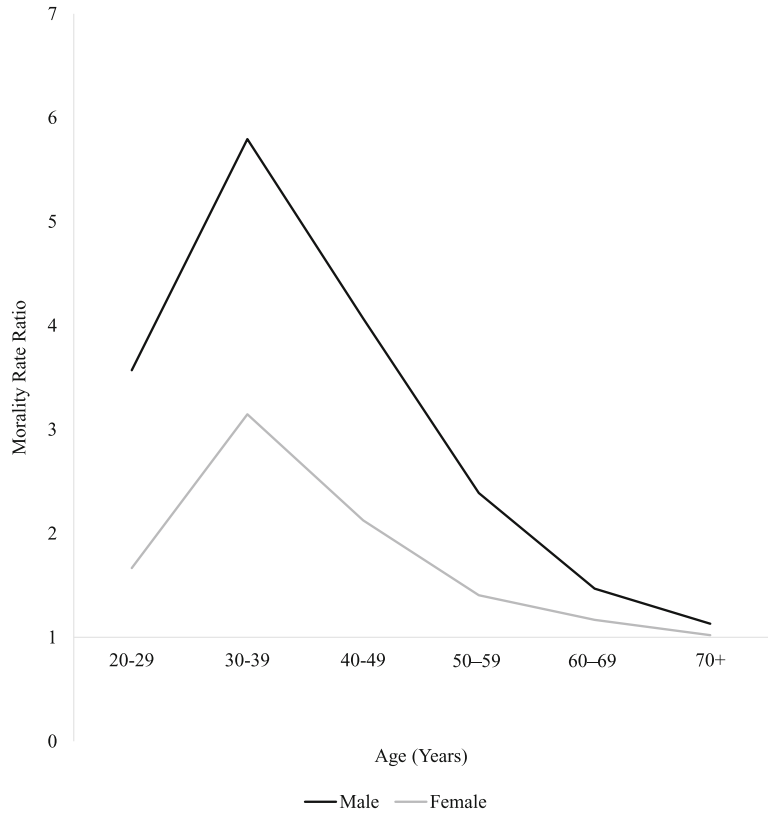

Fig. 2 Black African-to-white liver cancer mortality rate ratio in men and women, 1999–2015

younger (<50 year old) and (>70 year) old men and women from 1999 through 2015. Interestingly, joinpoint analysis identified two different periods for older black Africans (50–59 and 60–69 year old). After an initial period of decreasing ASMRs, a marked non-significant increase was found thereafter in men (5.4 and 2.5% respectively, $p > 0.05$), but was significant in women (2.6 and 6.3% respectively, $p < 0.05$). In whites, age-group analysis revealed that, throughout the entire study period, ASMRs either remained stable (men: 40–69 years; women: 40–49 years) or decreased (men: >70 years; women: >50 years). For both the black African and white populations, mortality rates were higher than incidence rates reported to the South African National Cancer Registry, yielding an average MIR of 4 and 3.3 in black African men and women, and 2.2 and 1.8 white men and women (Fig. 3).

Discussion

The data-set used (from StatsSA) is considered to provide the most comprehensive and representative information on mortality patterns in the country. Our analysis highlights an overall sociodemographic shift in LC mortality in SA over the past two decades. More specifically, there was a shift from a decreasing to an increasing trend in LC mortality among older black Africans (50–69 years). In contrast, mortality trends were stable and decreasing in the white population of the same sex and age groups. Black Africans were more likely to die from LC compared to whites. These racial differences in LC mortality were highest in the 30–39 year old age group with black African-to-white mortality ratios of six in men and three in women. For both the black African and white populations, mortality rates were higher than incidence, yielding an average MIRs of 4 and 3.3 in men and women in the former, and 2.2 and 1.8 in the latter population. Data of most individuals with LC-related deaths were not captured in the South African National Cancer Registry's pathology-based cancer registry. To the best of our knowledge, this is the first such analysis of time trends that demonstrated a LC mortality differential between the black African and white populations by sex nationwide.

In SA, accounting for population-age subgroup disparities in LC mortality is likely multifaceted and may be a consequence of socio-cultural, biological and socioeconomic factors. While the establishment of policies in the mid-1990s dealing with better quality and more accessible health care services may have improved overall patient survival over time [17], and ultimately decreasing mortality as a consequence of increased utilization of

Table 2 Age-adjusted liver cancer mortality rates joinpoint trends by population group, sex and age group in South Africa; 1999–2015

Direction	Sex	Population Group	Age, Years	Trend 1		Trend 2	
				Period	APC (95% CI)	Period	APC (95% CI)
1. Significant Decrease							
	Men						
		Black	20–29	1999–2015	− 2.3* (− 4.2, − 0.4)		
		Black	40–49	1999–2015	− 1.1* (− 2.2, 0)		
	Women						
		White	60–69	1999–2015	−2.6* (− 4.9, − 0.3)		
		White	70+	1999–2015	−3.5* (− 4.9, − 2.1)		
2. Non-significant Decrease							
	Men	Black	70+	1999–2015	−1.3 (− 2.5, 0)		
		White	70+	1999–2015	−0.7 (− 2.2, 0.9)		
	Women						
		Black	20–29	1999–2015	−1.5 (−4.2, 1.4)		
		Black	40–49	1999–2015	−0.9 (− 2, 0.1)		
		White	50–59	1999–2015	−0.9 (−3.8, 2.1)		
3. Stable							
	Men						
		Black	30–39	1999–2015	−0.2 (−1.5, 1.1)		
		White	40–49	1999–2015	−0.5 (−4.5, 3.6)		
		White	50–59	1999–2015	−0.1 (−2.6, 2.5)		
		White	60–69	1999–2015	0.4 (−1.2, 2)		
	Women						
		Black	30–39	1999–2015	−0.2 (−1.9, 1.6)		
		Black	70+	1999–2015	−0.1 (−1.2, 1)		
		White	40–49	1999–2015	−0.2 (−8.9, 9.2)		
4. Non-significant Increase							
	Men						
		Black	50–59	1999–2010	−3.7* (−5.3, −2.2)	2010–2015	5.4 (− 0.1, 11.2)
		Black	60–69	1999–2009	−5.2* (−7.3, − 3)	2009–2015	2.5 (− 2.4, 7.6)
5. Significant Increase							
	Women						
		Black	50–59	1999–2004	−5.2 (− 10.4, 0.4)	2004–2015	2.6* (0.8, 4.4)
		Black	60–69	1999–2009	−4.9* (− 6.9, − 2.8)	2009–2015	6.3* (1.5, 11.3)

*The annual percent change (APC) is statistically significant ($p < 0.05$)

these services and the better application of early detection strategies, other factors may impede the ability to seek and obtain timely services [18]. For example, evidence from earlier studies in SA show that the bottom segment of the distributions in income [19], education/literacy [20], dependence on public health care programs [21] are dominated by black Africans and health insurance coverage shares are far from proportionate with population shares (73.3% and 10.6% of white and black Africans with health insurance in 2015, respectively) [20]. In a study in SA, the authors reported that of the 22 patients with HCC who received surgical treatment, the surgical rates were lower for black Africans [22]. While it is possible that cultural beliefs and attitudes may have impacted on the appropriate treatment decision-making [23], other reports have attributed this disparity to differences in the biology and presentation of the disease [24–26]. Previous studies have suggested that black Africans are more likely to be diagnosed symptomatically in the absence of any screening

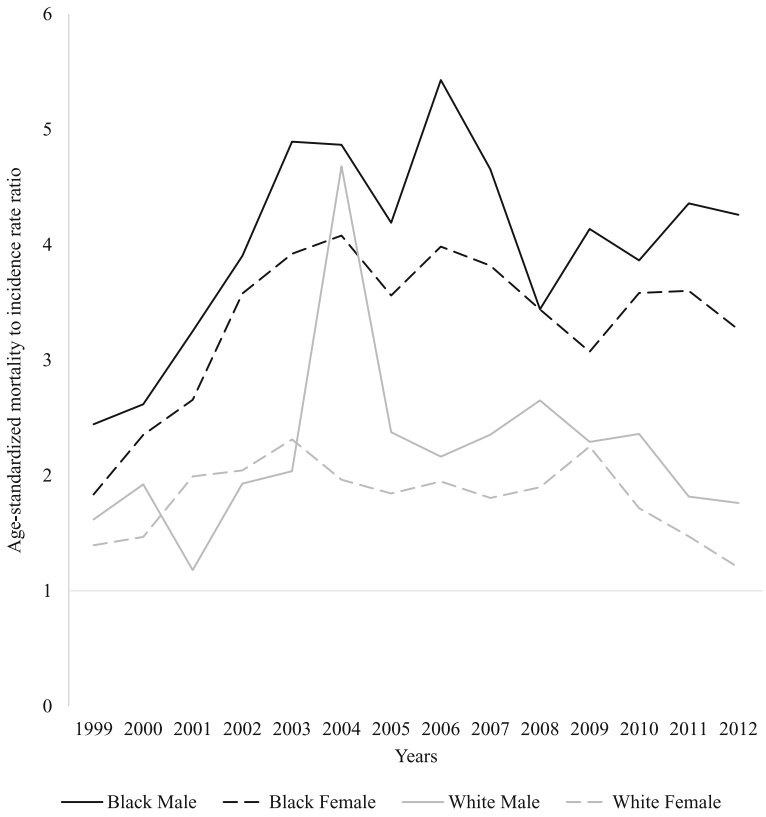

Fig. 3 Liver cancer mortality-incidence rate ratio by population group and sex, 1999–2012

methods, at a later stage of disease with larger tumours (80–90%) [24, 25], and when curative treatments are no longer feasible [26].

Alternatively, changes in the prevalence of risk factors may play a role. For example it is well recognized that HBV is a primary risk factor and its prevalence differs among black Africans (4–16%) and whites (0.2%) in the same manner as does the burden of LC in SA [27, 28]. While the vaccine against HBV has been shown to be efficacious at reducing HBV infections, and ultimately HCC, among vaccinated cohorts of children [29, 30], they remain ineffective for most chronically infected adults born prior to its incorporation into the Expanded Programme on Immunisation in 1995. Comparisons of a study in the 1990s [31] to that of the 2000s [32] showed that HBV-infected HCC cases are generally diagnosed in their 30s, and the HBsAg rates in HCC patients have increased in men (38.2% versus 43.2%, respectively) and women (24.1% versus 35.9%, respectively). In contrast, HCV infection is relatively uncommon in SA (0.75% and 0.16% in black Africans and whites, respectively) [33], but affects older patients who are in their 50s [31]. Unlike HBV infection, HCV-positivity in HCC patients decreased (28.7% versus 5.4%) from the 1990s [31] to 2000s [32].

Data on other potential factors associated with lifestyle behaviours (obesity, diabetes, alcohol consumption and dietary exposure to aflatoxin) influencing LC rates are limited in SA, with our recent study showing minimal effects [32]. Thus it is difficult to determine which of these factors predominantly influenced the trends in LC burden, because the population attributable fraction of these factors is not as large as hepatitis infection. Future studies should explore the impact of these aetiological factors on LC mortality and incidence at an individual level.

In SA, it is clear that human immuno-deficiency virus (HIV) epidemic has had a major demographic and health impact particularly in black African population. Findings from previous studies from SA and Uganda that have failed to show increases in HCC risk among HIV-infected persons through the pre-ART years (pre-2004 in SA), possibly as a result of the competing risks of AIDS-related deaths [34, 35]. However, an increase in LC cases has been noted in the ART era in developed countries [36]. Our results show an upswing in LC mortality rates in black Africans during the inception of ART in 2004 [37] (number of adults accessing ART was 4.9%) [38] and the massively scaled up program in 2009 (61%) [39]. In Uganda, a study reported that with each

10% increase in ART coverage, LC incidence increased by 12% [40]. It has been suggested that the change in the spectrum of liver disease is shifting from opportunistic infections to sequelae of chronic HBV/HCV infections, medication toxicities, alcoholism, and fatty liver [41], possibly as a result of increased longevity. Although these associations need to be further investigated in SA, it is conceivable that results presented here may be interpreted as revealing an effect of ART given that both the number of people in SA living with HIV, and those with access to ART are among the highest in the world [42]. Findings from this study raise considerable concern that the burden of LC may expand concurrently with the implementation of an universal ART eligibility to all 7.1 million HIV-positive South Africans from 2016 [43], further highlighting the need for a comprehensive program focused on population-based cancer surveillance and control including LC in SA [44].

In a region characterized by the poor surveillance systems, tracking the emerging trends in the death rates is an essential tool to assist with cancer-control planning, early detection, and prevention efforts. Additionally, because preventing deaths must be an important part of any competent cancer prevention and control effort, the statistics described in this study also address an important need – to describe and highlight the knowledge gap of LC mortality in relation to incidence. The MIRs of LC in men and women in our study (black African: 4 and 3.3; white: 2.2 and 1.8, respectively) was significantly great compared that reported in another HBV endemic region, China (0.91 and 0.92, respectively) [45]. Given the high MIR ratios reported in our study, pathology-based cancer registration is inadequate to characterise LC epidemiology in SA. The establishment of a nationally representative population-based cancer registration should be a priority for cancer control in SA as it will capture cases of LC diagnosed clinically and radiologically in addition to laboratory based diagnoses.

Several potential limitations should be acknowledged. There have been considerable improvements in the national coverage and completeness of death registration, but shortcomings of the quality of death certification have been suggested in ill-defined and misattributed causes of single-cause data [46]. Considering that the liver is a frequent site of cancer metastasis, cause of death determination using death certificates may result in false-positives in the absence of biopsy [47]. Additionally, failure or under-ascertainment of cause-of-death could lead to bias in death registration, particularly in resource-limited regions [39]. Despite regular census data being available, concerns about the accuracy of earlier population estimates may suggest the possible introduction of uncertainty in estimated mortality rates [12]. The number of LC-related deaths may be overestimated because StatsSA collects and processes death information irrespective of the deceased's citizenship. The livelihood conditions in SA make it an attractive destination for migration and therefore cross border migrations are frequent [48]. On the other hand, the inability of the SA health-system to meet the health needs of the migrants means that when their health deteriorates they return to their place of origin to seek healthcare and support [49]. Trends analyses on LC rates in the current study were descriptive analyses at population levels without inference at individual levels. Because data at individual level on socioeconomic status and various potential confounders were not available, interpretations from the results do not necessarily hold true for individuals.

Conclusion

In summary, this is the first study to estimate the total burden of liver cancer deaths in SA. Our results indicate a rising trend for this disease in middle to older aged black African men and women in the recent decade. The reasons for this are likely manifold, but may be a consequence of improved life expectancy and rising prevalence of risk factors related to lifestyle behaviours. Further research into the aetiologic factors contributing to population-age sub-group mortality patterns are needed, to provide informative interventions to curb the rising burden of liver cancer deaths and to reduce the population group disparities. Our findings may be used to inform national health and social development policies in SA and other regions of southern Africa.

Abbreviations

AAPC: Average annual percentage change; APC: Annual percentage change; ASIR: Age-standardized incidence rate; ASMR: Age-standardized mortality rate; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HIV: Human immuno-deficiency virus; LC: Liver Cancer; MIR: Mortality-to-incidence ratio; SA: South Africa; StatsSA: Statistics South Africa

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Availability of data and materials

The datasets supporting the conclusions of this article are available in the Statistics South Africa and South African National Cancer Registry repository, [http://www.statssa.gov.za (upon request) and http://www.nicd.ac.za/index.php/centres/national-cancer-registry/, respectively].

Authors' contributions

DM has been involved in all stages of the study including the conception and design of the study, literature review, data management and analysis, data interpretation, and the writing, review and/or revision of the manuscript. MS and WCC were involved in the acquisition of the data, data interpretation and the critical review and revision of the manuscript. CBdV initiated the research idea and was involved in the data interpretation critical review and revision of the manuscript. AK and ES were involved in the study

supervision, data interpretation critical review and revision of the manuscript. All authors gave final approval of the version to be published.

Ethics approval and consent to participate
Ethical approval (#M150239) was obtained from the Human Research Ethics Committee (Medical), the University of Witwatersrand, Johannesburg, South Africa.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher's Note

Author details
[1]Hepatitis Virus Diversity Research Unit (HVDRU), Department of Internal Medicine, School of Clinical Medicine, Faculty of Health Sciences, University of Witwatersrand, Johannesburg, South Africa. [2]National Cancer Registry, National Health Laboratory Service, Johannesburg, South Africa. [3]School of Public Health, University of the Witwatersrand, Johannesburg, South Africa. [4]Division of Human Genetics, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa. [5]Sydney Brenner Institute for Molecular Bioscience, University of the Witwatersrand, Johannesburg, South Africa.

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— CHAPTER 5 —

Analysis of Risk Factors Associated with Hepatocellular Carcinoma in Black South Africans: 2000 - 2012

Daniel Mak, Chantal Babb de Villiers, Charles Chasela, Margaret I. Urban, Anna Kramvis

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References for literature cited in the following paper appear within the references section of the paper itself, and not necessarily in the references section of this thesis.

