HEPATITIS B VIRUS-RELATED HEPATOCELLULAR CARCINOMA IN SOUTH AFRICA:

INVESTIGATIONS INTO THE RISK PROFILE OF A PREVIOUSLY UNSCREENED POPULATION FROM THE WESTERN CAPE

by

Bibi Nafiisah Chotun

December 2018

Dissertation presented for the degree of

Doctor of Philosophy in Medical Virology in the

Faculty of Medicine and Health Sciences at

Stellenbosch University

Supervisor: Professor Monique Ingrid Andersson

Co-supervisors: Dr Pedro Fernandez and Professor Wolfgang Preiser

Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

December 2018

TABLE OF CONTENTS

Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AASLD	American Association for the Study of Liver Diseases
ABCB7	ATP Binding Cassette Subfamily B Member 7
AFB-1	aflatoxin B1
AFP	alpha-fetoprotein
AFP-L3	Lens culinaris agglutinin-reactive AFP
AIDS	acquired immunodeficiency syndrome
ALT	alanine aminotransferase
ANNOVAR	Annotate Variation
anti-HBc	antibody to the core antigen
anti-HBe	antibody to the envelope antigen
Anti-HBs	antibody to the hepatitis B surface antigen
APASL	Asian Pacific Association for the Study of the Liver
APRI	aspartate transaminase-to-platelet ratio index
ART	antiretroviral therapy
ASPHD2	Aspartate Beta-Hydroxylase Domain Containing 2
ASSURED	Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable
AST	aspartate transaminase
ATP	adenosine triphosphate
BAIAP2L2	Brain-Specific Angiogenesis Inhibitor 1-Associated Protein 2-Like Protein 2
BCP	basal core promoter
BLAST	Basic Local Alignment Search Tool
BRCA2	Breast And Ovarian Cancer Susceptibility Protein 2

BtHV	bat hepatitis B virus
C22orf31	Chromosome 22 Open Reading Frame 31
C6orf132	Chromosome 6 Open Reading Frame 132
CADD	Combined Annotation Dependent Depletion
cccDNA	covalently closed circular deoxyribonucleic acid
CCDC125	Coiled-Coil Domain Containing 125
CCDC22	Coiled-Coil Domain Containing 22
CDC	Center for Disease Control
CDKN2A	cyclin-dependent kinase inhibitor 2A
CDKN2A/INK4	cyclin-dependent kinase inhibitor 2A/inhibitor of CDK4 (commonly called *p16*)
CHB	chronic hepatitis B virus infection
CHST7	Carbohydrate Sulfotransferase 7
CLIA	Clinical Laboratory Improvement Amendments
CMS	Center for Medicaid Services
CpG	deoxycytidylyl-deoxyguanosine dinucleotides
cRNA	complementary ribonucleic acid
CT	computed tomography
DALYs	disability-adjusted life years
DANN	deleterious annotation of genetic variants using neural networks
dbSNP	Single Nucleotide Polymorphism database
DHBV	duck hepatitis B virus
DMSO	dimethyl-sulfoxide
DNA	deoxyribonucleic acid
EASL	European Association for the Study of the Liver

EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EPI	expanded programme on immunization
ESCRT	endosomal sorting complex required for transport
FAM	6-carboxyfluorescein
FASTA	FAST-All
FATHMM	Functional Analysis through Hidden Markov Models
FCS	fetal calf serum
FDA	Food and Drug Administration
FIB-4	fibrosis-4
FFPE	formalin fixed paraffin embedded
GATK	Genome Analysis Tool Kit
gDNA	genomic deoxyribonucleic acid
GDP	gross domestic product
GGT	gamma-glutamyl transpeptidase
gnomAD	Genome Aggregation Database
GPR	gamma-glutamyl transpeptidase-to-platelet ratio
GTPase	guanosine-5'-triphosphatase
GWAS	genome-wide association studies
H&E	haematoxylin and eosin
HAART	highly active antiretroviral therapy
HBeAg	hepatitis B envelope antigen
HBIG	HBV immune globulin

HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HBx	hepatitis B x protein
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HGH1	HGH1 homolog
HHBV	heron hepatitis B virus
HIV	human immunodeficiency virus
HLA-DRB1	Major Histocompatibility Complex, Class II, DR Beta 1
HLA-DRB5	Major Histocompatibility Complex, Class II, DR Beta 5
HREC	Health Research Ethics Committee
IARC	International Agency for Research on Cancer
ICER	incremental cost-effectiveness ratio
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IgG	immunoglobulin G
IgM	immunoglobulin M
ITIH6	Inter-Alpha-Trypsin Inhibitor Heavy Chain Family Member 6
KDM7A	Lysine Demethylase 7A
KLHL17	Kelch Like Family member 17
LAMP	Loop-mediated isothermal amplification assay
LINE-1	Long Interspersed Nuclear Elements-1
MAF	minor allele frequency
MARVELD2	MARVEL domain-containing protein 2
mRNA	messenger RNA

MTCT	mother-to-child-transmission
MYLK3	Myosin Light Chain Kinase 3
NAFLD	Non-alcoholic Fatty Liver Disease
NASH	Non-Alcoholic Steatohepatitis
NCBI	National Center for Biotechnology Information
NPV	negative predictive value
NTCP	sodium taurocholate cotransporting polypeptide
OCSA	Occupational Care South Africa
OD	optical density
OH	occupational health
OR11G2	Olfactory Receptor Family 11 Subfamily G Member 2
ORF	open reading frames
p16	cyclin-dependent kinase inhibitor 2A/inhibitor of CDK4 (*CDKN2A/INK4*)
PBMCs	peripheral blood mononuclear cells
PC	precore
PCR	polymerase chain reaction
PERM1	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 And Estrogen Related Receptor-Induced Regulator In Muscle 1
PHC	primary healthcare
POCT	point-of-care test
PPV	positive predictive value
PQ	prequalification
PTX3	Pentraxin 3
QALYs	quality-adjusted life years

RAB19	Member RAS Oncogene Family
RASSF1A	Ras association domain-containing protein 1
RCF	relative centrifugal force
RNA	ribonucleic acid
RNase	ribonuclease
RPMI	Roswell Park Memorial Institute
SAMD11	Sterile Alpha Motif Domain Containing 11
SANAS	South African National Accreditation System
SB	sodium borate
SD	standard deviation
SEC14L6	SEC14 Like Lipid Binding 6
SMARCA1	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A, Member 1
SNP	single nucleotide polymorphism
SPANXN2	Sperm Protein Associated with the Nucleus on the X chromosome N2
SSA	SubSaharan Africa
TAMRA	Tetramethyl-6-Carboxyrhodamine
TMEM14A	Transmembrane Protein 14A
TNN	Tenascin N
TP53	p53 tumour suppressor gene
TTLL10	Tubulin Tyrosine Ligase Like 10
USP26	Ubiquitin Specific Peptidase 26
UTR	untranslated region
VCF	Variant Call File

VEGFA	Vascular Endothelial Growth Factor A
VL	viral load
WES	whole exome sequencing
WHO	World Health Organization
WHV	woodchuck hepatitis B virus
WMHBV	woolly monkey hepatitis B virus
ZNRF3	Zinc And Ring Finger 3

ABSTRACT

Hepatocellular carcinoma (HCC) is a neglected major public health problem worldwide. In SubSaharan Africa (SSA), most HCC cases are diagnosed with advanced disease, well past the timing of possible treatment. Most HCC cases worldwide are caused by chronic infection with hepatitis B virus (HBV). Although the bulk of the burden of HBV is in SSA, there are no screening programmes implemented in the general African population so only 0.8% of HBV-infected individuals are diagnosed. Most research on HBV-related HCC has been conducted in Asia, where HBV is also endemic, but where there are differences in disease progression and presentation. The present study investigated HBV and HCC from an African perspective and tackled these public health issues by incorporating three key components for early diagnosis of HBV-related HCC: HBV screening, HCC biomarkers, and HBV-related HCC genomics.

The HBV screening study found the prevalence of HBV in a South African community-based cohort using a validated point-of-care test to be 2.2% (95% CI: 1.4%–3.3%). The test performed well in the field and had a sensitivity, specificity, negative and positive predictive values of 100%. The test was also accepted by the community (93% uptake) and health care providers. The results of the present study support the case for the implementation of HBV screening in South Africa by demonstrating the magnitude of the HBV health problem in South Africa and new evidence that the POCT test performs well in the field, is accepted by the community and health care providers, and that patients diagnosed with the test can be successfully linked to treatment and long-term follow-up.

The HCC biomarker study found significant differences in methylation expression levels in CpG islands in the promoter region of the tumour suppressor gene *RASSF1A* between HCC cases and normal liver tissue controls as well as a significant association between HBV genotype A and HCC. Although the sample size was small, it showed that there are biomarkers that may be used to identify HCC, paving the way for future studies looking into developing HCC risk scores for early diagnosis of HCC.

Using whole-exome sequencing, the HBV-related HCC genomics study identified two novel germline variants in the *SMARCA1* and *RAB19* genes that in the absence of other risk factors besides HBV infection, could have contributed to early-onset HBV-related HCC in their respective hosts.

Overall, these data provide evidence that early diagnosis of HBV-related HCC in an African setting is possible especially if a multi-targeted approach is taken. The simplest approach to minimise the incidence of HCC in SSA would be to implement HBV screening, at the very least in pregnant women, to break the transmission cycle of HBV. Moreover, the biomarkers of interest identified in the present study should be investigated further in larger cohorts and non-invasive patient samples to determine their utility in stratifying HCC risk. Lastly, the WES study showed that there are germline variants that

could predispose carriers to HCC although these results need to be further investigated in *in-silico* and proteomic studies.

OPSOMMING

Hepatosellulêre karsinoom (HCC) is ‘n omvangryke maar verontagsame publieke gesondheid probleem wêreldwyd. In sub-Sahara Afrika (SSA) word meeste HCC gevalle eers gediagnoseer met reeds-gevorderde siekte, lank nadat moontlike behandeling toegepas kon word. Meeste HCC gevalle word deur kroniese hepatitis B virus (HBV) infeksie veroorsaak. Alhoewel die grootste HBV las op SSA is, bestaan daar geen siftingsprogramme vir die algemene populasie in Afrika nie, wat veroorsaak dat slegs 0.8% van HBV positiewe individuele gediagnoseer word. Meeste HBV verwante HCC navorsing is tot dusvêr in Asië uitgevoer waar HBV ook endemies is, maar daar is verskille in siekte ontwikkeling en voordoening. Hierdie studie het HBV en HCC uit ‘n Akrika perspektief benader en die publieke gesondheid kwessies ondersoek deur drie sleutelkomponente in ag te neem om HBV verwante HCC vroeg te identifiseer, naamlik: HBV sifting, HCC biomerkers en HBV verwante HCC genomika.

In die HBV sifting studie, het ‘n geverifieërde punt-van-sorg toets gevind dat die voorkoms van HBV in ‘n Suid Afrikaanse gemeenskap gebaseerde kohort 2.2% (95% CI: 1.4%–3.3%) is. Die toets was suksesvol uitgevoer en het sensitiwiteit, spesifisiteit, negatiewe en positiewe voorspellende waardes van 100% behaal. Die toets is ook deur die gemeenskap (93% opname) en gesondheidsorgverskaffers aanvaar. Die bevindings van die huidige studie ondersteun die implementering van HBV sifting in Suid Afrika deur die omvang van HBV as ‘n meenigte gesondheids probleem te bevestug is en deur nuwe bevindings dat the punt-van-sorg toets gebruiklik in die veld is en aanvaar word deur die gemeenskap en gesondheidsorgverskaffers. Pasiënte gediagnoseer met die toets kan ook behandeling en langtermyn nasorg ontvang.

Die HCC biomerker studie het bekenisvolle verskille in metilering uitdrukking-vlakke in CpG eilande in die promotor area van die tumor onderdrukking geen RASSFIA tussen HCC gevalle en normale lewer weefsel kontroles gevind, asook ‘n betekenisvolle verwantskap tussen HBV genotype A en HCC. Alhoewel die steekproefgrootte klein was, het dit daartoe aanduiding gegee dat sekere biomerkers gebruik kan word vir die identifikasie van HCC, wat die pad voorberei vir verdere studies om HCC risiko-gradering te gebruik om HCC vroegtydig te diagnoseer.

Met die gebruik van heel-eksoom nukleïensuurvolgordebepaling het die HBV-verwante HCC genomika studie twee nuwe kiemlyn variante in die SMARCA1 en RAB19 gene geïdentifiseer, wat onafhanklik van ander risiko faktore, HBV uitgesluit, kon bydra tot vroeë HBV-verwante HCC aanvang in hulle onderskeie gashere.

In samevatting, verskaf hierdie data bewyse dat vroeë diagnose van HBV-verwante HCC in ‘n Afrika-omgewing moontlik is, veral wanneer ‘n multi-geteikende benadering geneem word. Die eenvoudigste benadering tot die vermindering van HCC in SSA is om HBV-sifting te implementer, minstens in swanger vroue om die oordragsiklus te verhoed. Verder moet die biomerkers wat in hierdie studie geïdentifiseer is, in meer omvattende studies ondersoek met gebruik van nie-indringende pasiënt monsters. Laastens het die WES studie aangedui dat daar sekere kiemlyn variante is wat draers kan vatbaar maak aan HCC, hoewel hierdie resultate verder ondersoek moet word in ‘in-silico’ en proteomiese studies.

ACKNOWLEDGEMENTS

C'est avec un cœur débordé de reconnaissance que j'adresse mes sincères remerciements à tous ceux qui ont de près ou de loin, consacré leur bonne volonté et surtout leur dévouement en me soutenant dans la réalisation de ce projet. En premier lieu, je remercie Dieu tout puissant pour sa bonne grâce et tous les responsables de mon université ainsi que mes professeurs et encadreurs, mes parents et moi-même, pour la force et l'énergie consenti pour terminer ce projet.

Je ne pourrais commencer cette dissertation sans adresser particulièrement ma vive reconnaissance aux personnes et aux organisations qui m'ont soutenu durant ce voyage, notamment la Poliomyelitis Research Foundation, la National Research Foundation, la National Health Laboratory Service Research Trust, la L'Oreal-UNESCO For Women In Science et la Stellenbosch University Merit Bursary, sans qui je ne serai aujourd'hui Dr Chotun. Je suis immensément reconnaissante à toutes ces personnes pour avoir si bien veillé sur moi du début jusqu'à la fin de ce travail, car mon doctorat est le fruit de plusieurs années de dur labeur.