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HCC remains a complex disease in that risk factors associated with this malignancy is at a constant flux. For instance, while HBV is historically associated with a large proportion of HCC cases in SA, the implementation of the hepatitis B vaccination programme has proven to be effective in reducing the prevalence of HBV infection among children (Burnett et al. 2012), and the rollout of ART for HIV during the same period may have also played a role in reducing the risk of developing HBV-related HCC in adults. Apart from these efforts to control HBV infection, the government has also made other efforts in minimizing aflatoxin intake, while little is known of the role in which lifestyle behaviours in the development of HCC. As such, the study described in this chapter provides for an up to date risk analysis of viral infection in the risk of developing HCC, while also assessing the risk associated with lifestyle behaviours..

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RESEARCH ARTICLE

Analysis of risk factors associated with hepatocellular carcinoma in black South Africans: 2000–2012

Daniel Mak[1], Chantal Babb de Villiers[2,3¤], Charles Chasela[4,5,6], Margaret I. Urban[2], Anna Kramvis[1]*

1 Hepatitis Virus Diversity Research Unit (HVDRU), Department of Internal Medicine, School of Clinical Medicine, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa, **2** Cancer Epidemiology Research Group, National Cancer Registry, National Health Laboratory Service, Johannesburg, South Africa, **3** Division of Human Genetics, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa, **4** Epidemiology and Strategic Information (ESI), HIV/AIDS, STIS and TB (HAST), Human Sciences Research Council (HSRC), Pretoria, South Africa, **5** Department of Epidemiology and Biostatistics, School of Public Health, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa, **6** Right to Care, EQUIP HEALTH, Centurion, South Africa

¤ Current address: Department of Public Health and Primary Care, Primary Care Unit, University of Cambridge, Cambridge, United Kingdom
* Anna.Kramvis@wits.ac.za

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Abstract

Objective

The aims of this study were to determine the prevalence of risk factors associated with hepatocellular carcinoma (HCC) in black adult South Africans and to estimate the size of the associated risks.

Methods

A case-control analysis of 150 black South African patients (aged 18–75 years) with HCC—who were a subset of patients recruited for the Johannesburg Cancer Case Control Study 2000 to 2012—was undertaken. The association between this tumour and hepatitis B/C virus infections, and human immunodeficiency virus (HIV) mono- and co-infections was investigated. Odds ratios (ORs) and 95% confidence intervals (CI) adjusted for age, year of diagnosis, marital status, place of birth and selected modifiable risk factors were calculated.

Results

HCC was significantly associated with a rural birthplace ($p<0.05$), being male and living in an urban area for 14 years or less. The Odds Ratio (OR) for HCC increased significantly with HBV DNA+/HBsAg+ (OR 34.5; CI:16.26–73.13), HBV DNA+/HBsAg- (OR 3.76; CI:1.79–7.92), HBV DNA level >2000 IU/ml (OR 8.55; CI:3.00–24.54) to ≥200,000 (OR 16.93; CI:8.65–33.13), anti-HCV (OR 8.98; CI:3.59–22.46), HBV DNA+/HIV+ co-infection (OR 5.36; CI:2.59–11.11), but not with HBV DNA-/HIV+ (OR 0.34; CI:0.14–0.85). We did not find a synergistic interaction between HBV and HIV. Modifiable risk factors (alcohol

consumption, tobacco smoking, number of sexual partners, diabetes and hormonal contraceptive use) were nonsignificant.

Discussion

A considerable portion of the HCC burden in Johannesburg and surrounding provinces falls on rural migrants to urban areas, most of whom are men. The HBV will continue to contribute to HCC incidence in older age-groups and in others who missed vaccination. Although we did not find an increased risk for HCC in HIV positive individuals this may change as life expectancy increases due to greater access to antiretroviral therapies, necessitating the addition of hepatitis virus screening to preventive medical care.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and second leading cause of cancer-related mortality worldwide (745,000 deaths or 9.1% of the total cancer deaths) [1]. Approximately 80% of HCC cases occur in developing regions including sub-Saharan Africa and southeast Asia [1], and is usually associated with poor prognosis (5-year survival of 14% from diagnosis) [2]. Surgical interventions reportedly have a high recurrence (81%) of HCC and a low 5-year survival rate (38%) in South Africa (SA) [3].

In sub-Saharan Africa, epidemiological studies show that the most common aetiological risk factors for HCC include hepatitis B virus (HBV), hepatitis C virus (HCV), and dietary exposure to the fungal hepatocarcinogen aflatoxin B_1 [4]. Total aflatoxin levels in SA commercial foods are regulated by Section 15(1) of the Foodstuffs, Cosmetics and Disinfectants Act, 1972. Regardless of this, significant aflatoxin contamination does occur in stored crops such as maize and groundnuts grown and stored for family or local consumption; the problem is weather-dependent and expected to increase with increasing climate variability [5].

The World Health Organization (WHO) estimated that, in 2015, 257 million people were infected with HBV and 70 million people with HCV, of whom 60 million and 11 million resided in Africa, respectively [6]. In SA HBV is a well-recognized risk factor for HCC. HBV infection is characterized by childhood acquisition via horizontal transmission, male predominance and a high prevalence in rural-born black Africans [7]. Hepatitis B vaccine, introduced into the SA Expanded Programme on Immunisation in 1995, has been shown to effectively control the spread of HBV in children [8].

In recent decades human immunodeficiency virus (HIV) infected persons co-infected with hepatitis virus (HBV/HCV) have become a serious public health concern internationally due to a significantly higher risk of HCC development [9] and mortality [10]. Most of these studies were carried out in regions of low HBV endemicity where infections with HIV and hepatitis B/C viruses occur at more or less the same age (sexual maturity) and are mostly limited to certain high-risk groups [11]. It is unknown whether the effects of HIV on HCC risk are the same when it is acquired years after a chronic HBV infection is established, as is the case in sub-Saharan African countries [12].

Thus, the aim of this study was to determine the prevalence of risk factors for HCC development in black South African adults born prior to the introduction of routine HBV vaccination in 1995, as part of the Expanded Programme on Immunisation [8]. These factors included both viral factors (HBV/HCV; HIV) and potentially modifiable risk factors including alcohol consumption, tobacco smoking, self-reported diabetes and hormonal contraception use.

Materials and methods

Study design and setting

This retrospective analysis consisted of a subset of participants enrolled in the Johannesburg Cancer Case Control Study (JCCCS), a study that recruited self-identified black African (not mixed ancestry) cancer patients in the greater Johannesburg, Gauteng province, public referral hospitals that offer oncology services. Prior to commencement of cancer treatment, a written or witnessed verbal consent was obtained for interview and for having blood drawn for future assessment of factors of interest. Face to face interviews were conducted by trained research nurses who assigned a study number to participants. Each participant also gave consent to have records reviewed by the research nurses who collected the required information for the cancer diagnosis confirmation. Blood specimens were independently anonymously tested for HIV infection and stored at the National Health Laboratory Service as previously described [13]. All patient information provided to researchers was only identified by the study number.

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval granted by the University of the Witwatersrand Human Research Ethics Committee (Medical) (M150239; M140271).

Eligibility criteria

Newly diagnosed with primary HCC (ICD-O: 8170/3 and ICD10: C22), with no prior history of any cancer, was enrolled from 1 September 2000 to 31 December 2012. A total of 158 HCC cases, all with available stored pretreatment serum, were included. Fifty-one (34%) were confirmed by histology, 31 (20.7%) by cytology, 57 (38%) by elevated alpha fetoprotein levels (>450 ng/ml) and/or ultrasound (4) or CT scan (1), with the remaining cases (7.3%) confirmed as HCC by physicians. In the time period of the analysis only 8 potential HCC cases were excluded: 4 because patient files for confirmation of diagnosis could not be located, 3 because only a discharge or transfer letter indicating the HCC diagnosis was available, and 1 because the tumour was a secondary cancer.

For each HCC case three matched controls were randomly selected from the JCCCS database, matched by recruitment hospital, sex, and age at the time of interview (± 3 years). Following previously published criteria controls were included on the basis of confirmed malignant neoplasms that are not known to have a possible infectious cause [14]. Cancers with ill-defined, secondary and unspecified sites of carcinoma were also excluded. Twelve (2.7%) potential controls could not be used because sera were depleted.

It is highly unlikely that any of the participants were vaccinated against HBV because all participants were born prior to 1995, the year HBV vaccination, at 6, 10 and 14 weeks of age was introduced into the South African Expanded Programmed on Immunisation [8].

Laboratory testing

Enzyme-linked immunosorbent assay kits were used to test for serological markers of anti-HBc (Murex® Anti-HBc Total, Abbott Diagnostics, UK) and anti-HCV (Murex anti-HCV 4.0 ELISA, Abbott Diagnostics, UK). All HBV-DNA positive samples were tested for HBsAg (Murex HBsAg v3.0, Abbott Diagnostics, UK), and all HBsAg-positive further tested for HBeAg (ETI-EBK PLUS; DiaSorin).

HBV-DNA was extracted from 200 μl of serum using a QIAamp® DNA Blood Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. HBV-DNA detection and quantification were performed as described previously [15]. Briefly, quantitative real-time PCR (RT-PCR) was performed in a 25 μl total volume using 12.5μl of 2X EagleTaq Universal

Master Mix with ROX (Roche Applied Science, Basel, Switzerland), 10 μM HBV-Taq1 forward primer, 10μM HBV-Taq2, 10 μM BS-1 probe and 2 μl of DNA. Thermal cycling was performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Inc., Hercules, CA) as follows: 1 cycle of 2 min at 50˚C and 10 min at 95˚C, followed by 45 cycles of 95˚C for 15 sec and 60˚C for 1 min. HBV-DNA concentration was calculated using a standard of cloned plasmid full-genome HBV-DNA, with concentrations ranging from $5x10^2$ to $5x10^7$ IU/ml. Calibration of this standard was confirmed by comparison with an World Health Organization (WHO) International HBV-DNA Standard (product code 97/750 NIBSC; Hertfordshire, UK). The averaged copies/ml value was divided by 4.7 to convert copies/ml to IU/ml, with a detection limit of ~20 IU/ml or 94 copies/ml [16]. The blank, positive controls, negative controls, and samples were all tested in duplicate; the standard curve samples were tested in triplicate.

Risk factor classification

Modifiable risk factors—alcohol consumption, tobacco smoking and hormonal contraceptive use—included in this study have been previously described [17, 18]. Briefly, current smokers, including those who had stopped smoking within 5 years of date of interview, were classified according to the daily number of cigarettes smoked, and were subdivided into light (1–14 g/day) and heavy (≥15 g/day) smokers. Those who stopped smoking more than 5 years prior to the date of interview were classified as former smokers. Current alcohol drinkers were subdivided into categories based on the frequency of alcohol consumed per week assuming 1 serving of an alcoholic beverage contains 10g of ethanol: non-drinkers (<1 drink/week); moderate drinkers (1–7 drinks/week for women, 1–14 drinks/week for men; heavy drinkers– 8 or more drinks/week or 4 or more drinks in a single occasion for women, 15 or more drinks/week or 5 or more drinks in a single occasion for men [19]. HBV-DNA levels were divided into HBV-DNA categories according to the South African guidelines for the management of chronic HBV infection [20]. Occult HBV infection (OBI) was characterised by the presence of HBV-DNA in HBsAg-negative individuals with or without serological markers of HBV infection [21]. Use of antiretroviral therapy (ART) was only included in the questionnaire from November 2004; thus this information was excluded from analysis.

Statistical analyses

Continuous variables such as the age of participants were summarized as means (standard deviation); HBV-DNA levels (>20 IU/mL) were summarized as medians (interquartile range). Chi-square and Fisher's exact (for sparse data) were used to evaluate differences among and between the case and the control groups. The Shapiro-Wilk test was used to test for normal distribution of quantitative values. All non-normal distributions were analysed with the Mann-Whitney test. Conditional logistic regression models were used to evaluate the impact of independent predictors of the outcome as odds ratios (OR) with 95% confidence intervals (CI) and assessment of potential confounding factors including sex, age group, country and province of birth, number of years lived in urban area (0–14, 15–29 and ≥30 years), place of birth (urban or rural), alcohol consumption, HIV status and anti-HCV positivity. Adjustment of other potential HCC confounders, including marital status, smoking, self-reported diabetes, hormonal contraception use and the number of lifetime sexual partners, did not significantly change the estimates significantly and were therefore not included in the model. Statistical significance was defined as p-value<0.05. All analysis was done using STATA software, version 13.1 (StataCorp. 2013. *Stata Statistical Software*: *Release 13*. College Station, TX: StataCorp LP).

Results

Table 1 shows the demographic characteristics and viral infection status of the HCC and control participants. The majority of participants were born in SA (88.9%) and close to two thirds were born outside the Gauteng Province (60.4%). HCC cases were more likely to be male (74%) and rural-born (65.3%, p = 0.03). The male-to-female ratio was higher for the rural-born (4.2:1) than urban-born participants (1.6:1), and in the older population (>40 years old) born in rural areas (5:1), compared to their younger counterparts (≤40 years old) (3:1). In contrast, urban-born HCC cases reported a more equal male-to-female ratio in the two age groups (1.5:1 and 1.7:1 for >40 and ≤40 years old, respectively). The difference between the HCC cases and controls in terms of the number of years recently lived in an urban environment was also statistically significant, with approximately half of all HCC cases (48%) having lived in an urban area for only 0–14 years, compared to about a third of controls (34.9%, p = 0.01). The prevalence of HBV DNA and anti-HCV antibodies was significantly higher in HCC cases than in the controls (p<0.001), while HIV-positivity did not differ statistically between the groups (18.7% vs 25.8%, p = 0.08).

Of all the 588 participants, 315 (53.6%) were anti-HBc-positive and 136 (23.1%) were HBV DNA-positive (14.1% and 8.8% of HBV DNA-positive were HBsAg-positive HBsAg-negative, respectively). Of the 150 HCC cases, HBsAg positivity was significantly higher in men than in women (77.5% vs 22.5%, respectively). HBsAg- and HBeAg-positivity was significantly different between HCC cases and controls (HBsAg: 41.3% and 4.8%, respectively, p<0.001; HBeAg: 12% and 0.9%, respectively, p<0.05). Table 2 suggests that the increase in the risk for HCC was associated with some combination of HBV markers—anti-HBc, HBV-DNA and/or HBsAg. The multivariate analysis of HBV markers did not meaningfully differ from those observed in univariate analysis. Relative to those who tested negative for all markers, the risk for HCC among HBV DNA-positive participants who were HBsAg-positive or HBsAg-negative, was estimated at OR 34.48 (95% CI: 16.26–73.13) and OR 3.76 (95% CI: 1.79–7.92) respectively, with or without detectable anti-HBc. The risk was highest when all three HBV markers (HBsAg, HBV DNA and anti-HBc) were present (OR 46.71, 95% CI: 21.00–103.90).

To investigate the relationship between HBV-DNA levels and HCC development participants were divided into HBV-DNA categories according to the South African guidelines for the management of chronic HBV infection [20] and as shown in Table 3. Of the 150 HCC cases, 80 (53.3%) had detectable levels of serum HBV-DNA, of which 68 (85%) had HBV-DNA levels ≥20,000 and less than 200,000 IU/mL and 41 (51.3%) had greater than or equal to 200,000 IU/mL. Comparable levels in the control participants accounted for 12.8%, 62.5% and 37.5%. An increasing trend in OR was noted with increasing viremia, and was statistically significant from ≥2000 IU/mL (OR 8.55, 95% CI: 3.00–24.54) to ≥200,000 IU/mL (OR 16.93, 95% CI: 8.65–33.13) (Table 3).

Table 4 shows the independent and combined effect of HBV and/or HCV, with and without the presence of HIV infection. The excess estimated OR, as risk for HCC development, in individuals with HBV-DNA positivity and HIV infection did not exceed the sum of the relative excess risks for each risk factor alone: 5.36 < (9.09–1.00) + (0.39–1.00). Using our data HBV/HIV co-infection has little effect on risk for the development of HCC. As few HCC and control participants were anti-HCV-positive, the joint effect of HCV with HBV and/or HIV could not be accurately estimated. Irrespective of HBV serological markers HCV-positive participants from the current study were, on average, close to 20 years older than those who were anti-HCV negative (61.9 vs 44.9 years) whereas HBsAg-positive participants were approximately 10 years younger than those who were HBsAg-negative (39.4 vs 46.7 years). HIV-positive HCC cases were significantly older than the HIV-positive controls (43.3 vs. 38.1 years, respectively,

Table 1. Demographic and virological characteristics: HCC cases and age- and sex-matched control participants from the Johannesburg Cancer Case Control Study, 2000 to 2012.

Characteristic	Total	HCC	Control	p-value
	N (%)	N (%)	N (%)	
Age				0.97
18–29	65 (11.1)	15 (10.0)	50 (11.4)	
30–39	150 (25.5)	37 (24.7)	113 (25.8)	
40–49	142 (24.1)	37 (24.7)	105 (24.0)	
50–59	120 (20.4)	33 (22.0)	87 (19.9)	
≥ 60	111 (18.9)	28 (18.7)	83 (18.9)	
Missing Data	0 (0.0)	0 (0.0)	0 (0.0)	
mean (SD)	45.8±13.3	46.1±13.3	45.7±13.3	
Sex				1.00
Female	153 (26.0)	39 (26.0)	114 (26.0)	
Male	435 (74.0)	111 (74.0)	324 (74.0)	
Missing Data	0 (0.0)	0 (0.0)	0 (0.0)	
Year of Interview				0.51
2000–2004	192 (32.7)	48 (32.0)	144 (32.9)	
2005–2008	194 (33.0)	45 (30.0)	149 (34.0)	
2009–2012	202 (34.4)	57 (38.0)	145 (33.1)	
Missing Data	0 (0.0)	0 (0.0)	0 (0.0)	
Marital Status				0.37
Married/living as married	362 (61.6)	99 (60.0)	263 (60.0)	
Separated	60 (10.2)	10 (6.7)	50 (11.4)	
Single/ Never Married	111 (18.9)	28 (18.7)	83 (18.9)	
Widowed	53 (9.0)	13 (8.7)	40 (9.1)	
Missing Data	2 (0.3)	0 (0.0)	2 (0.5)	
Country of Birth				<0.001
Outside South Africa	65 (11.1)	29 (19.3)	36 (8.2)	
South Africa	523 (88.9)	121 (80.7)	402 (91.8)	
Missing Data	0 (0.0)	0 (0.0)	0 (0.0)	
Province of Birth				0.21
Gauteng	207 (39.8)	42 (28.0)	165 (37.7)	
Other Provinces	316 (60.4)	79 (52.7)	237 (54.1)	
Missing Data	0 (0.0)	0 (0.0)	0 (0.0)	
Place of Birth				0.03
Rural	346 (57.7)	98 (65.3)	241 (55.2)	
Urban	253 (42.2)	52 (34.7)	196 (44.9)	
Missing Data	1 (0.2)	0 (0.0)	1 (0.2)	
Place of Residence				0.15
Rural	55 (9.4)	11 (7.3)	44 (10.0)	
Urban	532 (90.5)	138 (92.0)	394 (90.0)	
Missing Data	1 (0.2)	1 (0.7)	0 (0.0)	
Years lived in Urban				0.01
0–14 years	225 (38.3)	72 (48.0)	153 (34.9)	
15–29 years	113 (19.2)	27 (18.0)	86 (19.6)	
≥30 years	247 (42.0)	50 (33.3)	197 (45.0)	
Missing Data	3 (0.5)	1 (0.7)	2 (0.5)	
HIV Status				0.08

(*Continued*)

Table 1. (Continued)

Characteristic	Total	HCC	Control	p-value
	N (%)	N (%)	N (%)	
Positive	141 (24)	28 (18.7)	113 (25.8)	
Negative	447 (76)	122 (81.3)	325 (74.2)	
Missing Data	0 (0.0)	0 (0.0)	0 (0.0)	
HBV DNA Status				<0.001
Positive	136 (23.1)	80 (53.3)	56 (12.8)	
Negative	452 (76.9)	70 (46.7)	382 (87.2)	
Missing Data	0 (0.0)	0 (0.0)	0 (0.0)	
Anti-HCV Status				<0.001
Positive	32 (5.4)	19 (12.7)	13 (3.0)	
Negative	550 (93.5)	131 (87.3)	419 (95.7)	
Missing Data	6 (1.0)	0 (0.0)	6 (1.4)	

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p = 0.01). Of the 39 HIV-positive individuals with detectable HBV-DNA, 25 (64.1%) were HBsAg-positive and 14 (35.9%) were HBsAg-negative (p = 0.69). HBV-DNA levels were significantly higher in HBV-HIV co-infected participants, compared to HBV mono-infected—median [IQR] log10 titre: 6.4 (5.5–7.3) vs 5.3 (4.9–6.7), respectively (p = 0.007).

Table 2. Hepatitis B viral markers and the associated odds ratio (OR) for HCC in black South Africans, 2000–2012.

No.	Anti-HBc	HBV DNA	HBsAg	HCC (N = 150) (%)	Controls (N = 437) (%)	OR* (95% CI)
1	-	-	-	27 (18.0)	207 (47.4)	1.00
2	+	-	-	43 (28.7)	175 (40.1)	1.59 (0.90–2.81)
3	-	+	-	6 (4.0)	18 (4.1)	2.60 (0.90–7.53)
4	±	+	-	18 (12.0)	34 (7.8)	3.76 (1.79–7.92)
5	+	+	-	12 (8.0)	16 (3.7)	5.10 (2.06–12.62)
6	-	+	+	7 (4.7)	7 (1.6)	10.19 (2.99–34.75)
7	±	+	+	62 (41.3)	21 (4.8)	34.48 (16.26–73.13)
8	+	+	+	55 (36.7)	14 (3.2)	46.71 (21.00–103.90)

* Adjusted for sex, age group, country and province of birth, number of years lived in urban area, place of birth, alcohol consumption, HIV status and anti-HCV positivity. Occult HBV infection is shown in rows 3–5. Hepatitis B viral marker status -: Negative; +: Positive; ±: Positive and/or Negative.

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Table 3. Hepatitis B virus DNA levels and the associated odds ratio (OR) for HCC in black South Africans: 2000–2012.

HBV DNA Levels (IU/ml)	HCC (%)	Control (%)	OR (95% CI)	OR* (95% CI)
≥200,000	41 (27.3)	21 (4.8)	10.65 (5.94–19.11)	16.93 (8.65–33.13)
20,000–199,999	27 (18.0)	14 (3.2)	10.52 (5.26–21.07)	10.97 (5.20–23.14)
2000–19,999	10 (6.7)	8 (1.8)	6.82 (2.60–17.89)	8.55 (3.00–24.54)
200–1999	1 (0.7)	12 (2.7)	0.45 (0.06–3.55)	0.68 (0.08–5.68)
<200	1 (0.7)	1 (0.2)	5.46 (0.34–88.27)	1.54 (0.08–28.22)
Undetected	70 (46.7)	382 (87.2)	1.00	1.00

* Adjusted for sex, age group, country and province of birth, number of years lived in urban area, place of birth, alcohol consumption, HIV status and anti-HCV positivity. P value <0.001.

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Table 4. Main effect of HBV-DNA positivity and interactive effects with anti-HCV- and/or HIV-positivity on the risk for HCC in black South Africans, 2000–2012.

Characteristics	All Participants	HCC	Control	p-value	OR* (95% CI)
	N (%)	N (%)	N (%)		
HBV DNA and anti-HCV				<0.001	
HBV DNA-/HCV-	421 (72.3)	52 (34.7)	369 (84.2)		1.00
HBV DNA+/HCV-	129 (22.2)	79 (52.7)	50 (11.4)		11.56 (7.10–18.85)
HBV DNA-/HCV+	28 (4.8)	18 (12.0)	10 (2.3)		10.60 (4.33–26.00)
HBV DNA+/HCV+	4 (0.7)	1 (0.7)	3 (0.7)		3.15 (0.31–32.40)
Missing	6 (1.0)	0 (0.0)	6 (1.4)		
HBV DNA and HIV				<0.001	
HBV DNA-/HIV-	350 (59.5)	62 (41.3)	288 (65.8)		1.00
HBV DNA+/HIV-	97 (16.5)	60 (40.0)	37 (8.5)		9.09 (5.27–15.66)
HBV DNA-/HIV+	102 (17.3)	8 (5.3)	94 (21.5)		0.39 (0.17–0.89)
HBV DNA+/HIV+	39 (6.6)	20 (13.3)	19 (4.3)		5.36 (2.59–11.11)
Missing	0 (0.0)	0 (0.0)	0 (0.0)		
Anti-HCV and HIV				<0.001	
Anti-HCV-/HIV-	412 (70.1)	105 (70.0)	307 (70.1)		1.00
Anti-HCV+/HIV-	29 (4.9)	17 (11.3)	12 (2.7)		6.47 (2.69–15.59)
Anti-HCV-/HIV+	138 (23.5)	26 (17.3)	112 (25.6)		0.48 (0.27–0.85)
Anti-HCV+/HIV+	3 (0.5)	2 (1.3)	1 (0.2)		3.69 (0.28–48.36)
Missing	6 (1.0)	0 (0.0)	6 (1.4)		
HBV DNA, anti-HCV and HIV				<0.001	
No Infection	321 (55.2)	46 (30.7)	275 (63.6)		1.00
HBV DNA only	91 (15.6)	59 (39.3)	32 (7.4)		10.29 (5.90–17.97)
Anti-HCV only	26 (4.5)	16 (10.7)	10 (2.3)		8.98 (3.59–22.46)
HIV only	100 (17.2)	6 (4.0)	94 (21.8)		0.34 (0.14–0.85)
2 or more infection	44 (7.6)	23 (15.3)	21 (4.9)		5.99 (2.99–11.97)
Missing	6 (1.0)	0 (0.0)	6 (1.4)		

*Adjusted for sex, age group, country and province of birth, number of years lived in urban area, place of birth, alcohol consumption, HBV DNA, HIV status and anti-HCV positivity

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No tested modifiable risk factors showed statistically significant differences between HCC and control groups; approximately half of the HCC participants were non-alcohol drinkers (51.3%) and 47.3% were non-tobacco smokers (Table 5).

Discussion

Almost a decade has elapsed since the most recent investigation of risk factors for and demographics of HCC in SA [22]. Consistent with previous hospital-based case-controls studies [22–26], we found a mean age of 46 years and a predominance of men in the HCC cases. Although our results show a higher prevalence of previous exposure to HBV in rural-born than in urban-born HCC cases (65% vs 35%, $p<0.001$), when compared to an earlier study in the 1980s [26], the lack of statistical significance in HBsAg positivity between rural- and urban-born cases was consistent ($p = 0.3$). Thus, HCC participants born in urban environments are just as likely to have been infected with HBV during early childhood as those who were born and in rural areas and subsequently moved to an urban area [27]. Although not investigated in the present study, it is possible that those born in rural areas are also vulnerable to co-carcinogens prevalent in rural settings, such as dietary exposure to aflatoxin B_1 and iron

Table 5. Modifiable risk factor characteristics of HCC cases and of control participants and the associated odds ratio (OR) in black South Africans, 2000–2012.

Characteristics	HCC	Control	p-value	OR (95% CI)	OR* (95% CI)
	N (%)	N (%)			
Alcohol Use			0.73		
Non-drinkers	77 (51.3)	225 (51.4)		1.00	1.00
Moderate	46 (30.7)	145 (33.1)		0.93 (0.61–1.41)	1.24 (0.73–1.10)
Heavy	27(18.0)	68 (15.5)		1.16 (0.69–1.94)	1.22 (0.64–2.30)
Missing Data	0 (0.0)	0 (0.0)			
Tobacco Smoking			0.30		
Non-smokers	71 (47.3)	215 (49.1)		1.00	1.00
Former-smokers	46 (30.7)	121 (27.6)		1.25 (0.74–2.12)	1.63 (0.81–3.29)
Current (1-14g/day)	7 (4.7)	39 (8.9)		1.15 (0.75–1.77)	1.63 (0.85–4.21)
Current (≥15 g/day)	26 (17.3)	63 (14.4)		0.54 (0.23–1.27)	0.62 (0.21–1.89)
Missing Data	0 (0.0)	0 (0.0)			
Diabetes(self-reported)			0.51		
Negative	145 (96.7)	415 (94.7)		1.00	1.00
Positive	5 (3.3)	23 (5.3)		0.62 (0.23–1.67)	0.83 (0.28–2.50)
Missing Data	0 (0.0)	0 (0.0)			
No. of Lifetime Sexual Partners			0.57		
0–1	17 (11.3)	48 (11.0)		1.00	1.00
2–5	80 (53.3)	216 (49.3)		1.05 (0.57–1.92)	0.97 (0.46–2.01)
6+	49 (32.7)	165 (37.7)		0.84 (0.44–1.59)	0.81 (0.36–1.82)
Missing Data	4 (2.7)	9 (2.1)			
Hormonal Contraception Use (women only)			0.09		
Never oral or injectable contraceptive user	13 (8.7)	41 (9.4)		1.00	1.00
Ever Oral and/or injectable contraceptive user	25 (16.0)	73 (16.7)		1.08 (0.50–2.34)	1.09 (0.43–2.79)
Missing Data	1 (0.7)	0 (0.0)			

* Adjusted for sex, age group, country and province of birth, number of years lived in urban area, place of birth, alcohol consumption, HBV DNA, HIV status and anti-HCV positivity

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overload [28, 29]. When they leave the rural areas for urban environments, their exposure to these co-carcinogens may decrease, thereby delaying the progression of HCC [27].

HBsAg prevalence in our study as a whole (14%) and in the HCC participants (41%), are in agreement with the prevalence found in the general SA population (from 8% to 10%) [30] and in HCC patients from previous SA case-controls studies (ranging from 40.3% to 70%) [27, 30–32]. Our study shows that in black South Africans HBV infection continues to be a primary risk factor for HCC development, followed by HCV infection. Our risk estimates for HBsAg (OR 34.5) and anti-HCV (OR 9.0) are consistent with a study of HCC patients attending a rural hospital in Mpumalanga, SA (OR 33.2 for HBsAg) [31], but are somewhat higher than another study conducted in urban Johannesburg hospitals (OR 23.3 for HBsAg and 6.6 for anti-HCV) [32]. The synergistic effect of HBV-HCV co-infection for risk of developing HCC observed by Kew *et al.* [32], was not found in the current analysis (Table 4), possibly because very few participants were infected with both viruses (Table 4).

To the best of our knowledge the current study is the first to determine an association between HCC risk and the 2000 IU/mL threshold of HBV-DNA levels in black South Africans, as well as the increased risk of HCC with increasing HBV-DNA level, in a dose-response relationship (Table 3). We found only one prior study that estimated HCC risk related to HBV-DNA viral load in SA: among 124 HBsAg-positive cases and 125 asymptomatic carriers

identified in a cohort of black southern Africans, the reported geometric mean viral loads were 553,618 copies/ml (approximately 118,000 IU/mL) and 16,084 copies/ml (approximately 3,400 IU/mL), respectively [22]. In the current study the risk of developing HCC in individuals with low HBV-DNA levels (<2000 IU/mL) was not significant, and differed in comparison to earlier studies conducted in Korea [33] and The Gambia [34] where five- and three-times higher respective risks were reported. This discrepancy, observed at low HBV-DNA levels, may result from marked differences in population characteristics and HBV genotypes both of which may influence the viral load. Moreover, environmental risk factors in West Africa such as aflatoxin B_1[35]—which is classified as a human carcinogen by IARC (International Agency for Research on Cancer)—may play an important role, especially at low viral loads. In the 1980s aflatoxin contamination in SA commercial foodstuffs was shown to be low (0.66 μg/kg) in comparison to Mozambique (range from 0.7–6.43 μg/kg) [36]; this was also confirmed in a more recent study (2004) that reported low rates of mycotoxin contamination in staple foods such as maize [37]. In any case suppression of HBV, even if only for a finite period, may significantly reduce risk for developing HCC [38]. Long-term follow-up studies are needed to further determine the role of low HBV-DNA levels as a risk factor for HCC in black South Africans.

In the current study, although the HIV prevalence in the case (18.7%) and control (25.8%) groups did not differ significantly (p = 0.08), both groups were higher than the background HIV seropositivity in this adult population in Gauteng province, SA (12.4%) [39]. Furthermore, we did not find an increased risk of developing HCC in relation to HIV infection (OR 0.4). This is consistent with an earlier case-control study from SA in the 1990s, that recruited 913 black African HIV cases and 325 controls comprised of patients with cancers other than those known to have an infectious cause (OR 0.9, 95% CI:0.3–2.9) [14]. Although it has been suggested that HIV infection accelerates liver damage related to HBV infection [40], a possible explanation for our finding a lack of higher HCC risk in HIV-positive individuals might be the reduction in the continual inflammation related to immune-mediated clearance of HBV-infected hepatocytes [41] as a result of impaired innate and adaptive immune response in HIV-coinfection [42]. Additionally, given the long induction period of HCC, many South African HIV-infected patients, in the absence of ART, died earlier, before symptoms related to HBV could manifest and in contrast to evidence from developed countries [43, 44]. Millions of relatively young HIV-infected SA adults are now surviving longer as a result of the widespread rollout of ART which began in 2009 [45] and is now available free of charge to all HIV infected people, regardless of CD4 count [46]. This may or may not be associated with a parallel increase in the burden of HCC depending on the coverage of infant HBV vaccination, the possible spread of HCV and also an increased prevalence of modifiable risk factors [47]. The shift in cancer burden from AIDS-defining cancers to non-AIDS defining cancers seen in developed countries is not yet evident in SA but is likely to occur in time [48]. Although both HIV and HBV are endemic in SA systematic studies investigating the association between HBV-HIV coinfection and HCC have not been done. This may reflect under-ascertainment and reporting due to the often late presentation with cancer, especially in resource-limiting settings thus further highlighting the need for a systematic approach to surveillance for HCC in the HIV-positive population in SA [4, 48].