Ainsi, j'ai une pensée spéciale pour tous mes amis et collègues de la Division of Medical Virology, qui comme moi, ont choisi ce parcours difficile et qui ont fait que toutes les années passées sur ce projet soient supportables et fructueuse. J'aimerai une fois de plus remercier particulièrement mon cher ami Ian pour avoir pris le temps d'écrire le résumé de ma dissertation en Afrikaans et qui pendant les derniers mois, les dernières semaines, et derniers jours avant la soumission de ma dissertation, a toujours été là pour me réconforter et m'encourager. Sans toi, j'aurai surement abandonné il y a longtemps. Je souhaite aussi remercier Natasha mon mentor et ma life coach, qui m'a encadré et soutenue jusqu'au bout et qui en fin de compte est devenu une de mes amies les plus proches. Je remercie Afia et sa famille pour leur accueille et leur amour inconditionnel. Aussi, une pensé spéciale à chère Shahieda et sa famille qui m'on accueilli comme un des leurs.

It always seems impossible until it is done.

~ Nelson Rolihlahla Mandela

1. INTRODUCTION

1.1 Background

Primary liver cancer is a neglected major public health problem (O'Hara, McNaughton, Maponga, Jooste, Ocama, *et al.*, 2017). Worldwide, it is the fifth most common cancer in men and the ninth most common cancer in women. In SubSaharan Africa (SSA), however, it is the second most common cause of cancer in men and the third most common cause of cancer in women (Ervik, Lam, Ferlay, Mery, Soerjomataram, *et al.*, 2016). Annually, 39 000 new liver cancer cases are diagnosed in SSA (Ervik *et al.*, 2016) with an age-standardized incidence of as high as 25.8/100 000 persons/year reported in Gambia (Ervik *et al.*, 2016). Although primary liver cancers include hepatocellular carcinoma (HCC), cholangiocarcinoma, and angiosarcoma, the most common type of primary liver cancer worldwide and in SSA remains HCC (Wong, Jiang, Goggins, Liang, Fang, *et al.*, 2017). Therefore, reported incidence of primary liver cancer is often considered to reflect incidence of HCC. HCC has a high case fatality rate so that its prevalence reflects incidence (Ervik *et al.*, 2016) and in SSA most HCC cases are diagnosed with advanced disease, well past the timing of possible curative therapy (Kew, 2013). In South Africa, liver cancer is the fifth most common cause of cancer mortality in men and the age-standardized annual incidence is estimated to be 4.8/100 000 persons (Ervik *et al.*, 2016).

Most HCC cases worldwide are caused by chronic infection with viral hepatitis, namely hepatitis B virus (HBV) and hepatitis C virus (HCV). However, while HCV is the main cause of HCC in developed countries, in contrast, in resource-limited settings, the main etiology of HCC remains HBV (Baecker, Liu, La Vecchia & Zhang, 2018; Maucort-Boulch, de Martel, Franceschi & Plummer, 2018). Baecker *et al.* 2018 reported a similar trend in SSA where a higher proportion of HCC cases are caused by HBV, ranging from 58% (95% CI 52%–65%) in East Africa to 69% (64%–74%) in West Africa. These proportions vary from country to country although neighbouring countries usually have similar prevalences. For example, a South African study showed that 68.2% (95% CI 54.3%-73.2%) of HCC cases were positive for hepatitis B surface antigen (HBsAg), indicating active HBV infection (Maponga, 2016). Similarly, in Zambia, the fraction of HBV-related HCC was reported to be 46.3% (95% CI 15.5%-73.2%) (Maucort-Boulch *et al.*, 2018).

Chronic hepatitis B virus infection (CHB) affects 257 million people worldwide, the majority of whom are in SSA and Asia. However, only 9% of affected individuals are aware of their status as CHB is usually asymptomatic (World Health Organization, 2017a). Moreover, although the bulk of the burden of HBV is in SSA, there are currently no HBV screening programmes implemented in the general population of SSA so only 0.8% of CHB cases get diagnosed. In contrast, although high-income countries have a lower HBV burden, 18% of their HBV cases get diagnosed (World Health Organization, 2017b). Current evidence suggests that SSA has a prevalence of CHB of 2.5% to 22.4% (Schweitzer, Horn, Mikolajczyk, Krause & Ott, 2015).

These data demonstrate that HBV-related HCC is a major health issue in SSA, although it is likely that these numbers are underestimations as HBV screening is not routinely practised, even after HCC diagnosis.

Moreover, HCC is underdiagnosed and underreported, especially since most SSA countries have registries that require histological diagnosis for an HCC case to be included within the registry and often lack recent data (Kew, 2013). Despite the high burden of disease observed in SSA, few studies to date have been conducted to establish whether targeted health approaches could be implemented in an African setting to enable early timeous diagnosis of HBV-related HCC, reflecting the need for more research in SSA on Africans to better understand the disease from an African perspective.

1.2 Study Rationale

Most research on early diagnosis of HBV-related HCC has been conducted in Asia, where HBV is also endemic but where there are differences in disease progression and presentation (Kew, 2013; de Martel, Maucort-Boulch, Plummer & Franceschi, 2015; Yang, Altekruse, Nguyen, Gores & Roberts, 2017). HBV-related HCC in SSA presents earlier, at a mean age of 38.9 years, compared to 54.5 years in Eastern Asia (de Martel *et al.*, 2015) and it is estimated that 40% of HBV-related HCC cases occur before the age of 40 (Yang, Gyedu, Afihene, Duduyemi, Micah, *et al.*, 2015). Moreover, the main genotypes in circulation in SSA are genotypes A, D, and E (Kramvis & Kew, 2007a) compared to genotypes B and C in Asia (Kao & Chen, 2006), which show differences in clinical disease progression (Lin & Kao, 2017). Therefore, although risk factors of HBV-related HCC have been identified and HCC risk prediction scores have been developed for Asian populations (Lee & Ahn, 2016; Wong, Chan, Mo, Chan, Loong, *et al.*, 2010; Yang, Yuen, Chan, Han, Chen, *et al.*, 2011; Yuen, Tanaka, Fong, Fung, Wong, *et al.*, 2009), they have not been validated elsewhere and may not be applicable to an African setting. The present study therefore does not only seek to understand the epidemiology and risk factors of HBV-related HCC but also apply the basic scientific results obtained through laboratory testing as part of a screening strategy to try to solve a public health issue.

1.3 Research statement

How can HBV point-of-care test (POCT) screening, biomarker testing, and genetic testing be utilised to contribute to the early diagnosis of HBV-related HCC in a South African population from the Western Cape?

1.4 Study Aims

The overarching goal of the study was to contribute to the body of knowledge on the epidemiology of HBV and HBV-related HCC in the South African population and to explore screening, biomarker, and genetic testing strategies that could be further utilised to contribute to early detection of HBV-related HCC in South Africans. Toward this main aim, three separate sub-studies were developed, each with its respective sub-aim.

Sub-aim 1: To assess the need for and feasibility of implementing HBV POC testing in a community setting in the Western Cape, South Africa.

Sub-aim 2: To identify biomarkers that can potentially be utilised clinically to discriminate between HCC and non-HCC cases.

Sub-aim 3: To identify genetic variants that can possibly predispose South African men to developing early-onset HBV-related HCC

1.5 Hypotheses

A separate hypothesis was developed for each study, corresponding to their respective sub-aim.

STUDY I: HEPATITIS B VIRUS POINT-OF-CARE TESTING STUDY

There is a need for implementing HBV POC testing in a community setting in the Western Cape, South Africa, and its implementation is feasible.

Null hypothesis: The implementation of HBV POC testing is not needed and/or is not feasible in a community setting of the Western Cape, South Africa

STUDY II: HCC BIOMARKER STUDY

Clinical biomarkers can be identified that may potentially be used to discriminate between HCC and non-HCC patients in the population of the Western Cape, South Africa.

Null hypothesis: There are no clinical biomarkers that can potentially be used to screen patients for HCC in the population of the Western Cape, South Africa

STUDY III: WHOLE EXOME SEQUENCING STUDY

Certain germline driver mutations potentially predispose South African men from the Western Cape to developing early-onset HBV-related HCC.

Null hypothesis: There are no germline driver mutations contributing to the development of early-onset HBV-related HCC in South African men from the Western Cape.

1.6 Study Objectives

STUDY I: THE HEPATITIS B VIRUS POINT-OF-CARE TESTING STUDY

1. To determine the prevalence of active HBV infection in a community-based South African population from the Western Cape using a validated HBsAg POCT.

2. To test the performance of the HBsAg POCT in terms of sensitivity, specificity, positive and negative predictive values.

3. To assess the perception of the tested population toward the HBV POCT and the reasons for accepting/refusing the test.

4. To assess the perception of the nursing staff toward the HBV POCT and the barriers encountered in administering the HBV POCT.

STUDY II: THE BIOMARKER STUDY

1. To determine the proportion of HCC caused by HBV in two tertiary hospitals in the Western Cape, South Africa.
2. To determine and compare HBV genotypes between HCC and non-HCC cases.
3. To compare the occurrence of aflatoxin exposure between HCC and non-HCC cases.
4. To determine the differences in methylation levels in tumour suppressor genes between HCC and non-HCC cases.
5. To determine the proportion of human immunodeficiency virus (HIV)-positive HCC patients.
6. To determine the proportion of HCC cases with cirrhosis
7. To evaluate the potential of the above-listed biomarkers to discriminate between HCC and non-HCC patients in terms of sensitivity, specificity, positive and negative predictive values.

STUDY III: THE WHOLE EXOME SEQUENCING STUDY

1. To use whole exome sequencing to identify novel rare germline variants predisposing young HBV-infected South African men to developing HBV-related HCC and confirm the presence of the identified variants by Sanger sequencing

2. To validate the identified potential genetic variants using Sanger sequencing in a retrospective cohort of HBV-infected patients without HCC

1.7 Chapter Overview

Chapter two is a literature review, whilst chapter three, four, and five detail the research design and methodology, results, and discussion for sub-studies one, two, and three, respectively. Each sub-study will have a conclusion in which the summary of research findings, recommendations, as well as suggestions for future research, will be provided. This dissertation will have a final conclusion in chapter six summarising the main findings of the overall research project.

2. LITERATURE REVIEW

2.1 Hepatitis B virus

Hepatitis B virus is a virus belonging to the family *Hepadnaviridae* (Gust, Burrell, Coulepis, Robinson & Zuckerman, 1985), with the genera *Orthohepadnavirus* and *Avihepadnavirus*. The former genus includes hepatitis B viruses that have been isolated from mammals, such as humans (HBV), non-human primates such as woolly monkeys (WMHBV; Lanford, Chavez, Brasky, Burns & Rico-Hesse, 1998), woodchucks (WHV; Summers, Smolec & Snyder, 1978), and bats (BtHV; Drexler, Geipel, Konig, Corman, van Riel, Leijten, Bremer, Rasche, Cottontail, Maganga, Schlegel, Muller, Adam, Klose, Borges Carneiro, Stocker, Franke, Gloza-Rausch, Geyer, Annan, Adu-Sarkodie, Oppong, Binger, Vallo, Tschapka, Ulrich, Gerlich, Leroy, Kuiken, Glebe & Drosten, 2013). The latter genus includes hepatitis B viruses that have been isolated from birds such as the Pekin duck (DHBV; Mason, Seal & Summers, 1980) and herons (HHBV; Sprengel, Kaleta & Will, 1988). A hepadnavirus was recently isolated from a white sucker (*Catostomus commersonii*), making it the first isolate to be identified in fish (Hahn, Iwanowicz, Cornman, Conway, Winton, *et al.*, 2015) and has been tentatively named a parahepadnavirus. Subsequently, other hepadnaviruses have been isolated from fish and amphibians and tentatively grouped as metahepadnaviruses and herpetohepadnaviruses, respectively (Dill, Camus, Leary, Di Giallonardo, Holmes, *et al.*, 2016).

2.1.1 Structure

Hepatitis B virus was first discovered in 1967 by Blumberg and was initially called the Australia antigen (Blumberg, Gerstley, Hungerford, London & Sutnick, 1967). Three distinct types of viral particles can be identified in the blood of an individual infected with HBV. The first one is the Dane particle, a mature spherical virion, 42 nm in diameter (Dane, Cameron & Briggs, 1970). The Dane particle is composed of two layers, an outer envelope composed of hepatitis B surface antigen proteins and an inner nucleocapsid made up of hepatitis B core antigens that can exhibit either a T3 or T4 symmetry, depending on the number of core proteins, which can result in core particles of 32 nm or 36 nm (Crowther, Kiselev, Böttcher, Berriman, Borisova, *et al.*, 1994). Within the nucleocapsid are the HBV genome and endogenous deoxyribonucleic acid (DNA) polymerase. Two sub-viral proteins are secreted and non-infectious and composed of hepatitis B surface proteins; one is spherical in shape and 17–25 nm in diameter and the other is filamentous and 20 nm in diameter and varying lengths. Their function is still unknown although it is speculated that they may act as immune decoys.

Hepatitis B virus has several peculiarities. Firstly, unlike most DNA viruses, it uses the enzyme reverse transcriptase for its replication (Seeger, Ganem & Varmus, 1986). Secondly, it has a partially-double stranded relaxed circular genome, with an incomplete plus strand to which the viral DNA polymerase is bound and a complete minus strand (Delius, Gough, Cameron & Murray, 1983). Thirdly, it is the smallest DNA virus to be identified to date, with a genome that varies in length, from 3181 to 3248 bases, according to the genotype, but whose length is also restricted because viral replication occurs within the nucleocapsid (Chirico, Vianelli

& Belshaw, 2010). Fourthly, to compensate for this restriction in genomic length, the minus strand encodes the entire viral genome as four overlapping open reading frames (ORF) (Nassal & Schaller, 1993). The longest ORF codes for the viral polymerase, the precore/core ORF code for the hepatitis B envelope antigen (HBeAg) and hepatitis B core proteins, the X ORF codes for the hepatitis B x antigen (HBx), and the surface ORF codes for the Pre-S1, Pre-S2, and S proteins.