OBI prevalence found in the HCC cases (12%) was significantly less than that found by an earlier SA study (48.4%) [25] but translated to a four-times higher risk of HCC (Table 2). Our findings show that OBI is associated with increased HCC risk (Table 2), and the highest OR of 5.10 (95% CI: 2.06–12.62) in OBI-infected participants with anti-HBc positivity was consistent with a previous meta-analysis of retrospective studies (OR 6.08, 95% CI: 3.45–10.72) [49]. Although the clinical implications of OBI are not clearly understood, this condition has been

extensively reported in HIV-infected ART-naïve patients in SA, with a prevalence ranging from 15.1% to 23% [16, 50], higher than that found in the current study (8.8%). The differences may be influenced by the ART use. Although OBI did not differ between HIV-positive and negative individuals in this study (9.9% vs 8.5%, p = 0.238), the presence of OBI poses a unique challenge in managing HBV disease, particularly against a backdrop of the high HIV prevalence in the general SA population (12% or 7 million are HIV-positive) (http://aidsinfo.unaids.org). Future research is required to determine the true risk of acquiring OBI as it is not detected by conventional HBsAg testing.

This study has limitations. Modifiable risk factors were self-reported, there was limited data on the prior use of antiretroviral treatment, absence of HIV viral and CD4 count, and an absence of sufficient clinical data evaluating liver function and/or the presence of cirrhosis, all of which may have influenced the estimates of HCC risk. Additionally, no aflatoxin exposure or HCV virological data were available. Using sera following prolonged storage could potentially introduce errors as a result of protein or HBV-DNA degradation. However, this possible effect was minimized by selecting control participants that were matched by time and hospital to the HCC cases enrolled. While the use of sonography in detecting HCC has its limitations [51], only four included cases were diagnosed by this method alone. In limited-resource settings HCC patients often present at an advanced stage where the finding of a mass using imaging techniques is rarely confirmed by histological examination [52].

A considerable proportion of HCC burden in northern SA is being borne by rural migrants to urban areas, the majority of whom are working-age men who may have had exposure to co-carcinogens prevalent in the rural environment. HBV-induced HCC will continue to occur in the older population group and in immigrants, and may increase in the foreseeable future especially when HIV co-infected individuals have an extended lifespan due to ART therapy, thus allowing sufficient time for HCC to develop [53]. Given the overburdened public health services in SA, the challenge is to put in place preventative measures through health education and early detection and screening programs targeting high risk individuals residing in rural areas. Poverty alleviation and the provision of cost-effective medical care will also assist in HCC treatment and in incidence reduction.

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Author Contributions

Conceptualization: Daniel Mak, Chantal Babb de Villiers, Charles Chasela, Margaret I. Urban, Anna Kramvis.

Data curation: Daniel Mak, Chantal Babb de Villiers, Margaret I. Urban.

Formal analysis: Daniel Mak.

Funding acquisition: Anna Kramvis.

Investigation: Daniel Mak.

Methodology: Chantal Babb de Villiers.

Project administration: Chantal Babb de Villiers, Anna Kramvis.

Resources: Anna Kramvis.

Supervision: Chantal Babb de Villiers, Charles Chasela, Anna Kramvis.

Writing – original draft: Daniel Mak.

Writing – review & editing: Daniel Mak, Chantal Babb de Villiers, Charles Chasela, Margaret I. Urban, Anna Kramvis.

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— CHAPTER 6 —

Molecular Characterization of Hepatitis B Virus isolated from Black South African cancer patients, with and without Hepatocellular Carcinoma

Daniel Mak[a], Anna Kramvis[a]

[a]Hepatitis Virus Diversity Research Unit (HVDRU), Department of Internal Medicine, School of Clinical Medicine, Faculty of Health Sciences, University of Witwatersrand, Johannesburg, SA

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Sub-Saharan Africa is ranked one of the most HBV endemic regions in the world (Global Burden of Disease Liver Cancer Collaboration 2017), but lacks a surveillance program for identifying and stratifying individuals at high risk of developing HCC (Razavi-Shearer and Razavi 2018). In contrast, guidelines for screening patients for HCC have been published in regions of lower HBV endemicity (America, Europe and Japan) (Marquardt et al. 2016), which provides guidelines for preventive measures and curative treatments, consequently improving survival rates (Fitzmorris and Singal 2015). Unfortunately, predictive algorithms may not always perform optimally especially when used in different population groups (Singal et al. 2013). Thus, screening high risk HBV carriers by using HBV biomarkers in particular has been

suggested as an alternative strategy that also has the benefit of being cost effective (Global Burden of Disease Liver Cancer Collaboration 2017; Fang et al. 2008). This chapter investigates a combination of molecular mutations in which they may be used to determine the risk of HCC development in South African black African individuals, a measure that is particularly important in a region where resources are limited.

6.1 Abstract

Purpose: In South Africa (SA), hepatitis B virus (HBV) infection is strongly associated with hepatocellular carcinoma (HCC). As HBV genotypes/subgenotypes and mutations can influence disease manifestation and progression, our aim was to molecularly characterize HBV in Black cancer patients, with and without HCC.

Methodology: The basal core promoter/precore (BCP/PC) and complete surface (S) regions of HBV isolates were amplified and sequenced from 55 HCC cases and 22 non-HCC cancer controls

Results: Phylogenetic analysis of 43 polymerase/complete S region isolates showed that majority (88.4%) clustered with subgenotype A1, 4.7% A2 and 7% A3. The following mutations were significantly higher in HCC cases than controls ($p<0.05$): 1753C/G (22.5.4% *versus* 0%), 1764A (69.4% *versus* 38.1%) and T64C (51.5% *versus* 20%) of the preS2 gene, which results in F22L. PreS1 and preS2 start codon mutants were detected only in HCC cases, occurring in two and 16 isolates, respectively. PreS deletion mutants were isolated from 11 HCC cases, which had HBV viral load > 10,000 IU/mL and were significantly younger than non-HCC controls (34±7.1 vs 41.2±9.5 years, $p=0.05$). The 1762T/1764A double mutation was detected in majority (90.9%) of the isolates from HCC cases with preS deletions.

Conclusion: Black HBV carriers were mainly infected with subgenotype A1, with HCC cases carrying BCP/PC and preS mutant strains that are associated with hepatocarcinogenesis. This is the first study to compare the molecular characteristics of HBV from HCC and non-HCC cancer patients in SA.

1. Introduction

Globally, the prevalence of hepatitis B virus (HBV) infection is closely mirrored by the incidence of hepatocellular carcinoma (HCC) and accounts for 60% of those diagnosed with this malignancy (1). In LMICs, HBV is responsible for approximately two out of three cases of HCC, whereas this is lower in HICs (one in four cases) (1). In particular, HBV shows a much higher degree of geographical aggregation in eastern Asia and sub-Saharan Africa, with an attributable fraction (AF) (2) (estimated by combining HBV prevalence with the risk for HCC) of 69% and 50%, respectively in each geographical region (1). The different genotypes prevailing in these two geographical regions may be responsible for this observed difference in AF (3).

Nine genotypes (A-I) and a putative 10th genotype of HBV have been identified in the world based on the intergroup divergence of >7.5% or more in the complete nucleotide sequence of HBV (3). The occurrence of certain basal core promoter/precore (BCP/PC) mutations and preS deletions in the HBV genome appears to be genotype specific (4) and have been linked to the pathogenesis of HCC. In Taiwan, the combination of preS deletion and BCP double mutations were shown to be associated with the development of HCC, rather than either mutation alone (5). Moreover, preS2 point mutations that are independently associated with 3- and 2-fold increased risk of developing HCC include amino acid (aa) substitution at codon 1 and 22 of this gene, respectively (6).

While the association between these HBV mutations and HCC in patients infected with genotype A has been demonstrated in Indian (7) and Kenyan patients (8), these mutations have rarely been investigated in South Africa (SA) where HBV infection and its sequelae are common (9). Such studies are important because previous reports have

demonstrated the clinical relevance of this genotype in this region (10). In the few reports that are available, studies were either conducted two decades ago (11) or did not investigate the frequency and patterns of BCP/PC and preS/surface gene mutations in combination (12). Furthermore, unlike patients who have been actively engaged in the health system, general population controls that are either HBV positive (10,13) or negative (11), may tend to overestimate the risk as a consequence of differences in their capability to recall and report exposures (14). On the other hand, another study did not include controls, but investigated HBV genetic variability amongst HCC patients only (12). Replacing population controls with patients with a disease that is neither confounded nor prevented by the exposure under investigation, may have a consistency in recall comparable to that of cases.

Thus, the aim of this study was to molecularly characterize HBV isolated from HCC cases and controls with cancer of other sites of the body (15), in order to determine the possible association of BCP/PC and/or preS mutations with the development of HCC.

6.2 Method

This study was a continuation of a previous case-control study that enrolled HCC (ICD-O: M8170/3 and/or ICD10: C22) cases and controls admitted to city hospitals in Johannesburg from 1 September 2000 to 31 December 2012 (16). This retrospective analysis consisted of a subset of participants enrolled in the Johannesburg Cancer Case Control Study (JCS), a study that recruited self-defined identified black African (not mixed ancestry) cancer patients in the greater Johannesburg, Gauteng province public referral hospitals that offer oncology services (17).

Inclusion criteria for the control group, included the absence of cancers associated with risk factors investigated in this study (alcohol consumption, smoking, diabetes, HBV, HCV and HIV infection). The majority (91%) of the controls were diagnosed with either skin cancer, multiple myeloma or tumours of the connective tissue. The study was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (M150239; M140271).

2.1 Serum Samples

A total of 136 HBV DNA+ve treatment-naïve participants (80 HCC cases and 56 controls) with viral loads >200 IU/mL were included in this study.

2.2 DNA extractions

The collected sera were incubated at -70°C followed by DNA isolation (from 200 µl of serum) using an extraction kit (QIAamp® DNA Blood Mini Kit, Qiagen, Germany), in accordance with the manufacturer's instructions, and eluted in 100 ml of elution buffer.

2.3 PCR and sequencing

Two regions of the HBV genome were amplified using a nested PCR procedure and primer sets as described before (18) in a SimpliAmp™ thermocycler (Life Technologies). The complete S open reading frame was amplified using: S1F 5′-TCAATCGCCGCGTCGCAGAAGATCTCAATC-3′ (2410-2439 from *Eco*RI site) and S1R5′-TCCAGACCXGCTGCGAGCAAAACA-3′ (1314-1291 from *Eco*RI site) for the first round and S2F 5′-AATGTTAGTATTCCTTGGACTCATAAGGTGGG-3′ (2451-2482 from *Eco*RI site) and S2R 5′-AGTTCCGCAGTATGGATCGGCAGAGGA-3′ (1280-1254 from *Eco*RI site) for the second round. The BCP/PC region was amplified

using: BCP1F 5′-GCATGGAGACCACCGTGAAC-3′ (1606-1625 from *Eco*RI site) and BCP1R 5′-GGAAAGAAGTCCGAGGGCAA-3′ (1974-1955 from *Eco*RI site) were used for the first round and BCP2F 5′-CATAAGAGGACTCTTGGACT-3′ (1653-1672 from *Eco*RI site) and BCP2R 5′-GGCAAAAAACAGAGTAACTC-3′ (1959-1940 from *Eco*RI site). After amplification, the final PCR products were visualized on a 1% agarose gel and sequenced by the Central Analytical Facility, Stellenbosch University, SA, using the ABI 3130XL Genetic analyser (Applied Biosystems, Foster City, CA). BCP/PC sequences were analysed in a single fragment, whereas the pol/complete S was analysed in three overlapping fragments (18) that was assembled using the Fragment Merger Tool (19). All sequences were deposited in Genbank (MK481953-MK481959; MK491799- MK491831; MK503729- MK503764).

Genotyping of HBV sequence was based on BCP/PC and pol/complete S genes. The pol/complete S gene sequences were translated, aligned, and the phylogenetic tree constructed using the Neighbor-Joining method using MEGA 7 software (20). Full genome sequences representing the different HBV genotypes were retrieved from the NCBI Genbank were used as references and the consensus subgenotype A1 (GenBank accession #AY233274.1) for comparison. Bootstrapping was performed using 1000 replicates in order to determine the support for the specific nodes.

Deletions and mutations in the BCP/PC and complete S regions were identified by visual analysis of the chromatograms. Mutations in BCP (T1753C, G1757A, A1762T, G1764A, C1766G, and T1768A) (21), and the PC regions associated with HCC (1802, 1803, 1809, 1810, 1811, 1812, 1814, 1815, 1816, 1858, 1862, 1888, 1896 and 1899) were also analysed (22,23). The preS regions were analysed for preS1/S2 start codon deletions/mutations and T64C of the preS2 gene, which results in F22L (22). PreS

deletion mutants were sequences in which one or more amino acid/s in the aligned protein sequences were missing.

2.4 Statistical Analysis

All data was recorded and analysed using the STATA software version 14.2 (StataCorp. 2013. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP). Chi squared and Fisher's exact test was used for the comparison of qualitative variables. A p value <0.05 was inferred to indicate statistical significance.

6.3 Results

6.3.1 HBV serology, amplification, and quantification of HBV DNA

By using nested PCR, we successfully amplified and isolated HBV BCP/PC and pol/complete S DNA sequences from the sera of 76 of 136 (55.9%) HBV DNA^{+ve} participants, of which more than two thirds were HCC cases (71.4%) and male sex (75.3%).

Table 6.1 details the demographic and virological characteristics of samples with and without sequences. While the mean age ± standard deviation (SD) of participants with and without sequenced HBV DNA did not differ significantly (41.8±10.9 years vs 46.4±13.6 years respectively, p>0.05), the median HBV DNA viral load (log_{10} IU/ml) was significantly higher in the former group compared to the latter group (5.68 (interquartile range of 6.99-4.81) vs 5.48 (interquartile range of 5.27-3.56), p>0.05). Moreover, this difference in viral load may reflect the HCC case to control ratio (2.5:1 in sequenced group vs 0.7:1 in non-sequenced group) as HCC cases had been shown to have significantly higher viral load (16).

Table 6.1 Demographic and virological characteristics of the Johannesburg Cancer Case Control Study participants, with and without BCP/PC and/or pol/complete S sequences.

Characteristic	Total (%)	BCP/PC and/or pol/complete S sequenced (%)	No sequence (%)	P-value
Age group				0.03
18-29	19 (14.0)	7 (9.1)	12 (20.3)	
30-39	40 (29.4)	29 (37.7)	11 (18.6)	
40-49	39 (28.7)	20 (26.0)	19 (32.2)	
50-59	24 (17.7)	16 (20.8)	8 (13.6)	
≥ 60	14 (10.3)	5 (6.5)	9 (15.3)	
mean (SD)	42.5±12.1	41.8±10.9	43.4±13.6	
Sex				0.72
Female	32 (23.5)	19 (24.7)	13 (22.0)	
Male	104 (76.5)	58 (75.3)	46 (78.0)	
HCC status				<0.05
Positive	80 (58.8)	55 (66.8)	25 (31.3)	
Negative	56 (41.2)	22 (39.3)	34 (60.7)	
Year of Interview				0.55
2000-2004	34 (25.0)	21 (27.3)	13 (22.0)	
2005-2008	53 (39.0)	27 (35.1)	26 (44.0)	
2009-2012	49 (36.0)	29 (37.7)	20 (33.9)	
Marital Status				0.45
Married/living as married	81 (59.6)	49 (63.6)	32 (54.2)	
Separated	14 (10.3)	9 (11.7)	5 (8.5)	
Single/ Never	26 (19.1)	13 (16.9)	13 (22.0)	

Married				
Widowed	14 (10.3)	6 (7.8)	8 (13.6)	
Country of Birth				0.57
Outside South Africa	26 (19.1)	16 (20.8)	10 (17.0)	
South Africa	110 (80.9)	61 (79.2)	49 (83.0)	
Province of Birth				0.88
Gauteng	44 (40.0)	24 (39.3)	20 (40.8)	
Other Provinces	66 (60.0)	37 (60.7)	29 (59.2)	
Place of Birth				0.51
Rural	81 (59.6)	44 (57.1)	37 (62.7)	
Urban	55 (40.4)	33 (42.9)	22 (37.3)	
Place of Residence				0.64
Rural	8 (5.9)	5 (6.5)	3 (5.1)	
Urban	127 (93.4)	71 (92.2)	56 (94.9)	
Years lived in Urban				0.04
0 - 14 years	72 (52.9)	46 (59.7)	26 (44.1)	
15 - 29 years	24 (17.7)	8 (10.4)	16 (27.1)	
≥30 years	39 (28.7)	23 (29.9)	16 (27.1)	
Diabetes Status				0.85
Positive	2 (1.5)	1 (1.3)	1 (1.7)	
Negative	137 (98.5)	79 (98.7)	58 (98.3)	
HIV Status				0.24
Positive	39 (28.7)	19 (24.7)	20 (33.9)	
Negative	97 (71.3)	58 (75.3)	39 (66.1)	
Anti-HCV				0.05
Positive	4 (2.9)	0 (0.0)	4 (6.8)	
Negative	129 (94.9)	76 (98.7)	53 (89.8)	

HBsAg				<0.001
Positive	83 (61.5)	59 (76.6)	24 (41.4)	
Negative	52 (38.5)	18 (23.5)	34 (58.6)	
HBeAg				0.5
Positive	23 (23.0)	19 (24.7)	4 (17.4)	
Negative	77 (77.0)	58 (75.3)	19 (82.6)	
Anti-HBc				0.24
Positive	97 (71.3)	58 (75.3)	39 (66.1)	
Negative	39 (28.7)	19 (24.7)	20 (33.9)	
Anti-HBs				0.44
Positive	35 (25.9)	18 (23.4)	17 (29.3)	
Negative	100 (74.1)	59 (76.6)	41 (70.7)	

Except for the age group and number of years lived in an urban area ($p<0.05$), no difference in sex, year of interview, marital status, place of birth, place of residence, was found between individuals from those that had HBV isolates that were sequenced and those that were not ($p>0.05$). Similarly, except for HBsAg prevalence ($p<0.05$), there was also no difference in the prevalence for viral serological markers including anti-HBc, anti-HBs, HBeAg, HIV and anti-HCV between the two groups ($p>0.05$) (Table 6.1).