2.1.2 Life cycle and replication

The host cells of HBV are human hepatocytes (Seeger, Mason, Seeger & Mason, 2000) although viral DNA has been found in peripheral blood mononuclear cells (PBMCs) (Bouffard, Lamelin, Zoulim, Pichoud & Trepo, 1990) and the human ovary (Yu, Gu, Xia, Wang, Kan, *et al.*, 2012). In 2012, the entry mechanism for HBV was elucidated and the sodium taurocholate cotransporting polypeptide (NTCP), a multiple transmembrane transporter predominantly expressed in the liver, was identified as the cellular receptor for the HBV pre-S1 receptor-binding region (Yan, Zhong, Xu, He, Jing, *et al.*, 2012).

After entering the hepatocytes, the nucleocapsids are transported to the nucleus where the enclosed relaxed circular genome is released and using host cell enzymes is converted to covalently closed circular (ccc) DNA resulting in a minichromosome (Bock, Schranz, Schröder & Zentgraf, 1994). The minichromosome acts as a template for transcription of all genomic and subgenomic messenger RNAs (mRNAs) using host polymerase (Bock *et al.*, 1994) and is also responsible for viral persistence. The genomic transcripts code for the viral polymerase and core proteins, as well as for the pre-core protein, which is subsequently modified to form HBeAg. The subgenomic transcripts are involved in the production of the X and surface proteins.

One of the genomic length mRNA transcripts, called the pregenomic RNA is packaged with viral polymerase forming core particles, in which reverse transcription occurs to form the partially double-stranded DNA. The viral envelope consisting of surface proteins is assembled independently of the nucleocapsids in the endoplasmic reticulum. Nucleocapsids with completed minus strand are preferentially enveloped with surface proteins and hijack the endosomal sorting complex required for transport (ESCRT) for release into the extracellular space. A proportion of nucleocapsids do not get enveloped and are instead transported to the nucleus using an intracellular conversion pathway thereby maintaining the number of cccDNA molecules in the hepatocytes (Wong & Locarnini, 2018). Viral DNA integration with host genome occurs randomly and although it is not a prerequisite for viral replication, it is an important hepatocarcinogenic pathway (Seeger & Mason, 2015).

2.2 Prevalence of HBV

2.2.1 Globally

A report by Schweitzer *et al*, 2015, where the authors performed mathematical calculations on worldwide HBsAg prevalence on a country-by-country basis, estimated that in 2010, 248 million people were living with

CHB, accounting for 3.61% of the global population (Schweitzer *et al.*, 2015). Although HBsAg prevalence varies across continents and between countries and regions, it is apparent that it is more prevalent in resource-limited areas, with the highest prevalence documented in Africa (8.83%; 95% CI: 8.82%–8.83%), followed by the Western Pacific region (5.26%; 95% CI: 5.26%-5.26%), where the highest prevalence observed in any country was 22.7% in the Kiribati (Schweitzer *et al.*, 2015).

Certain countries such as China have had significant success in controlling HBV by implementing health measures such as the administration of the birth-dose HBV vaccine. As a result, the prevalence of HBV in the Chinese general population has dramatically dropped from 13.99% (13.76%-14.23%) in the period 1957-1989 to 5.41% (5.40%-5.43%) in the period 1990-2013 (Schweitzer *et al.*, 2015). Another recent study modelled that the prevalence of HBsAg in China has decreased by 11% annually from the year 2000 onward (Ott, Horn, Krause & Mikolajczyk, 2017).

High-income countries tend to have a lower prevalence of HBV. Hence, in Europe, the overall prevalence of HBV observed is relatively low (Ott, Stevens, Groeger & Wiersma, 2012) although it varies across countries, from as low as 0.01% in the United Kingdom to 10.3% in Kyrgyzstan (Schweitzer *et al.*, 2015). In the North American mainland, the prevalence observed is less than 1% in Canada, the USA, and Mexico. Haiti, on the other hand, has the highest prevalence of HBsAg in the Americas region at 13.6%. Overall, the World Health Organization (WHO) Eastern Mediterranean Region has a low-intermediate prevalence of HBsAg, which varies from a low of 0.7% in the United Arab Emirates to a high of 14.8% in Somalia.

However, these overall prevalences do not provide an accurate picture of the HBV burden, because even within countries, certain groups, for example, refugees and immigrants, can be at higher risk of infection and transmission than the general population. For example, in Italy, while the countrywide HBsAg prevalence is 2.52% (95% CI: 2.49%–2.54%), a study found the HBsAg prevalence in undocumented refugees and immigrants to be 9.6% (Coppola, Alessio, Gualdieri, Pisaturo, Sagnelli, *et al.*, 2017). A good understanding of the epidemiology of HBV infection is required so that targeted public health policies can be implemented to prevent further transmission in high-risk groups.

2.2.2 Africa

It is estimated that more than 75 million Africans are currently living with CHB, making up approximately 8.83% of the African continent's population (Schweitzer *et al.*, 2015). It must be acknowledged that this number is likely an underestimation of the actual prevalence of CHB because it is based on either incomplete or unavailable data for some African countries. For example, half of the SubSaharan African countries included in the modelling calculations of Schweitzer *et al.* 2015 had less than five relevant studies that could be utilised. In contrast, Nigeria had 85 studies available, making it more likely that the overall calculated prevalence would

be more accurate. Furthermore, there are intra-country variations in HBsAg prevalence that could have influenced the reported prevalence.

Unlike the annual decrease in HBsAg prevalence observed in Asian countries endemic for HBV, several African countries, including Senegal, South Africa, Nigeria, and Uganda, had an annual increase in HBsAg prevalence of 1%, 2%, 2%, and 5%, respectively (Ott *et al.*, 2017). These data reflect the fact that the HBV epidemic in Africa is largely underestimated as a public health issue and is largely unknown to the public due to the fact that CHBs are usually asymptomatic and usually only manifest when they have progressed to end-stage liver disease. HBV is considered to be a disease on the decline because of the availability of a safe and effective vaccine that should prevent most horizontal transmissions of the virus. Horizontal transmission during childhood has long been assumed to be the principal transmission route in Africa, based on studies pre-dating the HIV epidemic. In Africa, the HIV and HBV epidemics form a potent combination and make mother-to-child-transmission (MTCT) a bigger problem than previously described. A meta-analysis of HBV MTCT studies in SSA showed that 367 250 newborns are infected at birth every year, which is twice the number of infants infected by HIV annually (Keane, Funk & Shimakawa, 2016). Unfortunately, research funding in much of SSA is geared toward research on malaria, tuberculosis, and HIV making HBV a neglected tropical disease (O'Hara *et al.*, 2017).

2.2.3 South Africa

South Africa is considered to be endemic for HBV and while the predominant mode of HBV transmission in South Africa was initially thought to be horizontal transmission (Botha, Dusheiko, Ritchie, Mouton & Kew, 1984; Prozesky, Szmuness, Stevens, Kew, Harley, *et al.*, 1983), vertical transmission from mother to child may have been underestimated as shown in a study by Vardas *et al.* 1999, before the HBV vaccine was introduced to the South African Expanded Programme on Immunization, where 8.1% of unvaccinated infants between the ages of 0 and 6 months were positive for HBsAg.

Certainly, recent studies from the different South African provinces have reported HBV MTCT and HBsAg prevalences of 0.4%, 7%, and 13% in HIV-exposed infants from the Western Cape (Chotun, Nel, Cotton, Preiser & Andersson, 2015), Gauteng (Hoffmann, Mashabela, Cohn, Hoffmann, Lala, *et al.*, 2014), and KwaZulu Natal (Mdlalose, Parboosing & Moodley, 2016), respectively. HIV-unexposed infants from KwaZulu Natal had a lower prevalence of HBsAg of 7.5% than HIV-exposed infants (Mdlalose *et al.*, 2016). These differences in HBsAg distribution also clearly demonstrate the variability in distribution of the disease which has previously been reported between South African provinces (Dusheiko, Conradie, Brink, Marimuthu & Sher, 1989; Ive, MacLeod, Mkumla, Orrell, Jentsch, *et al.*, 2013; Kew, 1996).

A pitfall of the numerous epidemiological studies conducted on HBV in South Africa is that they targeted pregnant women, blood donors, healthcare workers, and HIV-infected cohorts. These groups, although important to study, are not representative of the general South African population. Blood donors tend to be

healthy members of the population and the questionnaires administered to potential donors already weed out those considered at high risk of carrying blood-transmissible diseases. Moreover, like in developed countries, in South Africa, blood donations are voluntary and unpaid. In South Africa, pregnant women are used as proxies to determine the prevalence of the HIV-1 epidemic and antenatal surveys are conducted yearly to monitor the evolution of the epidemic. However, in the context of HBV infections, pregnant women are not a reliable proxy for the general prevalence of HBV and testing them will likely underestimate the extent of the silent epidemic. Healthcare workers are often used in studies as proxies for the general population but are often at higher risk of acquiring infections (nosocomially) than the general population (Fritzsche, Becker, Hemmer, Riebold, Klammt, *et al.*, 2013). Finally, unlike immunocompetent individuals, HIV-infected individuals are immunocompromised and more likely to develop CHB if exposed to HBV in adulthood and are also more likely to have HBV infections despite being HBsAg-negative which would necessitate more expensive molecular techniques for their detection (Lukhwareni, Burnett, Selabe, Mzileni & Mphahlele, 2009). As HIV and HBV share the same modes of transmission, HBV co-infection is usually more prevalent in HIV-1 infected individuals as has been shown previously (Andersson, Maponga, Ijaz, Barnes, Theron, *et al.*, 2013).

2.2.4 Distribution of genotypes and their clinical significance

There are ten genotypes of HBV, named A to J, that have been described worldwide and that are at least 8% genetically different (Arauz-Ruiz, Norder, Robertson & Magnius, 2002; Huy, Ngoc & Abe, 2008; Norder, Couroucé & Magnius, 1994; Olinger, Jutavijittum, Hübschen, Yousukh, Samountry, *et al.*, 2008; Stuyver, De Gendt, Van Geyt, Zoulim, Fried, *et al.*, 2000; Tatematsu, Tanaka, Kurbanov, Sugauchi, Mano, *et al.*, 2009). However, genotype I is contested by experts in the field as being a potential recombinant (Kurbanov, Tanaka, Kramvis, Simmonds & Mizokami, 2008) and genotype J has been identified in only one patient thus far (Tatematsu *et al.*, 2009). These genotypes can be further categorised into sub-genotypes differing genetically by between 4% and 8%. HBV genotypes and sub-genotypes tend to group in distinct geographical areas (Norder, Couroucé, Coursaget, Echevarria, Lee, *et al.*, 2004).

In SSA, genotypes are A, D, and E are the most common. Subgenotype A1 is the most commonly found in SSA, especially Eastern and Southern Africa, including South Africa (Kramvis & Kew, 2007b), while genotype D is the most prevalent in North Africa and the Mediterranean basin (Kramvis, Kew & François, 2005). Genotype D has also been described in South Africa (Chotun, Preiser, van Rensburg, Fernandez, Theron, *et al.*, 2017; Chotun, Strobele, Maponga, Andersson & Etienne De La Ray, in press; Maponga, 2016) and is considered to be the second most prevalent genotype in South Africa (Kimbi, Kramvis & Kew, 2004). Genotype E is found in Central and West Africa (Kramvis & Kew, 2007a) although some isolates have been identified in South Africa, originating from West African immigrants (Maponga, 2016).

These different genotypes can influence the management of affected individuals as they have been shown to be different in terms of disease progression (Lin & Kao, 2017) and susceptibility to antiviral therapy (Lin & Kao, 2013). Unfortunately, there is limited data on the influence of HBV genotypes on clinical outcomes in

SSA as most research on the topic has been conducted in Asia where the most prevalent genotypes are B and C. Research on genotype A1 in South Africa has shown that it was associated with a higher risk of HCC compared to genotype D (Kew, Kramvis, Yu, Arakawa & Hodkinson, 2005). In The Gambia, a significant association was also found between genotype A and increased risk of liver fibrosis although the study sample size was very small (Shimakawa, Lemoine, Njai, Bottomley, Ndow, *et al.*, 2016).

2.3 Natural history of HBV infection

2.3.1 Acute hepatitis

About 70% of individuals infected with HBV will have a self-limiting sub-clinical manifestation of the disease while the remaining 30% will develop clinical symptoms such as jaundice, termed icteric hepatitis (Oliphant, 1944). A severe form of acute hepatitis, termed fulminant hepatitis, is rare and will occur in about 1% to 2% of patients with acute HBV infection (Chu & Liaw, 1990). In patients with acute hepatitis, the liver enzymes alanine aminotransferase (ALT) and aspartate transaminase (AST) levels will initially be high and normalise within six months.

Persistently elevated ALT levels may be an indication that the acute infection is becoming chronic in nature. The risk of progression from acute to chronic infection is linked to age at exposure to HBV; perinatal exposure has a 90% risk of developing into a chronic infection (Beasley, Hwang, Lin, Leu, Stevens, *et al.*, 1982; Beasley, Trepo, Stevens & Szmuness, 1977), exposure between the ages of one and five years has a 20% – 50% risk (McMahon, Alward, Hall, Heyward, Bender, *et al.*, 1985), and exposure as an adult carries a less than 5% risk (Tassopoulos, Papaevangelou, Sjogren, Roumeliotou-karayannis, Gerin, *et al.*, 1987). There is no recommended treatment for acute hepatitis B and a Cochrane review showed no clear benefits of nucleos(t)ide treatment during acute infection (Mantzoukis, Rodriguez-Peralvarez, Buzzetti, Thorburn, Davidson, *et al.*, 2017). Mantzoukis *et al.* 2017 also reported that treatment did not appear to be efficacious in cases of fulminant hepatitis B although studies on infantile fulminant hepatitis B have shown that administering treatment does not worsen disease prognosis (Chotun *et al.*, in press; Laubscher, Gehri, Roulet, Wirth & Gerner, 2005).