Of the 18 $HBsAg^{-ve}$ individuals with sequences, three had viral loads <200 IU/ml (<2.3 $\log_{10}$ IU/ml), and thus according to the Taormina definition, were true occult HBV infection (24). The remaining eight had viral loads >200 IU/ml (>2.3 log10 IU/ml) and therefore were false occult HBV infection (25).

Table 6.2 shows a comparison of age, sex and virological characteristics between sequenced HCC cases and non-HCC controls. There was no statistical difference ($p>0.05$) between the two groups that were sequenced, except HBsAg, which was significantly higher in HCC cases (83.6%) compared to non-HCC controls (59.1%; $p<0.05$). Similarly, HCC cases and non-HCC controls that were not sequenced reported a higher prevalence of HBsAg in the former compared to the latter group and was statistically significant (data not shown; $p<0.05$).

Table 6.2 Demographic and virological characteristics of HCC cases and non-HCC controls with the HBV BCP/PC and/or pol/complete S regions sequenced.

Characteristic	HCC (%)	Controls (%)	P-value
Age group			0.07
18-29	1 (4.6)	6 (10.9)	
30-39	9 (40.9)	20 (36.4)	
40-49	3 (13.6)	17 (30.9)	
50-59	5 (22.7)	11 (20.0)	
≥ 60	4 (18.2)	1 (1.8)	
mean (SD)	40.5±9.7	45±13.1	
Sex			0.74
Women	13 (23.6)	6 (27.3)	
Men	42 (76.4)	16 (72.7)	
HIV Status			0.13
Positive	11 (20.0)	8 (36.4)	
Negative	44 (80.0)	14 (63.6)	
Anti-HCV			
Positive	0 (0.0)	0 (0.0)	
Negative	55 (100.0)	21 (100.0)	
HBsAg			0.02

Positive	46 (83.6)	13 (59.1)	
Negative	9 (16.4)	9 (40.9)	
HBeAg			0.40
Positive	15 (27.3)	4 (18.2)	
Negative	40 (72.7)	18 (81.8)	
Anti-HBc			0.04
Positive	45 (81.8)	13 (59.1)	
Negative	10 (18.2)	9 (40.9)	
Anti-HBs			0.93
Positive	13 (23.6)	5 (22.7)	
Negative	42 (76.4)	17 (77.3)	
Viral Load (IU/mL)			0.51
200-1999	1 (1.8)	2 (9.1)	
2000-19,999	4 (7.3)	2 (9.1)	
20,000-199,999	15 (27.3)	5 (22.7)	
≥200,000	35 (63.6)	13 (59.1)	

6.3.2 HBV genotyping and phylogenetic analysis

Using the criteria by Kramvis *et al*. (23), the BCP/PC region was used to differentiate between genotype A, A1, A2 and non-A in 70 of 76 isolates (92.1%). Four isolates (MK491800, MK503746, MK503753 and MK491828), with 1858T, did not belong to genotype A. Two isolates (MK503735 and MK491811) had Kozak sequence GCAC at 1809–1812, 1858C and 1888G, generally found in subgenotype A2. In 46 isolates with Kozak sequence TCAT (or mutant) at nucleotides 1809–1812 and 1858C/T, nineteen sequences with 1888A belonged to subgenotype A1. Of the remaining 27 isolates had 1888G and could not be classified using Kramvis *et al*. method.

Forty-three of 76 samples were genotyped using phylogenetic analysis of the pol/complete S region as shown in Figure 6.1. Majority (88.4%, 38/43) of isolates clustered with subgenotype A1, whereas two isolates (MK503760 and MK503735; 4.7%), clustered with subgenotype A2 and three (MK481954, MK503747 and MK503763; 7%) with quasi-subgenotype A3 (3, 26). Upon translation of the preS1/S2, the majority of the subgenotype A1 isolates showed distinct subgenotype A1 amino acids Q54, V74, A86 and V91 in the preS1 region and L32 in the preS2 region (27–29). Thirty-five of 43 isolates (81.4%) with pol/complete S sequences belonged to serological subtype *adw*2, five *ayw*1 (11.6%) and one *ayw*2 (2.3%).

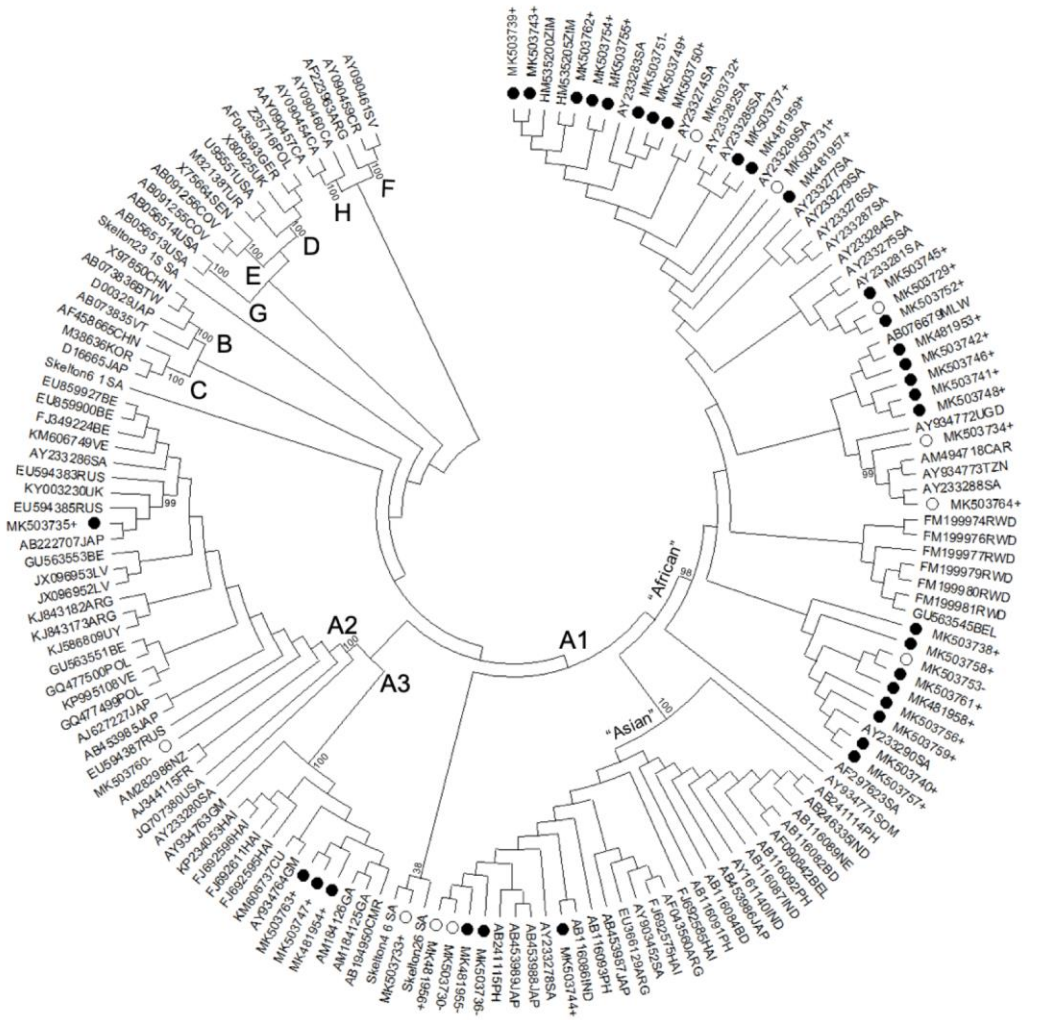

Figure 6.1 A rooted phylogenetic tree of 43 HBV obtained from isolates from HCC cases (black dot) and controls (white dot) (+: HBsAg^{+ve}; -: HBsAg^{-ve}) with 116 reference HBV sequences on the basis of a part of polymerase region (nt 2532-2854 from the *Eco*RI site) and the complete preS1/preS2/S sequences (nt 2854–835 from the *Eco*RI site), using neighbour-joining. Bootstrap statistical analysis was performed using 1000 datasets, indicated as percentages on the nodes under the Kimura 2-parameter substitution model with the program MEGA (28). The HBV sequences obtained from GenBank are labeled by their accession numbers and country for

genotypes/subgenotypes (represented by letters A-H and numbers): (ARG, Argentina; BE, Belgium; CA, Central America; CMR, Cameron; CAR, Central African Republic; CHN, China; CU, Cuba; FR, France; GA, Gabon; GER, Germany; GM, Gambia; HAI, Haiti; IND, India; JAP, Japan; KOR, Korea; LV, Latvia; MLW, Malawi; NE, Nepal; NZ, New Zealand; PH, Philippines; POL, Poland; RWD, Rwanda; RUS, Russia; SA, South Africa; SEN, Senegal; SOM, Somalia; SV, El Salvador; TNZ, Tanzania; TUR, Turkey; TW Taiwan; UK, United Kingdom; USA, United States; VE, Venezuela; VT, Vietnam; UGD, Uganda; UY, Uruguay; ZIM, Zimbabwe). Four isolates (labelled "Skelton") from HCC patients that were sequenced in another SA study by Skelton *et al*. (12), were also included in the rooted phylogenetic tree.

Subgenotype A1 isolates from individuals, with and without HCC, were compared to subgenotype A1 isolates from Asian and African countries using a rooted phylogenetic tree of the pol/complete S sequences (Figure 6.1). Five isolates (11.6%) clustered with the "Asian" cluster, while 33 isolates (76.7%) clustered with the African cluster. The majority of the isolates in the "Asian" cluster had S5, S6 and L25 in the preS1, while the "African" HBV subgenotype A1 strains displayed S5, A6 and F25 in the preS1.

A total of 76 HBV isolates were therefore successfully genotyped using one of the two methods. The genotype distribution of the 76 isolates with BCP/PC and pol/complete S was as follows: 27 (35.1%) subgenotype A1, two (2.6%) subgenotype A2, three (3.9%) subgenotype A3. Using the BCP/PC region, 41 (53.2%) were subgenotype A1 and two (2.6%) genotype non-A (because of 1858T). The genotype/subgenotype distribution did not differ between HCC and non-HCC controls, $HBsAg^{+ve}$ and $HBsAg^{-ve}$ sera, or between patients with true or false occult HBV infection ($p>0.05$).

A discrepancy in the subgenotype classification of two samples (MK503746 and MK503753) was identified. Based on the suggested classification system of the BCP/PC region, both sequences were identified as subgenotype non-A, whereas both clustered as subgenotype A1 following phylogenetic analysis of their respective pol/complete S sequences.

6.3.3 Genetic diversity of South African strains

The mean pairwise intragroup DNA divergence of the pol/complete S sequences was calculated using MEGA for the subgenotype A1 strains isolated from the cases and controls. There was no difference in the intragroup divergence between the cases and controls being 3.5% and 3.6%, respectively and below the 4% delineating subgenotypes.

6.3.4 Analysis of the BCP/PC region

Table 6.3 shows the prevalence of BCP/PC mutations that can either down-regulate or abolish the expression of HBeAg at the transcriptional, translational, or post-translational levels (9). Of the 19 isolates from $HBeAg^{+ve}$ sera, 12 had mutations in the BCP: one had T1753V (MK503763) and 9 had A1762T/G1764A (MK491814, MK491826, MK491818, MK491799, MK503736, MK503756, MK503763, MK503741, MK491825); Sample MK503747 had A1762T/G1764A whereas MK491813 had a deletion at position 1764; one had TCTT at 1809-1812 (MK491816). For the PC mutations: one had start codon mutation CTG at 1814-1816 (MK503755). The remaining isolate from $HBeAg^{+ve}$ participants (MK503749) had wild type BCP/PC.

Table 6.3 Prevalence of hepatitis B virus genotype and basic core promoter/precore (BCP) mutants in HCC and non-HCC controls with BCP/PC and polymerase/complete S sequences.

HBV characteristic		Total (%)	HCC (%)	Control (%)	P-value
Genotype	A	43 (61.4)	33 (67.3)	10 (47.6)	0.24
	A1	21 (30)	12 (24.5)	9 (42.9)	
	A2	2 (2.9)	2 (4.1)	0 (0)	
	non-A	4 (5.7)	2 (4.1)	2 (9.5)	
1753	C/G	11 (15.7)	11 (22.5)	0 (0)	0.02
	T (wild type)	59 (84.3)	38 (77.6)	21 (100)	
1762/1764	A/G (wild type)	27 (38.6)	14 (28.6)	13 (61.9)	0.03
	T/A	33 (47.1)	26 (53.1)	7 (33.3)	
	A/A	9 (12.9)	8 (16.3)	1 (4.8)	
	A/Deletion	1 (1.4)	1 (2.0)	0 (0)	
1766	C (wild type)	42 (60)	28 (57.1)	14 (66.7)	0.65
	T	27 (38.6)	20 (40.8)	7 (33.3)	
	Deletion	1 (1.4)	1 (2)	0 (0)	
1768	A	16 (22.9)	14 (28.6)	2 (9.5)	0.16
	T	53 (75.7)	34 (69.4)	19 (90.5)	
	Deletion	1 (1.4)	1 (2)	0 (0)	
1809-1812	CCAT	1 (1.4)	0 (0)	1 (4.8)	0.59

	TCAC	3 (4.3)	3 (6.1)	0 (0)	
	TCAT	46 (65.7)	32 (65.3)	14 (66.7)	
	TCAG	1 (1.4)	1 (2)	0 (0)	
	TCCT	3 (4.3)	2 (4.1)	1 (4.8)	
	TCTT	8 (11.4)	6 (12.2)	2 (9.5)	
	TTAT	1 (1.4)	1 (2)	0 (0)	
	TTCT	3 (4.3)	1 (2)	2 (9.5)	
	GCAC	4 (5.7)	3 (6.1)	1 (4.8)	
1814-1816	ATG	66 (94.3)	47 (95.9)	19 (90.5)	0.37
	CTG	4 (5.7)	2 (4.1)	2 (9.5)	
1858	C	65 (92.9)	46 (93.9)	19 (90.5)	0.61
	T	5 (7.1)	3 (6.1)	2 (9.5)	
1862	A/C/T	12 (17.1)	10 (20.4)	2 (9.5)	0.33
	G (wild type)	58 (82.9)	39 (79.6)	19 (90.5)	
1888	A/T	22 (31.4)	13 (26.5)	9 (42.9)	0.24
	G (wild type)	48 (68.6)	36 (73.5)	12 (57.1)	
1896	A	3 (4.3)	2 (4.1)	1 (4.8)	0.90
	G (wild type)	67 (95.7)	47 (95.9)	20 (95.2)	
1899	A	12 (17.1)	10 (20.4)	2 (9.5)	0.27
	G (wild type)	58 (82.9)	39 (79.6)	19 (90.5)	

Of the 51 isolates from $HBeAg^{-ve}$ individuals (35 $HBsAg^{+ve}$ and 16 $HBsAg^{-ve}$), 47 (92.2%) were of genotype A and 4 (7.8%) were non-A. Eight (15.7%) isolates had

A1762T/G1764A alone and three (5.9%) isolates had start codon mutations at 1814–1816. Only three (5.9%) isolates (two from HCC cases and one from a control) had G1896A. The variant 1858T was only found in the four (5.9%) genotype non-A isolates ($p<0.05$). As expected, a higher prevalence of C1858 was found more frequently in genotype A than non-A's allowing for a stronger base pairing and stem stabilization with the opposite guanine at position 1896. Nine (17.6%) isolates with T1753V all had the A1762T/G1764A double mutation and were all from HCC cases. In general, T1753V and A1762T/G1764A were significantly more prevalent in HCC cases compared to non-HCC controls in the 70 isolates with sequenced BCP/PC region ($p<0.05$) (Table 6.3).

6.3.5 Analysis of the preS1, preS2 and surface

The sequence of the pol/complete S region (2624–1240 from the *Eco*R1 site) was obtained for 43 HBV isolates from 33 (76.7%) HCC cases and 10 (23.3%) controls. Of the 43 isolates, 38 (88.4%) were from $HBsAg^{+ve}$ and 5 (11.6%) from $HBsAg^{-ve}$ patients. Of the isolates with pol/complete S sequences, preS deletions were identified in 11 (25.6%) isolates and are depicted in Figure 6.2. All the preS deletions were from HCC cases and in-frame deletions occurring between the aa 118 (C-terminal half of preS1) up to and including aa 23 of preS2, with deletions ranging from 1 to 26 aa (2 to 57 base pairs) in length. More specifically, most of the preS deletions encompassed HBV functional domains including B- and T-cell epitopes, the polymerized human serum albumin binding site (pHSA), the viral secretion domain (VS), the nucleocapsid binding site (Nb) and transactivator domain (Trans 2) (30). All deletion mutants were isolated from HCC cases with HBsAg positivity, except for MK503736, which was isolated

from a HBsAg^{-ve} HCC case. The difference between HBsAg seropositivity and the prevalence of deletion mutants was not statistically significant ($p>0.05$).

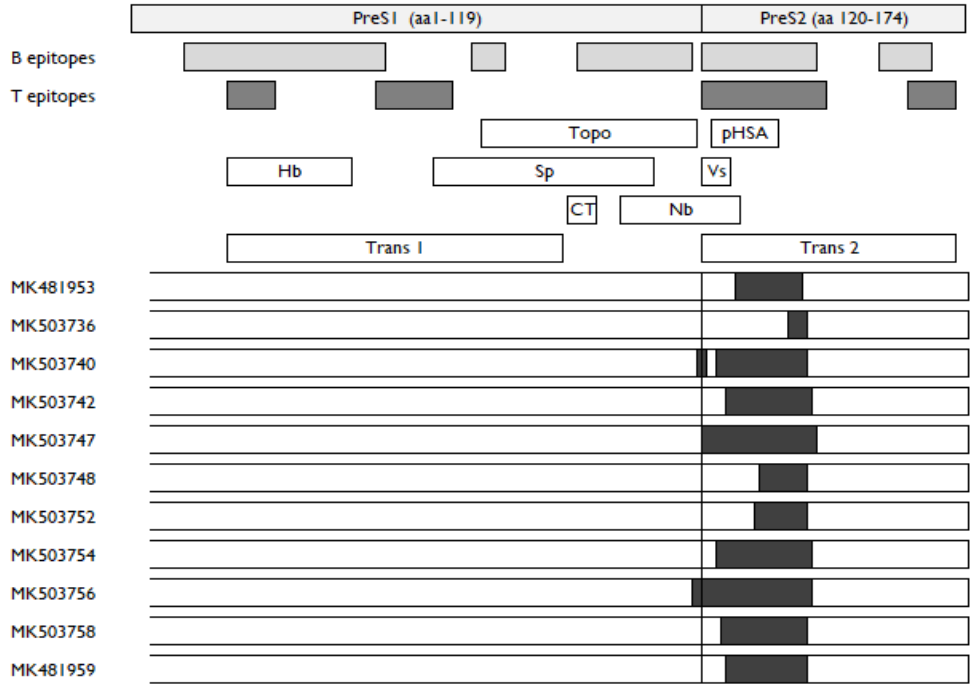

Figure 6.2 Mapping of preS mutations found exclusively in HCC cases. The HBV preS region contains immune epitopes and functional domains as adapted from (30): hepatocyte binding site (Hb), S promoter (Sp), CCAAT box (CT), topology domain (Topo) includes heat shock protein 70 binding site and cytosolic anchorage determinant, nucleocapsid binding site (Nb), viral secretion domain (Vs), polymerized human serum albumin binding site (pHSA), transactivator domain (Trans1/2) (30). The black blocks represent the deletions of amino acids in the HBV isolated from the respective patients.

A mutated ATG start codon in the preS2 gene (3211-3213 from *Eco*RI of subgenotype A) was found in 16 isolates, all of which were also from HCC cases. The methionine codon was replaced by one of the aliphatic, hydrophobic amino acids: isoleucine (3213 G→A: MK503736, MK503737, MK503743, MK481954, MK503749, MK503750,

MK503751, MK503755, MK503757, MK503763), threonine (3212 T→C: MK503744), valine (3211 A→G: MK503754 and MK503762) and a deletion (3211-3213 deletion: MK503740, MK503747 and MK503756). Another preS2 point mutation that was more frequently found in HBV isolated from HCC cases than in controls was the ps2F22L mutation (51.5% vs 20%, $p<0.001$).

6.4 Discussion

In our previous study of 150 SA Blacks with HCC, and 438 sex- and age-matched non-HCC cancer patients as controls, 56% (329/588) had serological evidence of past or current HBV infection (16). HBV DNA was detected in 23.1% (136/588): 61.5% with an overt $HBsAg^{+ve}$ infection and 38.5% were $HBsAg^{-ve}$ (16), and increasing HBV viral load was associated with the increased risk of developing HCC (16). Seventy-seven of 136 HBV DNA^{+ve} samples (56.6%) had sequences for BCP/PC and/or pol/complete S regions, of which 55 (71.4%) were HCC cases and 22 (28.6%) non-HCC controls. Here, statistically significant differences were observed for HBsAg and anti-HBc, which was higher in HCC cases compared to non-HCC controls (83.6% vs 59.1% and 81.8% vs 59.1%, respectively, $p<0.05$), but no difference was found for HIV, anti-HCV, HBeAg, anti-HBs and HBV viral load ($p>0.05$).

The determination of HBV genotypes was based the Kramvis *et al*. method (23) using the BCP/PC region and phylogenetic analysis of the pol/complete S region. Together, both regions showed that the majority of isolates belonged to genotype A (94.3% of the BCP/PC and 100% of the pol/complete S positive isolates), which corresponded to findings in previous SA studies (27,28). HBV genotype A is subdivided into subgenotypes A1, A2, quasi-subgenotype A3 (comprising HBV ‘subgenotype A3',

'tentative A4', and A5) and A4 (previously known as A6) (26,31). Accordingly, the present study found the majority belonged to subgenotype A1 (88.4%), followed by A2 (4.7%) and quasi-subgenotype A3 (7%) when using phylogenetic analysis of the pol/complete S region. In SA, subgenotypes A1, and to a lesser extent A2, have been reported to circulate in HCC patients (10,12), asymptomatic carriers (32), fulminant hepatitis patients (27), and HIV/HBV co-infected individuals (22). Consistent with a previous phylogenetic analysis of 26 complete HBV genomes in SA HCC patients, no isolates belonging to genotype D were identified (12).

In accordance with a previous study (9), subgenotype A1 strains differentially clustered into the Asian and African groups (Figure 6.1). Furthermore, HBV isolates belonging to quasi-subgenotype A3 have not been previously reported in SA, and we further found that these strains were isolated from three HCC patients born outside of SA (one from Democratic Republic of the Congo, while the remaining two were undeclared), which may correspond to the migration from central and western to southern Africa (33,34). As a consequence, immigration plays an important role in the geographic pattern of HBV distribution and the term "immigro-subgenotype" has been suggested to differentiate these imported strains from the indigenous ones (31). The implications of this immigro-subgenotype on HBV distribution and possible recombination between viral strains in SA has yet to be studied in more detail.

In agreement with a previous SA study (13), a higher prevalence of BCP T1753C/G (22.5% vs 0%) and 1762T/1764A double (53.1% vs 33.3%) mutations was reported in southern African Blacks with HCC than in controls. In HCC patients, Baptista *et al*. (13) reported the prevalence of 36% T1753C/G and 66% 1762T/1764A (12.8% and 11% in healthy controls, respectively), while another reported more than three quarters

of patients infected with HBV isolates with the 1762T/1764A double mutation (12). Although not investigated in the current study because of low frequency of non-A genotypes, Ochwoto *et al*. (8) demonstrated that Kenyans chronically infected with HBV genotype A had more frequent BCP/PC mutations (insertion, deletions, G1809T, A1811T/C, C1812T, G1862T/C, G1888A) and HBsAg aa substitutions within the antigenic determinant region (aa 100–160) than genotype D (8).

PreS analysis showed that deletions were more often located within the N-terminus half of the preS2, rather than the preS1 or the preS1/S2 boundary (Figure 6.2), and this is in accordance with a previous study (12). In contrast, acute and fulminant hepatitis SA patients had preS deletions accumulating at the C-terminus of the preS1 (27,35,36). While the preS2 region may not be necessary for virion assembly, infectivity and secretion (37), it may be a hot-spot region for the occurrence of deletions particularly in late stage chronic carriers (6).

In the current study, mapping of the preS functional domains showed that deletions occurred exclusively in isolates from HCC cases, and were specific to the preS2 region that coincided with the B- and T-cell epitopes, Vs domain, Nb domain, transactivator domain and pHSA binding site (30). As a result, these deletions may represent the selection of immune escape variants and the possibly increased hepatocarcinogenic potential thereof. The fact that the preS2 gene overlaps the spacer domain of the polymerase gene means that mutations and large deletions may be tolerated to an extent without compromising the activity of the polymerase. On the other hand, the retention of the preS2 mutant oncoproteins in the secretory pathway can induce an endoplasmic reticulum (ER) stress that results in a growth advantage, DNA damage, centrosome

overduplication, and genomic instability that drive the type II ground glass hepatocytes toward the pre-neoplastic and neoplastic lesions (38).

In the current study, mutations in the preS2 start codon that abrogate the expression of the M protein and result in preS2 defective variants were also found to be exclusive to isolates from HCC patients. These mutations have been reported to be associated with an increased risk of developing this malignancy among patients infected with genotype A, C and D (39,40). Furthermore, the aa substitution at codon 22 of the preS2 (ps2F22L) was also demonstrated to occur more frequently in HCC cases (51.5% vs 20% non-HCC controls, p=0.005), and is in accordance to previous reports in genotype A strains from India (7), and genotype E and D strains from Sudan (41). These aa substitutions in the preS and S genes and their relationship to HCC development have rarely been reported in SA subgenotype A1, thus further functional studies investigating their effect are needed, in order to further elucidate the biological pathway that leads to pathogenesis.

In summary, 1753V, 1762T/1764A, preS2 deletion, preS2 initiation codon and ps2F22L mutations were more frequent in strains isolated from HCC patients than non-HCC controls. The combination of BCP/PC and preS2 mutations may suggest the emergence of naturally occurring immune escape variants with a persistent HBV infection that leads to HCC development, and thus, should be explored further in carriers with HBV subgenotype A1. This information will be important for decision making in relation to the adoption of screening for HCC and HBV infections, as well as treatment strategies in SA. The use of these mutations described in the present study may be potential biomarkers that can be used for cost effective screening of carriers for the identification of those who are at high risk for developing HCC. Such screening as an alternative

strategy has the benefit of being cost effective as previously described in HBV endemic regions (39,40) which is important in resource-limited regions such as SA. However, confirmation of the usefulness of these mutations as biomarkers will require prospective, longitudinal studies consisting of a large sample size of HBV carriers.

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— CHAPTER 7 —

Synopsis and Future Direction

7.1 Background

LC is the 6th most commonly diagnosed cancer and second leading cause of cancer-related deaths globally. In developed countries, it is known that LC is a suitable target for surveillance programs as this disease has a well-defined set of risk factors that have been demonstrated by epidemiological and experimental evidence, and include: chronic viral infections (HBV and HCV) and other factors that depends on the behaviour and lifestyle of individuals (excessive alcohol consumption, aflatoxin exposure, iron overload, diabetes, obesity, etc) (Chapter 1). Furthermore, LC has a protracted preclinical phase, that is, the timely identification of this malignancy may lead to appropriate treatment of the individual while the disease is diagnosed at a curable stage. Despite this, LC screening (or surveillance) programs has not yet been established in the general population in SA, where historical literature show high number of cases.

Thus, in the absence of a surveillance program, the present study aimed to provide a comprehensive assessment of the epidemiology of LC in SA, by:

1. Investigating LC incidence and mortality trends using data that is currently available from two sources: the SA NCR and StatsSA, while taking sex and/or population group variations into consideration.

2. Describing the occurrence of viral (HBV, HCV and/or HIV infection) and non-viral (socio-demographics and lifestyle behaviours) risk factors associated with HCC in black African patients presenting with and without HCC in public hospitals in Johannesburg, SA.

3. Describing the combination of HBV mutations in strains that are predominantly found in the South African black African population, and the contribution of which may lead to the development of HCC.

7.2 Research questions

Efforts to reduce LC disparities begin with understanding the incidence and mortality trends between different population, age and gender groups, which have been documented in several countries/regions around the world. The incidence of HCC has been reported to have risen from 6.2 to 6.7 cases per 1000 person-years between 2000 (Bosch et al. 2004) to 2012 (Wong et al. 2017) in southern Africa, where HBV is endemic. Hitherto, studies of LC incidence in populations in African communities have been limited to regional-based cancer registries, whereas studies of LC mortality have been absent. Southern Africa has previously reported more than average incidence of LC (Kew and Geddes 1982; Kew 2013), thus is deserving of an in depth understanding. Furthermore, the present study aimed to provide an update of previously published literature. Until the present study, no previous study has systematically examined the LC disease burden in SA nationally, as well as in various groups within the population. Using the available incidence and mortality data that are provided by the SA NCR and StatsSA databases respectively, we described overall cancer incidence, mortality, age-adjusted temporal trends, and age-specific incidence patterns in the different sex-, age- and population- subgroups.

In addition, previous case-controls studies in SA utilising hospital-based patients have identified several lifestyle-related (alcohol abuse, smoking, dietary AFB_1 contamination, place of birth and hormonal contraceptive use) (Mohamed et al. 1992; Kew et al. 1985; Kew, Song, et al. 1990; Mandishona et al. 1998) and viral (HBV/HCV or HIV) (Kew 2006; Kew 1994; Kew et al. 2005; Sitas et al. 1997) risk factors in relation to HCC. However, these risk factors had not been validated more recently or in combination, as was addressed in this thesis. We have determined the prevalence of these risk factors and have provided risk analysis relative to non-HCC cancer patients, while adjusting for confounding factors. This was achieved by investigating a matched case-control study using secondary data from the JCS database located in Johannesburg, South Africa. Established in 1995, this large case-control study, which recruited self-defined black

cancer patients with all types of cancers, was conducted at the greater Johannesburg public referral hospitals that offered cancer treatments. Over 21 000 cancer patients have been recruited to the study with more than 90% having donated a blood sample.

Identification and stratification of individuals at high risk of developing HCC is important to guide future surveillance strategies, as well as providing preventive and curative strategies and consequently improving survival. While HCC surveillance in the HBV-infected population remains limited in SA, such programs would require studies investigating the cost-effectiveness of such programs and is particularly necessary in regions where monetary, human and infrastructural resources are scarce. The alternative strategy of screening high-risk HBV carriers with particular HBV mutations as biomarkers has been previously suggested in other HBV endemic regions such as China (Fang et al. 2008). Thus, analysis of HBV mutations in the BCP/PC and preS/S regions were undertaken in order to postulate how these mutational markers could provide inroads into point-of-care diagnostic and drug development effort for high-risk patients in SA.

7.3 Innovations and breakthroughs

7.3.1 Epidemiological study (LC incidence and mortality)

For the first time, by analysing all incident and death cases that were reported and recorded in SA, we identified variations in the pattern of LC incidence and mortality between the different population-age subgroups, using the most complete and representative data to date. The principal findings were that incidence and mortality rates of LC decreased from 1993 through 2012 (Table 3.5) and 1999 through 2015 (Chapter 4, Table 1) respectively, but with marked increases in both trends among middle-aged black African women from 2004, the year that corresponded with the massive roll-out of ART nationwide. The estimation of survival by using MIR calculations provided a dismal outlook for LC in the immediate term (Chapter 4, Figure 3) - a significant proportion of LC cases were only diagnosed during autopsy, and thus were not registered in the SA NCR. In addition, black African men in their early 30s were significantly at a higher risk of developing LC (Figure 3.7). Overall incidence and mortality rates for new LC patients increased with increasing age. While these findings

show similar rising LC incidence patterns to low-risk regions in HICs (Western Europe, North America, and Oceania) (Center & Jemal 2011), they differed to that of similar high-risk regions in sub-Saharan Africa and East Asia, where a decreasing trend in LC incidence and mortality has been observed in recent years (Figure 3.11). These trends however may also reflect the changes in the exposure to LC-subtype risk factors as discussed in Chapter 1. Age-period-cohort analysis identified period effect was statistically significant and this may result from increasing life expectancy (Figure 3.1). Accordingly, a consequence of population aging is that this may lead to the increase in the exposure to LC-related risk factors.