2.3.2 Chronic hepatitis

Chronic hepatitis B virus infection is defined as persistent positivity for HBsAg for more than six months (Terrault, Lok, McMahon, Chang, Hwang, *et al.*, 2018). Many patients with CHB are asymptomatic or may present with nonspecific symptoms, such as fatigue (Song, 2005). Their liver enzyme levels (ALT and AST) may be normal to slightly elevated although patients may experience flares sporadically.

2.3.2.1 Phases of chronic hepatitis B virus infection

The natural course of CHB is host- and virus-dependent and its progression is affected by factors such as age at acquisition, sex, alcohol consumption, and comorbidities (such as HIV co-infection). HBV is never truly cleared from the host's body even when HBsAg seroconversion is achieved and viral loads are undetectable

in the sera (Kuhns, McNamara, Mason, Campbell & Perrillo, 1992) and HCC can still develop years after HBsAg clearance (Simonetti, Bulkow, McMahon, Homan, Snowball, *et al.*, 2010). This is because HBV can still persist as cccDNA in hepatocyte reservoirs leading to low transcriptional levels and replication despite HBsAg seroconversion (Yuen, Wong, Fung, Ip, But, *et al.*, 2008).

2.3.2.1.1 Immune tolerance

Patients who have acquired HBV perinatally initially present with an immune tolerance phase that is generally asymptomatic. Clinically, this phase is characterised by high levels of HBV DNA and HBeAg in the blood, but no evidence of liver damage or immune response to the viral infection. Hence, patients may present with normal liver enzyme levels and minimal fibrosis (Fattovich, Bortolotti & Donato, 2008). The immune tolerance phase can last for 10–30 years with a low annual rate of clearance of HBeAg in adolescence and early adulthood (Lok, Lai, Wu, Leung & Lam, 1987). This phenomenon can lead to an increased risk of mother-to-child transmission, thus maintaining the cycle of viral transmission.

2.3.2.1.2 Immune-active, HBeAg positive

During this phase, patients undergo spontaneous HBeAg clearance, often accompanied by flares in levels of ALT and inflammation (Liaw, Pao, Chu, Sheen & Huang, 1983). This clearance seems to be preceded by an increase in viral activity (Liaw, Pao, Chu, Sheen & Huang, 1987), although how and why those changes occur are unknown. Clinically, patients in this phase rarely present with symptoms and will be diagnosed during routine follow-ups. However, patients with previously unknown HBV status may be mistakenly diagnosed with acute HBV when in this phase as it may be accompanied by antibody to the HBV core antigen (anti-HBc) IgM positivity (Chu, Liaw, Pao & Huang, 1989), which is often used as a marker of acute HBV infection. This phase may lead to HBeAg seroconversion and HBV DNA clearance from the blood, with a reported annual clearance rate of 10% to 20% (Alward, McMahon, Hall, Heyward, Francis, *et al.*, 1985; Lok *et al.*, 1987). In certain patients, however, viral clearance from the blood is not achieved and the flares may become recurrent (Liaw *et al.*, 1987), increasing their risk of cirrhosis and HCC as they age (Chen, Chu & Liaw, 2010).

2.3.2.1.3 Inactive chronic HBV

Subsequent to HBeAg clearance from the blood and seroconversion, patients enter the inactive CHB phase, where they are HBeAg negative and positive for antibodies to the envelope antigen (anti-HBe). These patients tend to have a low level or undetectable levels of HBV DNA in the blood and normal ALT levels, indicating that the liver disease is in remission. However, reports suggest that three consecutive normal ALT levels and HB viral loads below 2000 IU/ml over a one-year period are required to confirm that patients are truly in this phase of CHB (Lampertico, Agarwal, Berg, Buti, Janssen, *et al.*, 2017; Sarin, Kumar, Lau, Abbas, Chan, *et al.*, 2016; Terrault *et al.*, 2018). Histologically, patients may still present with liver inflammation and fibrosis although a meta-analysis has suggested that this was a rare occurrence in patients with repeatedly normal levels of ALT and low HB viral loads (Papatheodoridis, Manolakopoulos, Liaw & Lok, 2012).

2.3.2.1.4 Immune-active, HBeAg negative

Some patients, while having achieved HBeAg seroconversion, may be infected with HBV variants that cannot produce HBeAg due to viral precore (PC) or basal core promoter (BCP) mutations (Carman, Jacyna, Hadziyannis, Karayiannis, McGarvey, *et al.*, 1989). These patients still have active liver disease and will present clinically with increased ALT levels and moderate HB viral loads (Lok, Hadziyannis, Weller, Karvountzis, Monjardino, *et al.*, 1984). These patients are said to have HBeAg-negative chronic hepatitis and tend to be older, with more advanced liver disease, repeated peaks in ALT levels (Kumar, Chauhan, Gupta, Hissar, Sakhuja, *et al.*, 2009) and viral loads and are at risk of developing HCC (Hsu, Chien, Yeh, Sheen, Chiou, *et al.*, 2002).

2.3.2.1.5 Occult hepatitis B infection

Some individuals may test negative for HBsAg but still have other detectable markers of HBV infection such as anti-HBc, a low serum viral load (< 200 IU/ml), and detectable intrahepatic DNA which are indicative of an "occult" (hidden) HBV infection (Raimondo, Allain, Brunetto, Buendia, Chen, *et al.*, 2008). These individuals account for a small proportion of all CHBs (The Gambia – 4%; Shimakawa *et al.*, 2016) and are still at risk of HCC (Kew, Welschinger & Viana, 2008; Wong, Huang, Lai, Poon, Seto, *et al.*, 2011). Occult HBV infection seems to occur in higher proportions in HIV-infected individuals (Mayaphi, Rossouw, Masemola, Olorunju, Mphahlele, *et al.*, 2012) but this could be caused by atypical HBV serological presentations observed in HBV/HIV co-infected individuals due to their inherent immunosuppression, as demonstrated in previous South African studies where the authors reported HB viral loads above 200 IU/ml and up to 10^8 IU/ml in HBsAg negative and anti-HBc positive HBV/HIV co-infected patients (Lukhwareni *et al.*, 2009; Mphahlele, Lukhwareni, Burnett, Moropeng & Ngobeni, 2006).

2.3.2.1.6 Resolution of chronic hepatitis B virus infection

Some patients may spontaneously undergo HBsAg-to-antibody to the hepatitis B surface antigen (HBsAg) [HBsAg-to-anti-HBs] seroconversion, although this is a process that is as yet unclear and happens in 0.5%–2% of affected patients (Alward *et al.*, 1985; Liaw, Sheen, Chen, Chu & Pao, 1991; Liu, Yang, Lee, Lu, Jen, *et al.*, 2010; Simonetti *et al.*, 2010). This seroconversion may be preceded by a decrease in HBV DNA and HBsAg levels (Liu *et al.*, 2010). Lower DNA levels at baseline are associated with higher seroconversion rates (Liu *et al.*, 2010). Although these patients tend to have a better prognosis than those who are positive for HBsAg, they are still at risk of HCC, especially if the seroconversion occurred after the age of 50 (Simonetti *et al.*, 2010; Yuen *et al.*, 2008). Many patients may still test positive for HBV DNA years after HBsAg seroconversion as they may still have low levels of replicating HBV in their hepatocytes (Yuen *et al.*, 2008), or be infected with pre-S1 HBV variants that have suppressed HBsAg production (Cabrerizo, Bartolomé, Caramelo, Barril & Carreño, 2000), or they may have a reactivated infection due to immunosuppression (Shouval & Shibolet, 2013).

2.3.2.1.7 Reactivation of HBV

Patients with a resolved HBV infection (anti-HBc and anti-HBs positive) are at risk of HBV reactivation if they undergo immunosuppressive therapy (such as chemotherapy) (Shouval & Shibolet, 2013) due to the persistence of cccDNA in hepatocytes and other tissues despite seroconversion (Yuen *et al.*, 2008). This reactivation may either be asymptomatic and accompanied by a reversal of HBsAg seroconversion or may have severe consequences such as fulminant hepatitis leading to death (Gupta, Govindarajan, Fong & Redeker, 1990). Moreover, a transplanted liver from an anti-HBc and anti-HBs positive donor can cause *de novo* HBV infection in a recipient (even in an anti-HBs positive recipient) (Bortoluzzi, Gambato, Albertoni, Mescoli, Pacenti, *et al.*, 2013; Cholongitas, Papatheodoridis & Burroughs, 2010). Meta-analyses have shown that the administration of HBV antiviral therapy such as lamivudine and entecavir can reduce the risk of HBV reactivation (Huang, Hsiao, Hong, Chiou, Yu, *et al.*, 2013; Paul, Saxena, Terrin, Viveiros, Balk, *et al.*, 2016).

2.3.2.2 Long-term consequences of CHB

Patients with CHB may have different outcomes, from remaining clinically healthy inactive HBV carriers to developing cirrhosis and/or HCC. Chronic HBV infection is the cause of more than 75% of all HCC cases in SubSaharan Africa (SSA) and Asia (Baecker *et al.*, 2018; Di Bisceglie, 2009; Ferenci, Fried, Labrecque, Bruix, Sherman, *et al.*, 2010; Maucort-Boulch *et al.*, 2018).

In untreated CHB patients, the five-year progression rate from CHB to cirrhosis is estimated to be 8%–20%, from compensated cirrhosis to hepatic decompensation 20%–23%, and from compensated cirrhosis to HCC 6%–15% (Fattovich, 2003; Hadziyannis & Papatheodoridis, 2006; McMahon, 2009). However, these are estimates from studies conducted in Caucasian and Asian population groups, making them unreliable for African patients.

2.3.3 Management of chronic HBV infection

There are several guidelines published by major organisations worldwide available for the management of patients with CHB (Lampertico *et al.*, 2017; Sarin *et al.*, 2016; Terrault *et al.*, 2018; World Health Organization, 2015). South Africa has also published guidelines for the management of CHB patients but these have not been updated since their first publication (Spearman, Sonderup, Botha, Van der Merwe, Song, *et al.*, 2013).

At time of diagnosis, standard baseline evaluation including a thorough medical history and physical examination to evaluate the possibility of comorbidities, laboratory testing for platelet count, AST, ALT, total and conjugated bilirubin, albumin, viral markers of HBV infection (HBeAg, anti-HBe, and HBV DNA), and HIV coinfection, and if possible screening for fibrosis and cirrhosis using non-invasive tests and for space-occupying lesions using ultrasound is recommended for CHB patients. Liver biopsies are only indicated for patients older than 40 years with persistently elevated viral loads.

In HBV monoinfected patients, lifelong treatment with nucleos(t)ide analogues with a high barrier to drug resistance, such as tenofovir and entecavir, is recommended in those with clinical evidence of cirrhosis irrespective of viral load and ALT levels and in individuals without cirrhosis who have persistently elevated ALT levels and viral loads of > 20 000 IU/ml (World Health Organization, 2015). However, in SSA, assessing the eligibility of HBV-infected patients for treatment using these criteria, especially HB viral loads and cirrhosis status, remain expensive and limited due to lack of trained personnel (Lemoine, Eholié & Lacombe, 2015).

Non-invasive fibrosis tests have not been well-studied in SSA where there are different comorbidities compared to developed countries. The aspartate transaminase-to-platelet ratio index (APRI) score is recommended by the WHO (World Health Organization, 2015) but its validation for use in SSA remains limited (Spearman, Afihene, Ally, Apica, Awuku, *et al.*, 2017). A study from The Gambia has shown that gamma-glutamyl transpeptidase (GGT)-to-platelet ratio (GPR) was a more accurate test for significant fibrosis compared to the APRI and FIB-4 tests in HBV mono-infected patients (Lemoine, Shimakawa, Nayagam, Khalil, Suso, *et al.*, 2016). However, their cohort did not include individuals showing excessive alcohol consumption and may therefore not be appropriate for use in a South African setting where GGT levels are frequently elevated in patients, including non-drinkers (Pisa, Vorster, Kruger, Margetts & Loots, 2015). More recently, an algorithm to determine who qualifies for HBV antiviral therapy based on ALT levels and HBeAg was published (Shimakawa, Njie, Ndow, Vray, Mbaye, *et al.*, 2018), and could potentially be of better clinical value than the other tests once validated in this setting.

In those HBV monoinfected patients who do not qualify for treatment, continued follow-ups are required to monitor disease progression. HBV/HIV co-infected patients should be initiated on antiretroviral therapy (ART) containing tenofovir irrespective of CD4 count (Terrault *et al.*, 2018). In South Africa, all newly diagnosed HIV-infected individuals are immediately eligible for ART irrespective of CD4 count. First-line ART for patients with normal renal function in South Africa includes tenofovir thus inadvertently treating HBV infection in newly diagnosed HBV/HIV co-infected patients (Meintjes, Moorhouse, Carmona, Davies, Dlamini, *et al.*, 2017).

In both HBV monoinfected and HBV/HIV co-infected patients receiving treatment, baseline and annual renal function tests should be performed to monitor them for drug toxicity. Annual testing should also be conducted on both HBV monoinfected and HBV/HIV co-infected individuals for ALT, AST, HBsAg, HBeAg, and HBV DNA levels (Spearman *et al.*, 2013; Terrault *et al.*, 2018; World Health Organization, 2015). Annual non-invasive fibrosis tests should also be conducted to determine the presence of cirrhosis (World Health Organization, 2015). More frequent follow-ups may be advisable in patients with more advanced disease (World Health Organization, 2015).

2.4 HBV Diagnosis

2.4.1 Gold standard

Currently, the gold standard for the diagnosis of active HBV infection remains serological testing and confirmation by enzyme immunoassays (EIAs). Testing for HBsAg in the patient's blood is a reliable marker of active HBV infection as it is produced in large quantities during most HBV infections and secreted in the bloodstream from infected hepatocytes. Once the presence of active HBV infection is determined, other markers can be tested to give a better clinical picture of the stage of infection and to better monitor disease progression.

Acute HBV infection is diagnosed by positive HBsAg and anti-HBc IgM tests. It will resolve within six months and a patient receiving a repeat test after that timeframe will be negative for HBsAg, positive for anti-HBc IgG, which is a marker of past exposure to HBV, and positive for anti-HBs, which is a marker of immunity. The presence of anti-HBs alone (in the absence of anti-HBc) usually indicates artificial immunity, either actively acquired through vaccination or passively acquired through the administration of protective anti-HBV antibodies, commonly called HBV immune globulin (HBIG).