7.3.2 Viral and lifestyle-related risk-factors associated with HCC

In SA, previous case-control studies investigating lifestyle-related risk factors for HCC development in black Africans have done so by comparing HCC cases to controls that are either: healthy (Viana et al. 2009; Kew, Houghton, et al. 1990), asymptomatic chronic carriers (Skelton 2009; Kew et al. 2005), hospital patients suffering from a variety of non-HCC medical disorders that were either unspecified (Kew et al. 1986; Mohamed et al. 1992; Kew et al. 1997) or specified as the following: (chronic rheumatic valvular disease, malignant hypertension, congestive cardiomyopathy, obstructive airway disease, bronchial asthma, pneumonia, tuberculosis, diabetes mellitus, urinary tract infection, alcoholic liver disease, gastroenteritis, avitaminosis, schistosomiasis, renal failure, sarcoidosis, encephalitis and connective tissue disorders, or surgical diseases such as “acute abdomen,” pancreatitis, fractures, carcinoma of the oesophagus, carcinoma of the stomach, soft tissue tumours, breast abscess, burns, septic ulcers, peptic ulcer, lipoma, chronic otitis media, and perianal abscess (Kew, Song, et al. 1990); or bronchogenic carcinoma; chronic obstructive airways disease; ischemic heart disease, peripheral vascular disease, peptic ulcer disease, carcinoma of the mouth, and carcinoma of the oesophagus (Kew et al. 1985). Other studies investigated the malignancy by comparing HCC cases to other HCC patients based on: sex (men vs women) (Kew et al. 1983), place of birth (rural vs urban) (Kew et al. 2007), cirrhosis status (with or without) (Kew 1989b) or age (≤30 years vs ≥50 years) (Kew & Macerollo 1988). In contrast, several studies were carried out in the absence of controls (Kew & Geddes 1982; Skelton 2009). It should be noted, that the only study

investigating viral load in HCC cases (Skelton 2009) was not a case-control study and did not adjust for confounding risk factors. These South African studies utilising different ascertainment methods of the populations may differ in their exposures of the control population group. In the United States, a study by Hassan *et al*. (Hassan et al. 2002) consisting of 115 histologically confirmed HCC cases and 230 non–LC controls with histologically confirmed primary neoplasms (such as gastrointestinal tract [44.3%], urogenital tract [18.7%], respiratory tract [17.8%], and skin [19.1%]), synergistic interactions were observed between heavy alcohol consumption and chronic hepatitis virus infection (HBV and HCV) (OR, 53.9; 95% CI, 7.0-415.7) and diabetes mellitus (OR, 9.9; 95% CI, 2.5-39.3) (Hassan et al. 2002).

Thus, the present study is the only work to have determined the risk for both HCC-related viral and lifestyle-related risk factors while adjusting for compounding factors, between black South Africans with HCC and non-HCC cancer controls who were all treatment-naïve. These controls were hospital patients selected from a wide variety admission tumour diagnoses, all of which are believed to be neither caused by nor prevented by the viral and lifestyle-related risk factors that were examined. The HCC cases were normalised to the control group prior to univariate and multivariate analyses, using the case-control age-sex matched study design whereby HCC cases were age- and sex-matched to three non-HCC controls attending the same hospital in the greater region of Johannesburg.

This retrospective case-control study extensively investigated the demographics and serological markers of HBV infection of SA adults with regard to HCC risk: the association between demographics and the risk of developing this tumour was significant in those born in rural areas, male sex and having lived in urban area for fewer than 15 years (Chapter 4, Table 1). The group with HBsAg-positivity is significantly associated with increased risk of developing HCC when compared with those who were seronegative for this biomarker, and the risk increased with the presence of additional markers such as anti-HBc and HBV DNA (Chapter 4, Table 2). Additionally, this is the first study to show an association between HCC and occult HBV infection in Black South Africans (Chapter 4, Table 2), as well as an increased risk of developing HCC with increasing viremia from >2000 to ≥200,000 IU/ml

(Chapter 4, Table 3). HCV infection was associated with a risk of developing HCC whereas HIV was not. Moreover, co-infection between either of HCV or HIV and HBV was a risk factor (Chapter 4, Table 4). In contrast, the relationship between HCC development and potential lifestyle-related risk factors including alcohol consumption, smoking, hormonal contraception use and diabetes were not found to be significant (Chapter 4, Table 2).

7.3.3 HBV mutation markers in HCC patients

In a further nested study, HBV sequences from HCC patients were compared to HBV isolates from non-HCC controls. Both HCC cases and non-HCC controls were predominantly infected with HBV subgenotype A1. For the first time, subgenotype A3 was reported in a South African cohort (Chapter 6, Figure 1.2), implicating a recent introduction into SA possibly as a result of north-to-south migration in Africa. BCP mutations 1753C/G and 1764A were associated with an increased risk of HCC development (Chapter 6, Table 1.3). Moreover, we showed that mutations in preS2 start codon mutation, ps2F22L mutation and preS deletions in subgenotype A1 were significantly associated with HCC in SA Blacks. The preS deletion mainly occurred at the N-terminus half of the preS2 region, coinciding with the location of the B- and T-cell epitopes (Chapter 6, Figure, 1.4). Furthermore, these patients infected with the preS deletion mutant strains were presented in combination with the 1762T/1764A double mutation, high HBV viremia and HCC disease at a significantly younger age when compared to those without the preS deletions.

7.4 Applications

7.4.1 Changes in epidemiology of Liver Cancer

The practical application of the cancer mortality data as reported is that they can be used to supplement the cancer registries in confirming the cases that have or have not been diagnosed. Furthermore, the up-to-date estimates of the increasing LC trends in SA alongside the disparities in LC burden between the different sex-, age- and population-subgroups at a national level provides a basis for establishing priorities for LC control and future healthcare provision in SA. If evidence from developed countries are an indication, the decrease in AIDS-defining cancers (ADC) in the ART-era followed by

the concomitant increase in non-AIDS-defining cancers (NADC), such as LC, should raise an alarm (Deeken et al. 2012). This is particularly important in SA, where the proportion of the HIV-infected population and those with free access to ART are amongst the largest in the world. This is not surprising and actually would be expected because HIV-infected individuals are now living longer, with age being a potential epidemiological risk factor for NADCs, none more so than LC. Thus, the predilection of middle-aged black Africans with the projected overall worsening outcomes of LC, carries several implications. First, efforts directed towards control of LC-related risk factors should be intensified. In addition, the development of adequate surveillance tools aimed at early diagnosis of high-risk patients, particularly in rural communities should be carried out, in order to provide curative treatments. More specifically, surveillance should also focus on creating awareness and identifying age- and population-specific causes.

7.4.2 HBV infection in a South African cohort attending urban public hospitals

HCC is one of the most fatal human cancers, and is largely amenable to primary prevention with the currently available knowledge and technology. The current study revealed that with such a high prevalence of HBV in HCC patient cohort, HBV continues to play a major role in the development of HCC in Black SA adults. Despite the implementation of the universal infant HBV vaccination program (Burnett et al. 2012), there will be a several-decade gap before the impact on the reduction of the HBV-related sequelae will take place in adults. Thus, the incidence of HCC will continue to increase in HBV-positive individuals mainly in their late 30s and early 40s. While the adult vaccination coverage rate in SA is unknown, it is likely to be low as a consequence of limited awareness among the public of the benefits of this vaccine as well as payment challenges. Furthermore, vaccination programs targeting adults in resource-limited regions may prove ineffective as most adults in SA are infected with HBV early in their life. Thus, strategies to improve adult immunization in uninfected persons at risk for HBV infection and for whom the vaccine is recommended include primarily infants and children. Moreover, key goals should include provider recommendation and convenient access to vaccination not only among children but also adults without the protection of anti-HBs, while also raising awareness among decision

makers and rural communities of the risks and consequences of HBV infection through educating and engaging the communities. Additionally, we reported for the first time that high viral load increases the risk for HCC development and that occult infection also carries a risk for hepatocarcinogenesis. These findings suggest the importance of nucleic testing in prioritising high-risk patients for treatments as well as preventing false negatives in the diagnosis of HBV infections. Provision of antiviral treatments for HBV monoinfected individuals to decrease viral loads may also contribute to reducing the risk of HCC development.

7.4.3 HBV mutations as biomarkers for the detection of HCC

In the present study, 23 major HBV mutations associated with HCC risk was evaluated. The mutational patterns found may serve as prognostic factors as they represent a strategy of HBV to effectively replicate while becoming undetectable by the host immune system. Thus, the accumulation of preS mutations combined with BCP/PC mutation(s) may ultimately lead to the persistence of the virus in the liver, which is important for hepatocarcinogenesis. Therefore, possible applications of the current HBV mutational research could improve case ascertainment of high-risk patients and also prioritize them for ART with more frequent follow-ups, particularly for those patients with HBV with co-existing 1753C/G, 1762T/1764A, ps2F22L and preS2 start codon mutations and preS2 deletions. This is particularly important in SA where AFP is still relied upon for diagnosis of HCC and raised levels often corroborate with late stage disease and poor prognosis. Multivariate risk assessment profiles for HCC, when integrated with current HBV treatment guidelines, may enable practicing physicians to better manage HBV-patients with different HCC risks. Understanding the different factors and their associated contribution to the risk of developing HCC may also allow for risk modification through ART, and thus lead to the prevention of disease progression and eventually reducing the risk of HCC development even amongst high-risk groups.

7.5 Future directions

7.5.1 Changes in the epidemiology of LC

In a region characterised by the poor cancer surveillance systems, we show here that tracking the emerging trends by utilising both the incidence and mortality rates is an essential tool that can assist with developing policies that include cancer-control planning, early detection, and prevention efforts. On the other hand, more adequate screening and diagnostic tools should be prioritised given the poor detection of cases shown currently. This finding particularly highlights inadequacies and flaws in either the awareness of the public of the LC or access to diagnostic facilities and resources that may otherwise enable the diagnose of this malignancy in a timely manner. Thus, current trends in LC further supports the need for the development of a population-based cancer registry in SA.

The current undertaking of a population-based registry at the Ekurhuleni region by the SA NCR suggests that, in the future, it may be possible to describe the distribution of LC incidence more accurately amongst the South African population. The population-based cancer registry, unlike the current pathology-base cancer registry, will not solely rely on histological diagnosis of LC in order to be recorded in the NCR database. Histological diagnosis of LC is limiting in several aspects, particularly in cases whereby patients present with end-stage liver disease that are no longer amenable to curable treatments and a liver biopsy would confer no additional benefit to the patients well-being. Furthermore, unlike liver, imaging technologies such as ultrasound are both readily available and cost effective in most developing regions where pathology laboratories and skilled technicians are also scarce. First year data (2017) from the Ekurhuleni population-based registry has already been analysed and the results of which would inform clinicians by providing targeted LC surveillance for at-risk population groups. Additionally, this will also inform national health and social development policies in SA to strengthen cancer policies, cancer planning programs, monitoring and evaluation of interventions in the general population in order to curb the rising burden of LC, and consequently reduce the disparities in disease burden between the population group.

Lastly, the impact of adherence to surveillance of LC and the consequent survival rates associated with the tumour stage at diagnosis are further studies that are required in SA. While the reasons for the observed population-age subgroup trends observed in the present study are likely manifold, the underlying causes remain poorly understood and should also form the basis for future investigations. Studies investigating LC-related risk factors in the population born during the HBV vaccine-era will also constitute a worthwhile study area in the future; as the development of future policies regarding prevention and disease control will depend on such findings.

7.5.2 HBV infection in SA

In SA where both HIV and HBV are endemic, HIV/HBV co-infection is not uncommon and the population with ART-treatment is one of the largest world, well-designed cross-sectional studies investigating the incidence of HCC in HIV/HBV patients compared to HBV or HIV-monoinfected patients have been absent in this population. If the HCC trends in developed countries should be of any indication, lifestyle-related risk factors for HCC will progress towards a more prominent role in an ageing population and a concurrent decreasing role of HBV as a result of infant vaccination programs. Despite this, characterisation of HCC cases with regards to lifestyle-related risk factors was noted to be poor in the present study and thus suggests that prospective studies consisting of larger cohorts selected from the general population may yield higher quality data if suspected cases of HCC are investigated.

The efficacy of the universal HBV immunization has been shown to be effective in reducing HBV carriage in children, however, greater effort is required to overcome the social and economic hurdles that the adults are experiencing with regards to this infection. Moreover, a HBV management guideline that is specific to South Africans would be necessary in order for early screening and detection of the infection. Future studies should investigate the feasibility of implementing HBV surveillance programs, availability of the vaccine and management of chronic infection in the SA adult population. Thus, future programs that identify HBV-monoinfected individuals should link them to regular care and treatment based on the currently available network of ART programs for HIV infected patients.

Fortunately, safe and effective ART is available SA, and incorporating HBV-monoinfected individuals into the treatment programmes would not require additional infrastructure. The method of incorporating these HBV-infected individuals into the existing ART-programs should form the basis of future studies, as to prevent imposing further strains on the already stretched governmental healthcare resources. Notwithstanding this, HBV control in the Black African adult population in the near future is possible, but only if we can overcome these challenges by clearly identifying them, while also ensuring that resources that are necessary are both made available and easily accessible.

7.5.3 HBV mutations

The final goal of the current endeavour was to identify the risk of developing HCC associated with the different HBV mutations. Whether the different HBV mutations represent an element required for, or are consequent upon hepatocarcinogenesis are subjects that deserve further investigation. On the other hand, future work in SA is needed to validate the present findings in larger cohorts, as well as providing means in which the diagnostic technique (sequencing of HBV isolates) may be more readily available and applicable to larger at-risk populations, most of whom reside in rural communities. However, to do so would require the external validation of the cost-effectiveness and long term follow-up prospective studies in chronic HBV-patients. Additionally, corroboration of the various HBV mutations determined in HBV isolates from the sera and liver tissues of infected patients would provide evidence of consistency and reliability.

Most of the mutations have been described separately in previous studies between HBV-HCC patients and healthy controls, while the current research comparing HBV mutational profiles to HBV-non-HCC tumours will need further elucidation in the context of cirrhosis and tumour staging. Further studies are needed to analyse the effect of variations of preS2 mutations for the immunogenicity in HBV vaccines, and whether this has any implications on current HBV vaccines. In addition, studies comparing HBV mutations between HBsAg-negative occult HBV patients with groups of vaccinated individuals would provide a basis of elimination of the infection in groups that may not

be detected using commercially available diagnostic kits, as well as providing information to further understand the pathogenesis of HCC.

7.6 Conclusion

In SA, temporal trends in LC incidence and mortality rates increased at the end of the first decade of the 21st century. While these observed trends may be the consequence of various factors, in SA, HBV remains a major risk factor for developing HCC in adult black African men who were born in rural areas.

This work provides a critical assessment of current control strategies in the prevention and intervention of HCC, while also potentially assists in the development of more targeted screening of this disease in high risk groups. It therefore provides for a much needed data that is lacking in sub-Saharan Africa as a result of underreporting and the lack of LC surveillance. Importantly, exploration of the epidemiological trends in LC within SA provides a unique vantage point to scrutinize various factors at work. First, SA is a middle-income country that is more developed than its neighbouring nations but less so than most Western countries. Second, SA is ethnically diverse, while also home to the world's largest population with HIV infection. The consequence of the latter is that the impact is largely felt in the black African population, particularly women, and contributes to a younger demographic than most developed regions. Third, nationwide hepatitis B vaccination programs have been successfully implemented since 1995, while the world's largest ART program has been available to the public since 2004/5. Both these programs have been shown to positively contribute to the decline in hepatitis B seroprevalence in children and persons infected with HIV, respectively. Despite this, there remains a group of unvaccinated adults who were born prior to the HBV vaccine introduction and are ageing in significant proportions. Fourth, HBV in SA is mainly acquired horizontally during adolescence and the strain (subgenotype A1) differs to those identified in other Eastern regions (genotype C and B) that also have high HBV prevalence. The use of diagnostic biomarkers specific to the South African HBV strain may guide clinicians in effectively treating the infection, while also enable the

identification of occult HBV infections that are missed when using conventional diagnostic kits. Finally, with the rapid development, urbanization, and changing of lifestyles in SA, an increasing prevalence of metabolic disease (e.g. obesity and diabetes) has been reported. As metabolic risk factors are commonly associated with NAFLD, the prevalence of which would also be expected to be on the upsurge in the coming decades, particularly as a large proportion of the South African population are now living longer, and thus, are more likely to be exposed to NAFLD-related risk factors.