A person positive for HBsAg for more than six months is said to have a chronic HBV infection. In this person, serum HBeAg provides an indication that the virus is actively replicating and that the infected person is highly infectious. Antibodies to the HBV core antigen indicate whether the patient has an acute infection (positive for IgM antibodies) or past exposure which could have resolved or become chronic (positive for IgG antibodies), although some CHB patients may present with flares of anti-HBc IgM antibodies. Antibodies to the envelope antigen usually develop in the later phase of CHB and are associated with lower levels of viral replication, although some anti-HBe positive patients may be infected with HBV mutant strains that are highly replicative (Jammeh, Tavner, Watson, Thomas & Karayiannis, 2008; Tong, Li, Vitvitski & Trépo, 1990). In addition to serological testing, molecular testing can also be used to monitor patients by testing for the serum HB viral load using quantitative real-time PCR.

While these tests are reliable and robust, they are not widely available in SSA, partly because of the level of technical expertise that is required to conduct such tests and also because of the prohibitive cost of performing such tests. In many SubSaharan African countries, centres with such facilities are limited to cities and the tests may not be routinely performed or may not be accessible to all (Andriamandimby, Olive, Shimakawa, Rakotomanana, Razanajatovo, *et al.*, 2017). Therefore, to increase diagnosis of CHB cases in this endemic setting, the utilisation of point-of-care tests that can be used as screening tools is highly desirable.

2.4.2 Hepatitis B surface antigen POC testing

Point-of-care testing refers to the use of a test that will provide a result quickly so that a diagnosis can be provided to the patient being tested during the same session or on the same day. The field of POCT has rapidly progressed in the past forty years as research has focused on translational medicine, from the laboratory to the bedside and beyond, especially in resource-limited countries.

The ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable) criteria were developed by the WHO to evaluate POCTs (Peeling, Holmes, Mabey & Ronald, 2006). According to the ASSURED criteria, the ideal point-of-care test needs to have a short turnaround time (typically less than 30 minutes) so that the patient can be linked to care immediately if required and therefore follow-up losses minimised (rapid). It also needs to be stable at room temperature and not need refrigerated storage for at least one year (robust). This is especially relevant to SSA, where ambient temperatures in certain countries can be around 30°C all year round. The test itself needs to be sensitive so that the number of false negatives and, therefore, the number of undiagnosed infected patients, is minimised and specific so as to minimise the number of false positives and so that the results can be confidently communicated to the patient. The tests should also require minimal training so that they may easily be performed by non-technical staff and therefore be accessible to a larger number of end users (user-friendly). Moreover, the POCT should be simple enough to be used in resource-limited settings and therefore be portable/hand-held (deliverable) and not require any complex equipment at any stage of its use, especially to read the result. This is an important requirement especially in SSA where some countries have a poor health infrastructure and access to electricity is unreliable (equipment-free). Lastly, even if a test meets all the above requirements, it still needs to be affordable so that it can be widely used in resource-limited settings where the need for such tests is the greatest.

These requirements often seem onerous, if not impossible, to achieve for a POCT. However, while some of the test requirements are non-negotiable, others may be more flexible. A survey of health care providers on the use of POCTs for sexually transmitted infections revealed that the majority of healthcare providers reported their ideal POCT needed to have at least a sensitivity of above 90%, low cost, and short turnaround time (Hsieh, Gaydos, Hogan, Uy, Jackman, *et al.*, 2011).

In the context of HBV POC testing, although several commercial tests are currently available for some of the HBV markers listed in section 2.4.1, few have been validated for use in SSA. For example, a test for HBeAg detection has been shown to have poor sensitivity when tested on West Africans, limiting its use in SSA (Seck, Ndiaye, Maylin, Ndiaye, Simon, *et al.*, in press). A loop-mediated isothermal amplification assay (LAMP) assay has also been described for the semi-quantification of HBV DNA across genotypes A–F and had sensitivity and specificity of 75.7% and 92.0%, respectively, for viral loads of 2,000 IU/ml and sensitivity and specificity of 98.0% and 92.6%, respectively, for viral loads of 200,000 IU/ml but still needs to be validated in the field (Vanhomwegen, Kwasiborski, Sauvage, Boizeau, Hoinard, *et al.*, 2018).

For HBV screening purposes, however, HBsAg testing remains sufficient. There are several commercial tests available, although the Determine™ HBsAg test remains the best-studied one and appears to fulfil most of the ASSURED criteria. It provides a result after 20 minutes of incubation, has a shelf-life of 18 months and can be stored at 2–30°C (rapid and robust). It is inexpensive (< $2), does not require sophisticated equipment to be operated, is portable, and user-friendly (World Health Organization, 2001). Moreover, a meta-analysis of different POCTs for HBsAg reported that the Determine™ HBsAg test had a very high sensitivity (97.6 %

(95% Crl: 96.3–98.6 %) and specificity 99.7 % (95% CrI: 99.2–99.9 %) (Shivkumar, Peeling, Jafari, Joseph & Pai, 2012). In contrast, in the same study, the authors found that the other HBsAg POCTs assessed (Dainascreen, Serodia, Hybritech, DRW, Virucheck, Hexagon, Cypress, Hepacard, Genedia, Daewoong, SD, Asan, One Check, Accurate, Acon, Atlas, Intec, Blue Cross, DIMA, and Cortez) had a lower pooled sensitivity (94.8%, 95% CrI: 90.1–98.2) and specificity (99.5%, 95% CrI: 99.1–99.9), making them less attractive options for HBV screening. The Determine™ HBsAg test retains its high sensitivity with samples containing HBV variants with mutations that usually make their detection difficult (Hirzel, Pfister, Gorgievski-Hrisoho, Wandeler & Zuercher, 2015). However, its sensitivity has been found to be lower in field trials in The Gambia (88.5%, 95% CI; 80.7–93.9%) (Njai, Shimakawa, Sanneh, Ferguson, Ndow, *et al.*, 2015) and Zambia (87.9%, 95% CI; 77.5–94.6) (Chisenga, Musukuma, Chilengi, Zürcher, Munamununugu, *et al.*, 2018), although the false negatives were all patients with low HBsAg titres and low HB viral loads, respectively. These findings can be further explained by the limit of detection of the Determine™ HBsAg test, which is between 1 and 2 IU/ml (Servant-Delmas, Duong, Hamon, Houdah & Laperche, 2015) compared to the limit of detection of the EIA gold standard which is 0.05 IU/ml.

It should be mentioned that the WHO procurement eligibility for HBsAg POCTs requires that such tests have a sensitivity of ≥ 99% and specificity of ≥ 98%. With regard to analytical sensitivity, tests submitted to WHO prequalification (PQ) for use as screening tests in asymptomatic and symptomatic individuals require an analytical sensitivity of at least 4 IU/ml using the WHO 1st International Reference Preparation for HBsAg as a measure of HBsAg concentration. Those tests that pass this first PQ round are then tested on the WHO HBsAg specimen reference panel, HBsAg positive lot-to-lot variation panel, HBsAg seroconversion panel, HBsAg low titre performance panel, HBsAg mutants panel (World Health Organization, 2010). If an HBsAg test meets those laboratory requirements, the WHO recommends its use for screening of symptomatic and non-symptomatic individuals if it satisfies other bureaucratic requirements. In South Africa, the Determine™ HBsAg test has been validated for use in a laboratory setting (Kariem, Chotun, Preiser & Andersson, 2015) at the Division of Medical Virology, Stellenbosch University, which is currently accredited as a WHO PQ evaluation laboratory (World Health Organization, 2018) but its performance in the field has not been fully investigated.

In addition to conforming to the ASSURED criteria, it is also imperative to investigate the barriers to testing before a POCT is introduced so that they may be addressed prior to or at the time of implementation. A survey conducted on healthcare providers suggests that the biggest barriers to implementation of a POCT are time constraints, interruption of workflow, and reluctance of some patients to getting a finger prick (Hsieh, Hogan, Barnes, Jett-Goheen, Huppert, *et al.*, 2010). However, the majority of respondents in that survey belonged to high-income countries where the perceived barriers may be different from the ones seen in low-and-middle income countries such as South Africa (Pai, Vadnais, Denkinger, Engel & Pai, 2012). It is therefore important for such surveys to be conducted in this setting to evaluate the feasibility of implementing HBV POC testing.

2.4.2.1 Implementation

Before a screening test is implemented, it also needs to conform to certain criteria, known as the Wilson-Jungner principles, devised in 1968 (Wilson, Jungner & World Health Organization, 1968). These principles are:

2.4.2.1.1 The health problem needs to be an important issue

The importance of a health problem can be measured in terms of its prevalence in the community where the screening test is to be implemented as well as its long-term consequences for the affected individual. However, even if the disease in question has a low prevalence but has severe consequences for the affected individual, it is still regarded as a relevant and important public health problem.

Hepatitis B virus in South Africa fits both these descriptions. HBV is endemic to South Africa although its prevalence differs between the different provinces (Dusheiko *et al.*, 1989; Ive *et al.*, 2013; Kew, 1996). In addition, it can have severe consequences if not detected early as described in section 2.3.2.2 (Long-term consequences of CHB).

2.4.2.1.2 Screening needs to improve the prognosis of those diagnosed

This second criterion looks at whether treatment of those patients in whom the disease is detected at an earlier stage will result in a better prognosis (alters the outcome of the disease) compared to waiting for the patients to come forward of their own accord once they experience signs and symptoms of the disease. If early detection and management do not lead to an improved prognosis, then the test is of little use.

In the case of HBV, it must be noted that the screening test could detect the presence of active infection at the pre-symptomatic stage before it progresses to cirrhosis or HCC. What should also be considered is that HBV is a largely asymptomatic disease and that symptoms only manifest when disease progression is under way. Even then, the symptoms tend to be so non-specific that HCC is rarely diagnosed in the early neoplastic stages, especially in resource-limited settings such as South Africa. In fact, the incidence of HCC mirrors mortality (Ervik *et al.*, 2016) as most cases are diagnosed too late for any curative therapy (Kew, 2012). Even if HCC is diagnosed in the early stages and treated, more than half of patients undergoing liver resection will suffer from a recurrence within two years of surgery (Tabrizian, Jibara, Shrager, Schwartz & Roayaie, 2015) and five-year survival rates can be as low as 32% (Fong, Sun, Jarnagin & Blumgart, 1999). Therefore, testing for HBV becomes particularly important in the light of those observations and the POCT has a high prognostic value if used on a population where HBV is known to be endemic.

2.4.2.1.3 There should be appropriate facilities for diagnosis and treatment of the disease

This criterion is critical to the success of a screening programme. Once a patient is found to be positive on a screening test, there must be appropriate healthcare facilities where patients can be followed-up and receive

treatment once their diagnosis is confirmed. In the context of HBV in South Africa, EIA testing is available to confirm the HBV POCT results serologically and HB viral load testing is also available to help the clinical evaluation of the patient and to monitor disease progression (Chotun *et al.*, 2017). The treatment for CHB is lifelong and consists of 300 mg tenofovir tablet daily. This treatment is also available to South Africans with CHB at the same healthcare facilities where they are followed-up (Chotun *et al.*, 2017).

The management of CHB patients in South Africa is undertaken by gastroenterologists at tertiary healthcare facilities countrywide to monitor CHB patients using implemented treatment protocols (Chotun *et al.*, 2017). However, access to such specialists is limited in much of SSA and restricted to specific centralised health centres, raising the concern that there may be insufficient personnel to properly manage all newly diagnosed CHB patients if HBV POC testing is implemented (Andriamandimby *et al.*, 2017). A potential solution to this problem would be the development of guidelines for management of CHB patients at primary health care (PHC) level by registered nurses or general practitioners, as has been successfully demonstrated for HIV in South Africa (World Health Organization & Médecins Sans Frontières South Africa, 2003) and SSA (Kredo, Adeniyi, Bateganya & Pienaar, 2014; Kredo, Ford, Adeniyi & Garner, 2013). In Australia, CHB management guidelines have been developed and successfully implemented as part of a larger programme for HBV education and CHB screening and monitoring at PHC level. However, this programme is guided by a stratification algorithm that allows primary PHC providers to determine the HCC risk of their CHB patients (Robotin, Kansil, Porwal, Penman & George, 2014). Before such guidelines can be developed for SSA, a better understanding of the risk factors of HCC in this setting is therefore required.

2.4.2.1.4 The disease should be detectable at an asymptomatic stage

There must be a latent period during which the disease can be identified and successfully managed or treated. In the case of chronic diseases, there will be a phase during the early stages of the disease where it is asymptomatic but can still be detected. This is the golden opportunity phase where the disease can be detected early enough so that it can be managed and treated before the appearance of symptoms and further disease progression.

HBV has an asymptomatic stage during which the virus produces large quantities of HBsAg that can be detected using a POCT. In fact, this latent period can last for many decades without significant liver damage or disease progression (Chang, Hwang, Hsu, Lee & Beasley, 1988; Hui, Leung, Yuen, Zhang, Leung, *et al.*, 2007; Lok & Lai, 1988). A test screening for active HBV infection can therefore help in the detection of the disease decades before it progresses to cirrhosis and HCC.

2.4.2.1.5 There should be a suitable test available to detect the disease

There is indeed, as has been described in detail under section 2.4.2 (HBsAg POC testing).

2.4.2.1.6 The test should be acceptable to the population

The test available for screening should be acceptable to the population both in terms of what it is testing for and the way in which it is performed. A Gambian study has shown that the test was accepted and uptaken by 68.9% (95% CI, 65.0%–72.4%) of approached community members (Lemoine, Shimakawa, Njie, Taal, Ndow, *et al.*, 2016). Although no such studies have been carried out in South Africa, a study of HIV-1 POC testing offered to community members attending the emergency department showed that 72.8% of approached individuals accepted to be tested (Hansoti, Stead, Parrish, Reynolds, Redd, *et al.*, 2018), suggesting that if a similar test, such as an HBV POCT, was offered, it would be readily accepted. However, since HBV is generally unknown in an African setting (Malungu Ngaira, Kimotho, Mirigi, Osman, Ng'ang'a, *et al.*, 2016), informational and educational campaigns would be required prior to the introduction of any HBV POCT as well as appropriate training programmes for the nursing staff and other healthcare providers who will provide the test so that they may provide adequate counselling to their patients.