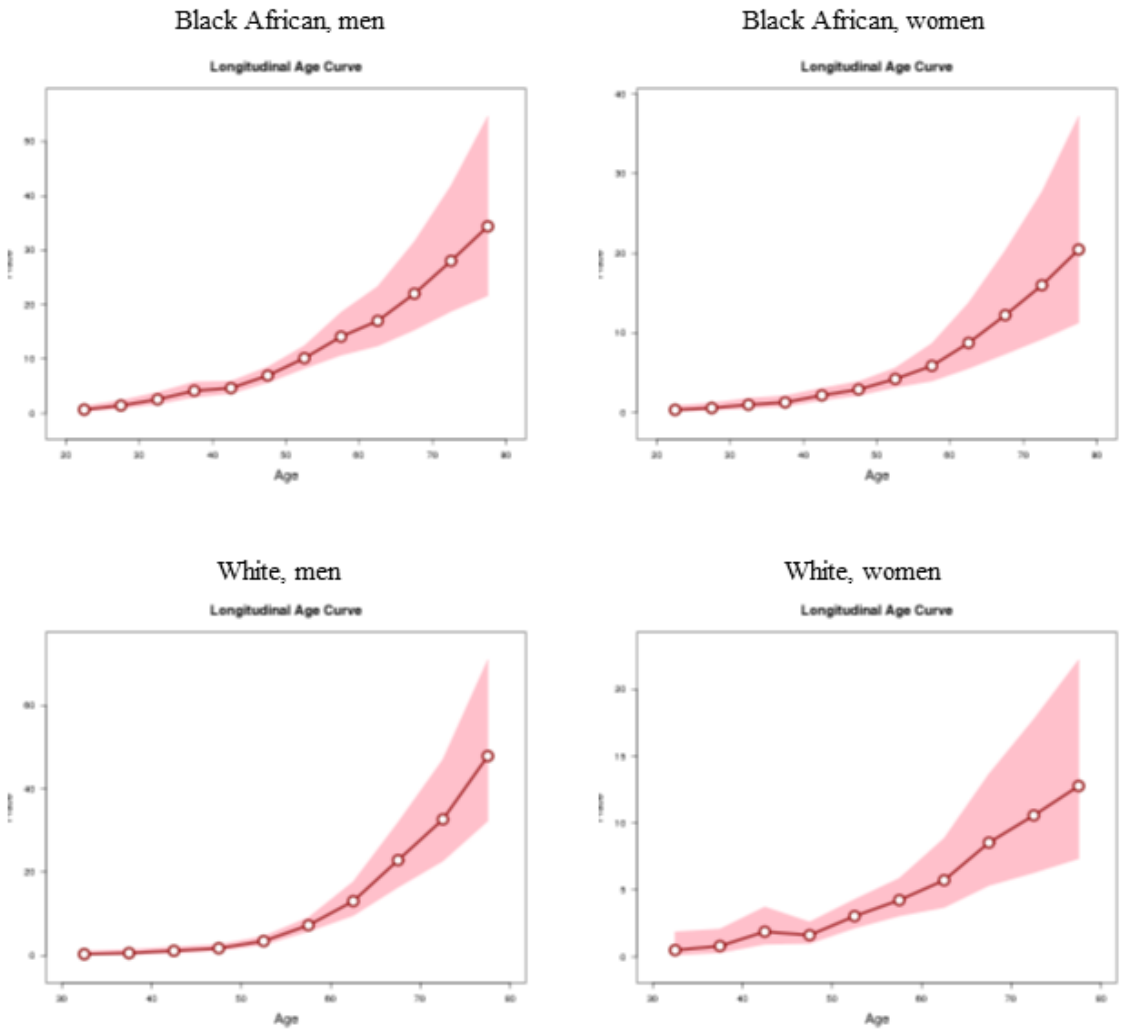
Changes in the population demographics and prevalence of HCC-related risk factors will dictate the temporal trends in epidemiology of this malignancy in the coming decades. it is hypothesized that the decline of HBV as a risk factor for HCC may be offset by the emergence of NAFLD and ageing as risk factors for HCC development in the coming decades. Indeed, future prospective cohort studies are required to confirm this. Equally, the foreseeable adoption of imaging diagnostic modalities and the population-based cancer registry may contribute to an increase in LC incidence rates that may be more in-line with the current mortality rate, and thus, positively impacting the outcome of LC. Lastly, appreciation of the epidemiological trends will permit clinicians and public policy makers to anticipate and adapt accordingly in terms of public health perspectives, resource allocation, and formulation of management guidelines for patients with HCC.

Appendix

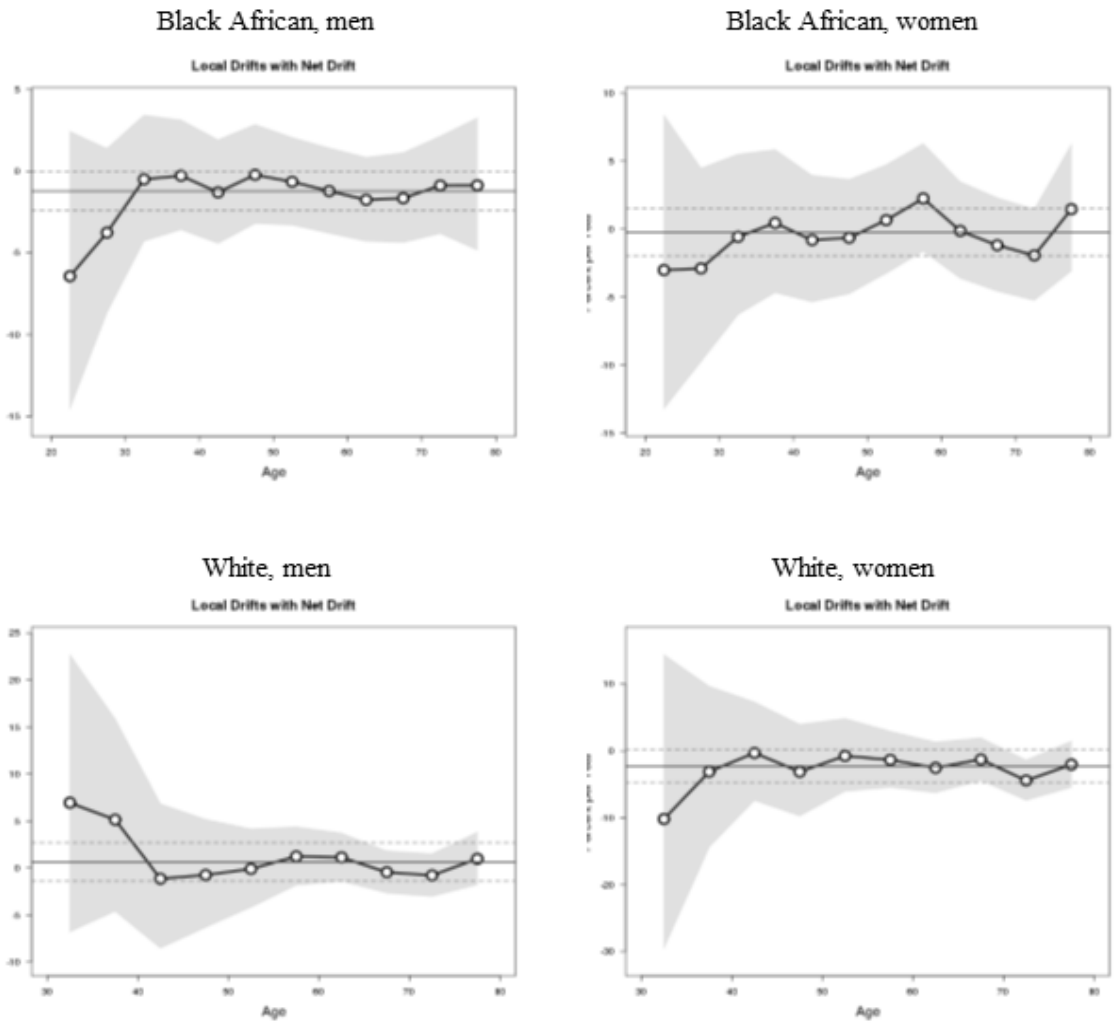
APPENDIX A. The number of primary liver cancer cases reported to the SA pathology based National Cancer Registry (NCR), years 1993–2012, by population group.

Year	Indian/Asian	Black	Coloured	White	Total
1993	7	572	29	133	741
1994	9	331	26	67	433
1995	8	374	15	54	451
1996	11	340	15	56	422
1997	4	363	20	67	454
1998	5	347	30	58	440
1999	6	318	17	70	411
2000	10	315	22	42	389
2001	9	258	20	73	360
2002	12	246	22	59	339
2003	8	205	24	58	295
2004	7	212	20	42	281
2005	6	196	17	49	268
2006	3	152	19	69	243
2007	2	189	22	36	249
2008	10	217	21	50	298
2009	6	172	20	42	240
2010	8	158	26	51	243
2011	9	194	21	65	289
2012	13	204	22	63	302
Total	153	5363	428	1204	7148

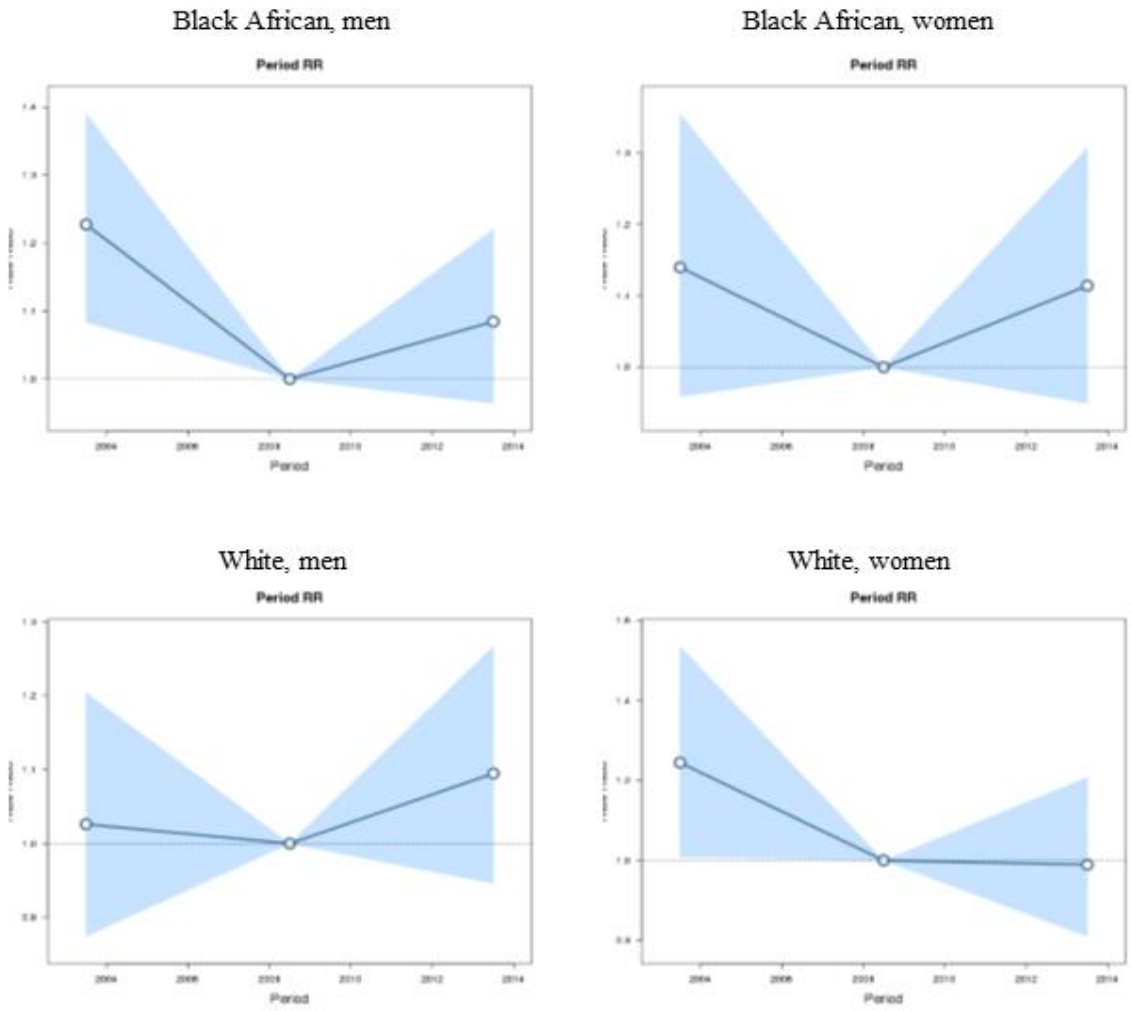
In *1996 and 1997 whites and coloureds were reported together; NCR provided the individual figures that are not in the printed report.



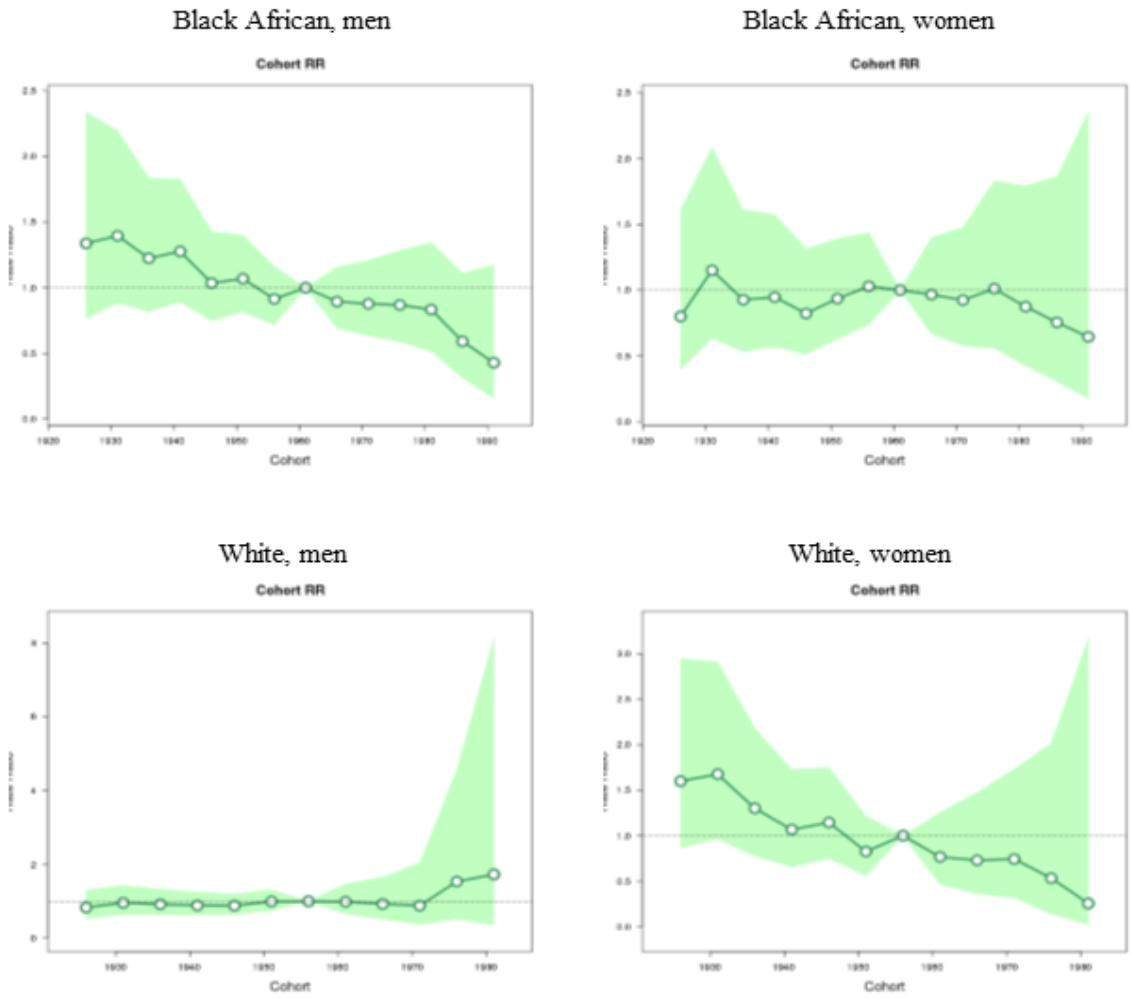
APPENDIX B. Longitudinal age curves of the mortality rates (per 100,000 person-years) of LC and the corresponding 95% confidence intervals in black African and white population by sex.



APPENDIX C. Local drift values for LC mortality rates. Age group specific annual percent change (%) in the mortality rates of LC and the corresponding 95% confidence intervals in black African and white population by sex.



APPENDIX D. Period effects on LC mortality rates. Period effects obtained from age-period-cohort analyses for the rates of LC and the corresponding 95% confidence intervals in black African and white population by sex.



APPENDIX E. Cohort effects on LC mortality rates. Cohort effects obtained from age-period-cohort analyses for the rates of LC and the corresponding 95% confidence intervals in black African and white population by sex.

5

Ethics Clearance Certificate No. M150239

R14/49 Prof Anna Kramvis and Mr Daniel Mak et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150239

NAME: (Principal Investigator)	Prof Anna Kramvis and Mr Daniel Mak et al
DEPARTMENT:	Internal Medicine Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	The Interaction of HIV with Hepatitis Viruses as Risks for Hepatocellular Carcinoma in the Black South African Population 1998-2011
DATE CONSIDERED:	27/02/2015
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	

APPROVED BY: Professor P Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 02/03/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**

Principal Investigator Signature Date 10-03-2015

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Ethics Clearance Certificate No. M170292

R14/49 Prof Anna Kramvis and Mr Daniel Mark et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M170292

NAME: (Principal Investigator)	Prof Anna Kramvis and Mr Daniel Mark et al
DEPARTMENT:	Internal Medicine Hepatitis Virus Diversity Research Unit University of the Witwatersrand Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	The Epidemiology and Risks Factors Associated with Liver Cancer in South Africa
DATE CONSIDERED:	Adhoc
DECISION:	Approved unconditionally
CONDITIONS:	Sub-Study(M150239)
SUPERVISOR:	
APPROVED BY:	Professor P. Cleaton-Jones,Chairperson, HREC (Medical)
DATE OF APPROVAL:	10/03/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004,10th floor, Senate House/2nd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. in this case, the study was initially review in February and will therefore be due in the month of February each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

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