2.4.2.1.7 There should be an accepted and effective treatment for those diagnosed

There is currently no cure for CHB but there is effective lifelong therapy available for treatment of patients with CHB. Nucleos(t)ide inhibitors such as lamivudine, tenofovir, and entecavir are accepted treatments for HBV (Lok, McMahon, Brown, Wong, Ahmed, *et al.*, 2016). Antiviral treatment has been shown to reverse cirrhosis and to improve the lifespan of CHB sufferers (Marcellin, Gane, Buti, Afdhal, Sievert, *et al.*, 2013).

2.4.2.1.8 The natural history of the disease should be understood

The natural history of HBV infection is well understood (section 2.3) although there are still gaps in the knowledge. However, based on current knowledge, patient monitoring and management are feasible and justifiable (section 2.3.3).

2.4.2.1.9 There should be a policy on whom to treat as patients

There are several guidelines available from international organisations such as the WHO (World Health Organization, 2015), the European Association for the Study of the Liver (EASL) (Lampertico *et al.*, 2017), the American Association for the Study of Liver Diseases (AASLD) (Terrault *et al.*, 2018), and The Asian Pacific Association for the Study of the Liver (APASL) (Sarin *et al.*, 2016) describing standard of care of treatment and the criteria of those who qualify for treatment as has been described in detail in section 2.3.3 (Management of chronic HBV infection).

2.4.2.1.10 The screening programme should be cost-effective

Economic evaluation studies comparing the costs of implementation with the health benefits (short- and long-term) to the tested population are able to give an approximation of the cost-effectiveness of a screening programme. This information can then be used to compare different possible health interventions to determine which ones should be implemented. However, carrying out cost-effectiveness studies in countries with limited resources can be tricky as every cost needs to be researched and calculated individually at the location of

implementation since these data are rarely available online (as is the case in more developed countries). In the case of HBV screening, the costs would involve the rapid test, the additional resources required to implement testing (although these can be mitigated by merging HIV and HBV testing, hence existing resources including the nurses can be used as both HIV and HBV POCTs follow the same testing principles), the cost of follow-up testing every six months if found positive, the cost of lifelong treatment if qualifying for treatment, and the initial information campaigns needed to inform the public about hepatitis B and its long-term consequences. The benefits can be measured in terms of disability-adjusted life years (DALYs) and quality-adjusted life years (QALYs). In The Gambia, a cost-effectiveness study, where the HBsAg prevalence is 8.8%, showed an adult screen-and-treat programme had an incremental cost-effectiveness ratio (ICER) of $540 per DALY averted, $645 per life-year saved, and $511 per QALY gained, compared with current practice, which was considered to be cost-effective as it was within one times the country's gross domestic product (GDP) per capita ($487) per DALY averted (Nayagam, Conteh, Sicuri, Shimakawa, Suso, *et al.*, 2016). A recent South African study has shown that the implementation of a Hepatitis Action Plan that includes HBV POC testing within its framework had an overall ICER of $3310 per DALY averted, which was considered acceptable value-for-money as it was below the more stringent benchmark of half GDP per capita ($3810) per DALY averted. Moreover, the screen-and-treat HBV intervention for pregnant women alone within this action plan had an ICER of $5021 per additional DALY averted which was still within one times of South Africa's GDP per capita ($7620) and therefore, still cost-effective (Hecht, Hiebert, Spearman, Sonderup, Guthrie, *et al.*, 2018).

2.4.2.1.11 Screening should be an ongoing process and not a one-off event

The screening test should not be offered as part of a one-off drive or campaign. Case finding should be introduced in such a way that it becomes part of normal health care services offered to the public.

WHO guidelines recommend integrating viral hepatitis testing with existing infrastructure (World Health Organization, 2017c). In South Africa, HBV POC testing could easily be integrated with HIV testing services already in place. Importantly, it can also be integrated with HIV PMTCT programmes to identify pregnant women at risk of transmitting HBV to their children. Counsellors already performing HIV testing could be trained to conduct HBV POC testing and the associated pre- and post-test counselling, as has previously been done in India, where counsellors with experience in HIV testing were also successfully trained to provide HCV pre- and post-test counselling and testing (World Health Organization, 2017c). Long-term, to be more cost-effective, it is also possible to envisage using multiplex POCTs combining testing for both HIV and HBV (Robin, Mboumba Bouassa, Nodjikouambaye, Charmant, Matta, *et al.*, 2018).

2.5 HBV-related HCC

Hepatocellular carcinoma is the most common form of primary liver cancer that arises from liver diseases such as cirrhosis and chronic viral hepatitis (B and C). It is a major cause of morbidity and mortality worldwide–it is the seventh most common cause of cancer and the second leading cause of cancer deaths (Ferlay,

Soerjomataram, Dikshit, Eser, Mathers, *et al.*, 2015). It accounts for 5.6% (782 500/14 090100) of all new human cancers (excluding non-melanoma skin cancer) with an estimated 782 500 incident cases annually (Ferlay *et al.*, 2015). HCC cases are often diagnosed during the terminal stages of diseases, such that incidence reflects mortality rates (Ervik *et al.*, 2016). However, these values largely underestimate the real incidence of this particular cancer in SSA because of the lack of data from most African countries but also the quality of data utilised for those estimates. Cancer registries for example, only record histologically diagnosed cases of HCC (Kew, 2012). As a result, HCC is underreported because it is often diagnosed too late to necessitate a biopsy for confirmation of the diagnosis.

2.5.1 Epidemiology of HBV-related HCC

2.5.1.1 Globally

The global incidence of HBV-related HCC varies geographically. For example, in the developed world, HBV-related HCC is overshadowed by lifestyle risk factors contributing to HCC, such as excessive alcohol consumption, obesity, and diabetes. However, in the developing world, HBV-related HCC plays a far more important role and accounts for more than 75% of all HCC cases (Baecker *et al.*, 2018; Di Bisceglie, 2009; Ferenci *et al.*, 2010; Maucort-Boulch *et al.*, 2018).

In Europe, a discordant trend is observed, and in countries such as Italy and Spain, chronic HCV infection is the main cause of HCC, whereas in countries such as Germany and Austria non-viral hepatitis causes of HCC lead, while Greece has a high prevalence of HBV-related HCC (Baecker *et al.*, 2018; Maucort-Boulch *et al.*, 2018).

In Asia, the incidence of HBV-related HCC seems to be higher in countries such as China, Taiwan, and South Korea but in contrast, HCV appears to be the primary aetiology of HCC in Japan (Baecker *et al.*, 2018; Maucort-Boulch *et al.*, 2018). Taiwan has experienced a remarkable reduction in HCC rates ever since the introduction of HBV vaccination in 1984. A nationwide study in Taiwan conclusively showed a significant decrease in annual incidence of HCC in children aged 6–14 years who were immunised against HBV at birth, from 0.70/100 000 prior to the introduction of the HBV vaccine to 0.36/100 000 10 years after its introduction (Chang, Chen, Lai, Hsu, Wu, *et al.*, 1997). This success story demonstrates that the implementation of appropriate health measures to prevent HBV infection in areas where it is endemic will lead to a significant reduction in incidence of HCC.

2.5.1.2 Africa

In SSA, the data suggest that the highest burden of HCC is in West Africa, with an annual age-standardised incidence of 12.1/100 000, and the lowest in Eastern Africa, with an annual age-standardised incidence of 4.0/100 000 (Ervik *et al.*, 2016). In West Africa, The Gambia seemed to have the highest age-standardised incidence rate (25.8/100 000), although this could be an artefact caused by “over-reporting” in comparison to

other neighbouring West African countries. Studies on HBV have been carried out in The Gambia since 1973 and the Prevention of Liver Fibrosis and Liver Cancer in Africa consortium has been present in The Gambia since 2011 (Howell, Ladep, Nayagam, Lemoine, Garside, *et al.*, 2016), suggesting they have better diagnostic and reporting mechanisms for HCC than other African countries.

2.5.1.3 South Africa

Countrywide data on HCC in South Africa are sparse and incomplete as only histologically diagnosed liver cancer cases are recorded. In fact, a study from the Western Cape, South Africa, showed that only 30% of HCC cases were diagnosed histologically (Maponga, 2016).

Moreover, the South African cancer registry provides annual incidence of liver cancer combined with bile duct cancer. In 2014, 248 cases of liver and bile duct cancers were histologically diagnosed in South African males and 165 in South African females. Of those, 148 were diagnosed in Black males, 22 in black Coloured males, 63 in Caucasian males. In Black males, the highest frequency was observed in the 45-49 years age group and in Caucasian males, the 75-79 years age group (South African National Cancer Registry, 2014).

2.5.2 Diagnosis of HCC

HCC is often diagnosed in the late stages because the lack of characteristic signs and symptoms makes its diagnosis difficult (Kew, Dos Santos & Sherlock, 1971). Consequently, patients tend to present at a late stage where only palliative treatment can be offered (Kew, 2012). The median survival time following diagnosis is 6–20 months (The Cancer of the Liver Italian Program (CLIP) Investigators, 1998). Methods of HCC diagnosis depend on the size of the liver lesion at time of diagnosis and whether imaging modalities are sufficient for diagnosis or need to be confirmed histologically by performing a biopsy of the lesion.

2.5.2.1 Clinical features

Patients with HCC commonly present with symptoms indicative of their underlying chronic liver disease, for example, splenomegaly, ascites, jaundice, variceal bleeding. Some patients may present with mild abdominal pain, weight loss, feeling full after eating a few bites, or a palpable mass in the abdomen (Barghini, Donnini, Uzzau & Soardo, 2013).

Laboratory testing for HCC is often non-specific although abnormal liver function test results will be observed. Some patients present with low serum albumin levels, high bilirubin levels, high aminotransferase, alkaline phosphatase, and gamma-glutamyl transpeptidase levels, but not consistently (Lopez, Balasegaram, Thambyrajah & Timor, 1996). The benefit of these tests is to monitor disease progression over time once a diagnosis has been made.

2.5.2.2 Imaging methods for HCC diagnosis

In SSA, including South Africa, where HCC patients present at a late stage, HCC is usually diagnosed using imaging techniques. The imaging tests most commonly used in HCC diagnosis are ultrasounds and computed tomography (CT) scans (Kew, 2012).

Ultrasonography is not considered a sensitive method of diagnosis as it cannot differentiate between HCC and other solid liver tumours. However, it is widely available in an African setting, non-invasive, and less expensive than other imaging modalities. Suspicious lesions observed on an ultrasound still need to be confirmed by other radiologic methods. A computed tomography (CT) scan of the liver is usually performed to confirm the nature of suspicious lesions observed on ultrasound. A meta-analysis has shown that the sensitivity and specificity of CT scans in the diagnosis of HCC were 83% (95% CI 46%-90%) and 91% (95% CI 86%-96%), respectively (Chou, Cuevas, Fu, Devine, Wasson, *et al.*, 2015).

Magnetic resonance imaging (MRI) can be used in the diagnosis of HCC and provides high-resolution images of the liver without the use of contrast agents or ionizing radiation. However, its use in an African setting remains limited due to its high cost.

2.5.2.3 Serological markers of diagnosis

The most commonly used marker in HCC diagnosis is alpha-fetoprotein (AFP). AFP is a glycoprotein normally produced during gestation by the foetal liver and yolk sac and its serum concentration is often elevated in HCC patients. However, elevated levels of AFP in adults are not specific to HCC. Elevated AFP levels are also observed in pregnant women and in patients with chronic liver disease. AFP is also not produced by all HCCs (Chen, Sung, Shed, Lai, How, *et al.*, 1984). Although AFP has been largely considered to be an unreliable marker of HCC elsewhere (Forner, Reig & Bruix, 2009; Sherman, 2001), in SSA, where most HCC cases are caused by HBV, it remains an important non-invasive marker (Kew, van Staden & Bellingan, 1995). Moreover, if used in conjunction with ultrasound, the diagnostic sensitivity is increased from 71% to 79% (Sherman, Peltekian & Lee, 1995). The ability of AFP to predict the presence of HCC therefore depends on the population being tested and appropriate cut-off values have to be developed according to the population at risk of HCC development and the underlying cause of HCC.

Other non-invasive markers are currently being investigated although none are recommended for diagnostic use or surveillance. MicroRNAs have shown some promise with a panel of six microRNAs (miR-122, miR192, miR-21, miR-223, miR-26a, and miR-801) accurately identifying HCC patients with a sensitivity of 82% and specificity of 84% and capable of distinguishing patients with HCC from those who were healthy, had CHB, or had cirrhosis (Zhou, Yu, Gao, Hu, Wang, *et al.*, 2011). Other promising serological markers include Lens culinaris agglutinin-reactive AFP (AFP-L3), an AFP isoform and des-gamma-carboxy prothrombin (Carr, Kanke, Wise & Satomura, 2007). Des-gamma-carboxy prothrombin was shown to have a sensitivity of 74% and specificity of 86% in detecting early-stage HCC. When combined with AFP, its diagnostic sensitivity increased to 91% but the specificity decreased to 74% (Lok, Sterling, Everhart, Wright, Hoefs, *et al.*, 2010).

These markers need to be externally validated in different populations so their diagnostic potential can be fully elucidated.

2.5.2.4 Histological diagnosis

Biopsies are not usually recommended unless the imaging results are inconclusive and non-invasive methods of assessing fibrosis are unavailable (Russo, Imondi, Lynch & Farinati, 2018; Terrault *et al.*, 2018). There are risks associated with performing biopsies such as bleeding and seeding of tumour cells along the biopsy tract although this is considered minimal (Durand, Regimbeau, Belghiti, Sauvanet, Vilgrain, *et al.*, 2001).

Core biopsies are considered to be more useful than fine-needle biopsies because of the increased amount of tissue and the potential of obtaining non-neoplastic liver tissue to aid diagnosis. The histologic appearance of HCC can range from well-differentiated with minimal cytologic atypia to undifferentiated with spindle or round-shaped cells (Martins-Filho, Paiva, Azevedo & Alves, 2017). In cases where lesions cannot be characterized by haematoxylin and eosin (H&E) staining and microscopy alone, tumour markers such as CD34, CK7, glypican 3, Hsp70, and glutamine synthetase can help with the diagnosis (Zimmermann, 2017).

2.5.2.5 HCC surveillance and models of early diagnosis

Whilst the HBV vaccine was introduced to the South African EPI in 1995, its long-term benefits, namely HCC prevention, will not be seen for at least another ten to twenty years when the first generation of vaccinated children reaches the at-risk age group of 30 years and older. Meanwhile, men who did not have access to the HBV vaccine and who have CHB are at high risk of developing HCC.

As HCC patients can be asymptomatic or present with non-specific symptoms until they reach an advanced stage of disease, screening for HBV infection and enrolling those who are HBV-positive in an HCC surveillance program could potentially identify those at high risk of HCC development and help to diagnose the cancer at an early stage. A previous randomized controlled trial found a 37% reduction in mortality rate after HCC surveillance with ultrasound and AFP over a five-year follow-up period in patients with CHB compared to patients with no surveillance. Moreover, 60.5% of HCC cases were diagnosed at the sub-clinical stage in the HCC surveillance group versus 0% in the non-surveillance group (Zhang, Yang & Tang, 2004).

Such surveillance programmes would be of particular importance in SSA where HCC presents mainly in African men early in life (Kew & Macerollo, 1988; Yang *et al.*, 2017; Yang & Roberts, 2010) and more than 40% of HCC patients will be diagnosed before the age of 40 (Yang *et al.*, 2015). This is in contrast to Asia, where the risk of HCC increases with age and is more prevalent in men in their fifties and above (Beasley, Hwang, Lin & Chien, 1981; Yang *et al.*, 2017). Developing cancer in mid-adulthood puts a drain on health care resources as well as income in low- and middle-income families, further adding to the importance of better understanding this malignancy and setting up screening procedures for HBV and HCC surveillance in high-risk groups.

Ideally, an individualised approach to assess a patient's HCC risk is desired. If HCC risk could be accurately predicted in HBV-positive patients, the surveillance procedures could be adapted to fit that particular patient's profile. In Asia, algorithms have been developed to predict risk of HCC within a 3-year, 5-year and 10-year period and have been validated in Asian populations (Hsu, Yip, Ho, Wong, Huang, *et al.*, 2018; Wong *et al.*, 2010; Yang *et al.*, 2011; Yuen *et al.*, 2009). These simple models use patient demographics (age, sex), disease factors (ALT levels) and virological factors (presence of core mutations, hepatitis B viral loads) that can be obtained through laboratory testing to predict risk of HCC. However, these models have not been externally validated and have been shown to have limited use in a non-Asian population (Arends, Sonneveld, Zoutendijk, Carey, Brown, *et al.*, 2015) as certain factors (such as older age and genotype B/C) that are considered major predictors of HCC development in Asian populations are not risk factors elsewhere.

As there is currently no such risk score available for an African population, there is a need to first identify HCC risk factors in this population that could in future be incorporated in similar prediction models for African patients.

2.5.3 Risk factors of HBV-related HCC

2.5.3.1 HBV-associated risk factors

There are several known viral risk factors associated with HBV infection such as HBeAg status, HBV genotype, viral load, and specific mutations within the viral genome that influence disease progression and risk of HCC.

Patients who remain HBeAg positive for long periods of time are 60.2 times more likely to develop HCC than those negative for both HBsAg and HBeAg (Yang, Lu, Liaw, You, Sun, *et al.*, 2002) and an increase in survival rate has been associated with HBeAg clearance (Chu, Hung, Lin, Tai & Liaw, 2004; Lin, Yu, Lee, Chien, Sheen, *et al.*, 2007; Niederau, Heintges, Lange, Goldmann, Niederau, *et al.*, 1996). This could be linked to the repeated exacerbations observed in certain patients during the immune-active HBeAg positive phase of CHB leading to hepatic inflammation and subsequent injury. HBV DNA levels have also been shown to be associated with an increased incidence of HCC, independent of other risk factors, including HBeAg (Chen, Yang, Su, Jen, You, *et al.*, 2006). In patients who are in the immune-active HBeAg negative phase of CHB, HBsAg levels $\geq$ 1000 IU/mL, even in patients with low HB viral loads, have been associated with increased risk of disease progression and HCC (Tseng, Liu, Yang, Su, Wang, *et al.*, 2012, 2013).

Studies on Asian subjects infected with HBV genotype B or C have found mutations in the BCP (Yin, Xie, Liu, Zhang, Han, *et al.*, 2011) and precore regions and pre-S deletions (Liu, Zhang, Gu, Yin, He, *et al.*, 2009) which are thought to play a role in the evolution of HCC. These mutations have also been described in African subjects in association with HCC (Baptista, Kramvis & Kew, 1999; Kramvis, 2008; Ochwoto, Chauhan, Gopalakrishnan, Chen, Ng, *et al.*, 2013).

Kew *et al.*, 2005 reported the impact of HBV genotype on HCC development in South Africa showing that genotype A presents 4.5-fold increased risk of HCC, as compared to non-A genotypes. Genotype A has also been associated with increased fibrosis in Gambian patients (Shimakawa *et al.*, 2016) although the sample size of patients with HBV genotype A in that study was small.

2.5.3.2 Environmental risk factors

Established environmental risk factors for HCC include aflatoxin exposure and dietary iron overload (Kew, 2013). Dietary iron overload is thought to occur due to the excessive consumption of traditional home-brewed beer prepared in non-galvanized iron drums and was associated with a 10.6-fold risk of HCC (95% CI: 1.5%–76.8%) (Mandishona, MacPhail, Gordeuk, Kedda, Paterson, *et al.*, 1998).

Aflatoxin is a metabolite produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* and together with HBV has a multiplicative impact on the risk of developing HCC (Kew, 2003). It contaminates foodstuffs such as corn, rice and peanuts. Aflatoxin B1 (AFB1) covalently binds to guanine and cytosine residues of DNA and forms AFB1-DNA, ribonucleic acid (RNA) and protein adducts which impair DNA, RNA and protein synthesis. Mutations of the p53 tumour suppressor gene (*TP53*) are the most frequently observed gene mutation in human cancers. The evidence suggests that aflatoxin exposure and HCC are associated with mutations in *TP53* such as a point mutation at the third position of codon 249 resulting in an amino acid change from arginine to serine due (commonly referred to as the R249S mutation) (Bressac, Kew, Wands & Ozturk, 1991; Hsu, Metcalf, Sun, Welsh, Wang, *et al.*, 1991). Mitotic recombinations and genetic instability are other mechanisms whereby aflatoxin may contribute to the development of HCC (Zhang, 2010). The highest prevalence of the mutation has been reported in Senegal (67% – Coursaget, Depril, Chabaud, Nandi, Mayelo, LeCann & Yvonnet, 1993), Mozambique (53% – Ozturk, 1991), and The Gambia (35% – Szymańska, Lesi, Kirk, Sam, Taniere, Scoazec, Mendy, Friesen, Whittle, Montesano & Hainaut, 2004). South Africa, on the other hand, may have a lower prevalence of the R249S mutation (18% – Kimbi, Kew, Yu, Arakawa & Hodkinson, 2005) because of the lower risk of exposure to dietary aflatoxins (Carr & Cooper, 1997). Another study from the Gambia reported on the co-occurrence of the R249S mutation and the full integration of the HBx sequence in the genome (Gouas, Villar, Ortiz-Cuaran, Legros, Ferro, *et al.*, 2012). In SSA, high prevalence rates of HIV, HBV and aflatoxin exposure overlap and may lead to a dramatic increase in the incidence of HCC.

2.5.3.3 Socio-economic/Lifestyle risk factors

Alcohol misuse is a well-known independent risk factor of HCC and can account for as many as half of all HCC cases in developed countries (Hellerbrand, Hartmann, Richter, Knöll, Wiest, *et al.*, 2001). A meta-analysis showed that consuming 25 g of alcohol per day was significantly associated with liver cirrhosis (relative risk [RR] = 2.90; 95% CI:2.71–3.09%) and liver cancer (RR = 1.19; 95% CI:1.12%–1.27%) and that these risks increased with higher daily alcohol consumption (Corrao, Bagnardi, Zambon & La Vecchia, 2004).

Similarly, a South African study has previously shown alcohol misuse to be a risk factor for HCC, independent of HBV status (Mohamed, Kew & Groeneveld, 1992). The same study also found that there was an additive risk for HCC between alcohol abuse and HBV infection. Similarly, a Taiwanese study reported an increase in annual incidence of HCC in patients with both HBV and alcoholism (7.8%) compared to patients with HBV alone (3.7%) or alcoholism alone (1.9%) (Lin, Lin, Mo, Chang, Perng, *et al.*, 2013). Alcohol-associated HCC has been shown to have different DNA methylation levels to HBV-related HCC in certain genes (Hernandez-Vargas, Lambert, Le Calvez-Kelm, Gouysse, McKay-Chopin, *et al.*, 2010; Lambert, Paliwal, Vaissière, Chemin, Zoulim, *et al.*, 2011) which may be one of the pathways affected during hepatocarcinogenesis. Other potential pathways include oxidative stress from alcohol metabolism (Seitz & Stickel, 2006) and acetaldehyde (a carcinogenic compound) formation (McKillop & Schrum, 2009).

The effect of smoking on HCC development is less clear especially because of the strong correlation between smoking and drinking. While studies have shown a significant association between the two (Chen *et al.*, 2006; Chen, Wang, Lu, Wu, You, *et al.*, 1996) that was dose-dependent (Koh, Robien, Wang, Govindarajan, Yuan, *et al.*, 2011), other studies have not found significant association between the two (Shimakawa, Lemoine, Bottomley, Njai, Ndow, *et al.*, 2015; Tanaka, Hirohata, Takeshita, Hirohata, Koga, *et al.*, 1992), including a South African study (Mohamed *et al.*, 1992). However, Mohamed *et al.* 1992 only tested Black South Africans and it is therefore possible that smoking is not a significant risk factor for HCC in this particular demographic, especially since they are the lowest consumers of cigarettes in South Africa (Reddy, Zuma, Shisana, Kim & Sewpaul, 2015). Meta-analyses have shown a significant odds ratio of 1.55 (95% CI: 1.46 to 1.65; $P < 0.00001$) for HCC development in smokers (Abdel-Rahman, Helbling, Schöb, Eltobgy, Mohamed, *et al.*, 2017) as well as an additive interaction between HBV infection and cigarette smoking (Chuang, Lee, Hashibe, Dai, Zheng, *et al.*, 2010).

Obesity and diabetes have also been reported to be independent risk factors of HCC (Dyson, Jaques, Chattopadyhay, Lochan, Graham, *et al.*, 2014; Regimbeau, Colombat, Mognol, Durand, Abdalla, *et al.*, 2004; Wolk, Gridley, Svensson, Nyrén, McLaughlin, *et al.*, 2001) and a synergistic relationship with HBV infection has also been shown with 100-fold risk of HCC in patients with concomitant HBV infection, obesity, and diabetes (Chen, Yang, Yang, Liu, Chen, *et al.*, 2008). These are serious risk factors to consider in the South African context, where besides the high HBV prevalence, 61% of the population is overweight, obese, or severely obese and 2 million have diabetes (Baleta & Mitchell, 2014).

2.5.3.4 Demographics

HCC affects men more than women worldwide (Akinyemiju, Abera, Ahmed, Alam, Alemayohu, *et al.*, 2017; Ervik *et al.*, 2016; Kirk, Lesi, Mendy, Akano, Sam, *et al.*, 2004). In 2012, of the 782 000 HCC incident cases, 554 000 occurred in men (Ervik *et al.*, 2016). In Africa, the annual age-standardised incidence in men was

9.8/100 000 and that for women was 5.2/100 000 (Ervik *et al.*, 2016). The same was observed in South Africa where the age-standardised incidence was 6.7/100 000 for men and 3.3/100 000 in women (Ervik *et al.*, 2016). The reason for this disparity is poorly understood and it has been suggested that estrogen could play a protective role in women (Naugler, Sakurai, Kim, Maeda, Kim, *et al.*, 2007).

In addition to sex, racial disparities have been reported in HCC development. In America, Black Americans were shown to be significantly more likely to develop HCC and have poorer survival rates than Caucasian Americans (Sloane, Chen & Howell, 2006). Similarly, in South Africa, previous reports suggest that Black and Coloured men are at higher risk of HCC compared to other races (Kew, 2013; Maponga, 2016). This has been attributed to their higher exposure to HCC risk factors such as HBV infection and dietary iron overload (Kew, 2008; Mandishona *et al.*, 1998).

In Asia and countries of low HBV endemicity, being older (> 50 years) is an additional risk factor for HCC development (Wong *et al.*, 2010; Yang, Sherman, Su, Chen, Liaw, *et al.*, 2010; Yang *et al.*, 2011; Yuen *et al.*, 2009). In SSA however, 40% of HBV-associated HCC cases develop before the age of 40 (Yang *et al.*, 2015) further emphasising the importance of better understanding this disease in an African setting.

2.5.3.5 HIV co-infection

There is little evidence to fully support HIV as an independent risk factor for HCC although a previous cohort study has shown that immunosuppression in HIV-infected individuals was significantly associated with HCC (Clifford, Rickenbach, Polesel, Dal Maso, Steffen, *et al.*, 2008). However, with the discovery and increased availability of highly active antiretroviral therapy (HAART), patients with HIV are living longer and liver-related mortality has become an important cause of death in this group. A large study of 23 441 HIV infected people from Europe, the United States and Australia has shown that liver-related death was the most frequent cause of non-AIDS-related death (Weber, Sabin, Friis-Møller, Reiss, El-Sadr, *et al.*, 2006). The French National Mortalité study showed that liver-related deaths from HCC increased from 15% in 2000 to 25% in 2005 (Salmon-Ceron, Rosenthal, Lewden, Bouteloup, May, *et al.*, 2009). (Thio, Seaberg, Skolasky, Phair, Visscher, *et al.*, 2002) describe an 18-fold increase in the death from liver-related mortality in men from the United States who were co-infected with HIV and HBV as compared to those monoinfected with HBV.

The evidence for such an increase is less clear in SubSaharan Africans. Southern African studies have reported higher prevalence of HBV infections in HIV-infected patients (Andersson *et al.*, 2013; Boyles & Cohen, 2011; Burnett, Francois, Kew, Leroux-Roels, Meheus, *et al.*, 2005; Firnhaber, Reyneke, Schulze, Malope, Maskew, *et al.*, 2008; Maponga, 2016) but previous findings from a meta-analysis suggested this apparent risk would probably be modest in comparison to that seen in Western countries (Barth, Huijgen, Taljaard & Hoepelman, 2010).

Theoretical mechanisms that might account for an increase in HCC risk include the loss of immune control associated with HIV, resulting in higher HBV viral loads and HBeAg positivity (Mayaphi *et al.*, 2012)

increasing the risk of HCC. In patients who are on HAART, liver toxicity (Sulkowski 2003; Núñez 2006), increased risk of metabolic syndrome (Barbaro & Barbarini 2006; Núñez 2006), Non-alcoholic Fatty Liver Disease (NAFLD) (Guaraldi, Squillace, Stentarelli, Orlando, D'Amico, *et al.*, 2008), Non-Alcoholic Steatohepatitis (NASH) (Núñez 2006) and immune restoration (Carr & Cooper 1997; Núñez 2006) may potentially result in an increase in liver damage, accelerating the development of HCC. It also has been postulated that HIV may directly facilitate malignant transformation (Kew, Smuts & Stewart, 2010). Among transgenic mice, the HIV trans-activator of transcription (tat) protein expressed in the liver appears to enhance the effect of hepatitis viruses. Tat-binding protein interacts with HBx gene to regulate HBV transcription, and HBx protein induces HIV-1 replication (Altavilla, Caputo, Lanfredi, Piola, Barbanti-Brodano, *et al.*, 2000).

2.5.3.6 Epigenetics

The development of HCC is a multistep process with accumulations of genetic and epigenetic alterations in regulatory genes, leading to activation of oncogenes and inactivation or loss of function of tumour suppressor genes (Lee, Lee, Kim, Lee, Jang, *et al.*, 2003; Nishida, Nagasaka, Nishimura, Ikai, Boland, *et al.*, 2008). Unlike the genetic events, the epigenetic processes are reversible, occur early in the process of evolution of malignancy and therefore may be used as markers of early malignant disease and even as targets of future therapeutic approaches. Epigenetic changes can occur through three main mechanisms (i) DNA hypermethylation leading to inactivation (ii) DNA hypomethylation causing genomic instability and, (iii) histone modifications affecting chromatin conformation (Tischoff & Tannapfel, 2008). Characteristically, DNA methylation differs from genetic mechanisms in that the DNA is not changed; methylation alters the readability of the DNA and results in inactivation of genes through mRNA transcript repression (Tischoff & Tannapfel, 2008).

The Ras association domain-containing protein 1 gene (*RASSF1A)* is one of the most frequently inactivated genes in HCC (Hernandez-Vargas *et al.*, 2010; Hu, Chen, Yu & Qiu, 2010; Yang, Guo, Herman & Clark, 2003), and hypermethylation of the promoter region of this gene is suspected to be a major inactivation pathway (Dammann, Schagdarsurengin, Strunnikova, Rastetter, Seidel, *et al.*, 2003). *RASSF1A* promoter hypermethylation is well-established in HBV-related HCC (Lambert *et al.*, 2011; Zhang, Gao, Yu, Liu, Lu, *et al.*, 2014; Zhong, Yeo, Tang, Wong, Lai, *et al.*, 2003) and has been proposed as a biomarker for early HCC diagnosis as the gene is rarely found in a methylated form in normal tissue and patients with chronic liver disease (Lambert *et al.*, 2011; Lee *et al.*, 2003).

The inactivation of cyclin-dependent kinase inhibitor 2A (*CDKN2A*) frequently occurs through promoter hypermethylation (Azechi, Nishida, Fukuda, Nishimura, Minata, *et al.*, 2001; Jin, Piao, Kim, Park, Shin, *et al.*, 2000) and has been shown to occur in the early stage of HCC (Lee *et al.*, 2003) as well as HBV-related HCC. This gene codes for two proteins, p16 and p14 by alternate splicing. So far, only *CDKN2A/INK4* (*p16*) has been shown to be a true tumour suppressor gene and its silencing has been associated with increased risk of

cancer (Rocco & Sidransky, 2001). The hypermethylation of *p16* has been suggested to be linked to HBx protein expression in patients with HBV-related HCC (Zhu, Zhu, Fan, Pan, Li, *et al.*, 2010). It has also been associated with multistep hepatocarcinogenesis in the published literature (Zhang, Ahsan, Chen, Lunn, Wang, *et al.*, 2002) and therefore has potential for use as a screening marker in HBV-related HCC (Lee *et al.*, 2003).

In contrast, global DNA hypomethylation leads to genomic instability, affects repeated DNA sequences (Wilson, Power & Molloy, 2007) and increases with tumour progression (Ehrlich, 2002). Long Interspersed Nuclear Elements-1 (*LINE-1*) hypomethylation is known to occur in several cancers including HCC (Gao, Qu, Chang, Lu, Bai, *et al.*, 2014; Lin, Hsieh, Sheen, Lee, Chen, *et al.*, 2001) and has been used as a surrogate for global hypomethylation which may be an integral part of hepatocarcinogenesis irrespective of aetiology (Shitani, Sasaki, Akutsu, Takagi, Suzuki, *et al.*, 2012).

Several studies have observed preferential hypermethylation of certain target genes, including *RASSF1A* and *p16*, in HCCs of differing etiologies, such as HBV and HCV infections, but these reports have not been consistent (Feng, Stern, Hawes, Lu, Jiang, *et al.*, 2010; Lambert *et al.*, 2011; Lv, Ye, Zhang & Huang, 2017). It is possible that this variability is due to differences in the population groups being tested, such as ethnic background, geography, and environmental exposures, that are known to influence methylation levels (Galanter, Gignoux, Oh, Torgerson, Pino-Yanes, *et al.*, 2017; Lambert *et al.*, 2011). In SSA, two studies comparing DNA methylation between South African HCCs and Australian HCCs showed promoter methylation in *p16* in both populations, although the methylation levels in the South African HCC cases were lower than those in the Australian HCC cases (Herath, Kew, Walsh, Young, Powell, *et al.*, 2002a; Herath, Purdie, Kew, Smith, Young, *et al.*, 2009). The authors speculated that age at presentation as well as host genetic factors could have contributed to the differences in methylation observed confirming that methylation studies in African populations are required in order to assess their usefulness as HCC diagnostic biomarkers in this setting.

2.5.3.7 Genetic predisposition

Besides changes to the epigenome, there is growing evidence supporting the hypothesis that point mutations in the genome can increase the risk of cancer (Stadler, Schrader, Vijai, Robson & Offit, 2014). This is represented in the high penetrance germline mutations in the breast cancer 1, early-onset (*BCRA1*) and breast cancer 2, early-onset (*BCRA2*) genes which increase the risk of familial breast cancer by 10 to 30 times (Foulkes, 2008).

The evidence for genetic predisposition to developing HCC is less clear although family history of HCC is considered to be a risk factor for HCC development in areas of low HBV prevalence (Donato, Gelatti, Chiesa, Albertini, Bucella, *et al.*, 1999; Turati, Edefonti, Talamini, Ferraroni, Malvezzi, *et al.*, 2012), where individuals with HBV or HCV and a family history of HCC were 61.9 times more likely to develop HCC than the controls (Hassan, Spitz, Thomas, Curley, Patt, *et al.*, 2009a). Although familial clustering of HBV-related HCC has

also been observed in Asia (Tong, Huynh & Siripongsakun, 2013; Yu, Chang, Liaw, Lin, Lee, *et al.*, 2000), it has been attributed to the high prevalence of HBV in the affected families (Volk & Lok, 2009). However, as early-onset cancers are more likely to have a strong hereditary component compared to sporadic cancers (Brandt, Bermejo, Sundquist & Hemminki, 2008a; Goldgar, Easton, Cannon-Albright & Skolnick, 1994a) and HCC is seen to develop in African men earlier than in Asia (Yang *et al.*, 2017, 2015; Yang & Roberts, 2017), it is highly likely HCC is associated with a genetic predisposition in SSA. Moreover, although CHB is a significant risk factor for HCC development, only a fraction of HBV-infected individuals will develop HCC, suggesting that there may be host genetic factors involved in disease progression. However, no study to date has investigated the presence of single nucleotide polymorphisms (SNPs) that could potentially genetically predispose Africans to developing HCC.

Elsewhere, several genome-wide association studies (GWAS) have investigated the potential role of SNPs in HCC in non-African populations (Chen, Wang, Xu, Liu & Zhao, 2013; Jiang, Sun, Cao, Liu, Lin, *et al.*, 2013; Sawai, Nishida, Mbarek, Matsuda, Mawatari, *et al.*, 2012). More recently, the use of whole exome sequencing (WES) where only the coding regions of the genome (which make up less than 2% of the whole genome) are sequenced and analysed for the presence of rare disease-causing variants, has gained favour (Bick & Dimmock, 2011). Whole exome sequencing has a greater coverage of the coding genes because of the smaller region being sequenced and can be done on fewer samples to identify rare cancer-predisposing variants. Moreover, WES will identify SNPs that are more likely to be clinically relevant (Popejoy & Fullerton, 2016). Exome sequencing has successfully identified inherited susceptibility genes in other cancers such as hereditary pheochromocytoma (Comino-Méndez, Gracia-Aznárez, Schiavi, Landa, Leandro-García, *et al.*, 2011) and pancreatic cancer (Jones, Hruban, Kamiyama, Borges, Zhang, *et al.*, 2009).

One whole exome sequencing study identified HCC driver genes and mutational signatures that were specific for certain HCC risk factors (Schulze, Imbeaud, Letouzé, Alexandrov, Calderaro, *et al.*, 2015). A small subset of their sample population (n = 5) was of African origin and was positive for both HBV and aflatoxin exposure and showed a mutational signature with a high rate of C>A that the authors speculate could be specific for AFB1. In contrast, another WES study conducted in Chinese HCC patients identified different sets of genes that were mutated and a T>A mutational signature (Zhan, Jiang, Sun, Ke, Hu, *et al.*, 2017), suggesting that different populations and aetiologies will present with different susceptibility genes. To date, no WES studies have been conducted on South African HCC cases so that the susceptibility genes relevant to this setting remain unknown.

3. STUDY I: HEPATITIS B VIRUS POINT-OF-CARE TESTING STUDY

3.1 MATERIALS AND METHODS

3.1.1 Study design

3.1.1.1 Choice of study design

This cross-sectional study was composed of two parts. In the first part, a point-of-care test (POCT) was used to determine the prevalence of active HBV infection in a community-based population from the Western Cape consisting mostly of manual workers and to a smaller degree (approximately 5%) of office workers. Individuals found to be positive were followed-up and linked to care at a tertiary healthcare facility.

In the second part of the study, the HBV POCT's performance and barriers to the implementation of HBV POC testing were investigated. Patient questionnaires were administered to one in five individuals approached for testing. A healthcare staff questionnaire was completed by the nursing staff administering the test to determine their perception of the test.

3.1.1.2 Rationale for study design

A cross-sectional design was selected for this study to determine the prevalence of active HBV infection at one time point. Study participants diagnosed with HBV infections through the study were linked to care and reviewed at least once by a gastroenterologist and radiologist to determine their baseline disease status.

3.1.2 Ethical approval and considerations

Ethical approval for this study was granted by the Human Research Ethics Committee of Stellenbosch University in 2016 (S15/08/179). This approval was renewed on a yearly basis after submission of a progress report (Appendix A).

Autonomy: Study participants were informed that participation in the present study was voluntary, that they could refuse testing at any point, and that refusal to participate in the study would not affect their medical care in any manner.

Respect for persons: Data collected in this study were stored in a password-protected Excel sheet on a university computer. For backup purposes, data were also stored off-site in a password-protected format. Patients were de-identified in the study database that was only accessible to the study researcher. Identifiers of the study participants were kept in a separate password-protected Excel sheet.

Beneficence: This study had direct benefits and effects on individual healthcare for those involved. Those found to be HBV-positive were reviewed by a gastroenterologist and radiologist for long-term follow-up and management at Tygerberg Hospital. The close contacts of HBV-positive study participants were offered free HBV testing and, if found to be positive, were managed in the same way as the HBV-positive study participants

enrolled in the present study. If found to be negative for both HBsAg and anti-HBc, they were advised to be vaccinated.

Non-maleficence: This was a minimal risk study as study participants only received a finger prick for the HBV POCT. Subsequently, venous blood samples were collected from a proportion of HBsAg negative study participants and from all HBsAg positive study participants for confirmation of results. There was therefore no risk for those involved besides some discomfort from the finger prick and phlebotomy.

Justice: All study participants were treated fairly and had equal access to the test being offered. Every study participant was also treated with the same level of respect and medical attention.

3.1.3 Sample size

Sample size was calculated using an estimated HBsAg prevalence of 5% based on a previous epidemiological study conducted in the Western Cape (Andersson *et al.*, 2013), a confidence interval width of 0.03 (3%) and a precision of ± 0.015 (3%). Based on these calculations, a sample size of 811 would provide sufficient statistical power to the study.

3.1.4 Study sites

This study was conducted at Occupational Care South Africa (OCSA), a private company providing occupational health (OH) services to private South African companies. OCSA OH clinics screen employees from companies in the Western Cape to ensure they legally conform to the health requirements necessary for their occupations. These clinics were selected as the employees screened there were considered representative of the adult Western Cape community.

The OH clinics targeted for this study were the stand-alone main office in Brackenfell, Cape Town, South Africa and additional clinics found on the premises of private companies in Parow and Paarl (Figure 3.1-1). Although OCSA has additional OH clinics located in Simon's Town and St. Helena's Bay, these clinics were excluded as they were located too far from the referral hospital (Tygerberg Hospital), which would have increased patient loss to follow-up.

3.1.5 Nursing staff training

Before the study commenced, clinic nurses received a three-hour theoretical and practical training session in performing the HBV POCT conducted by the Determine™ HBV POCT product specialists. An intensive training session was also conducted by the researcher on the aim and objectives of the present study, HBV epidemiology and lifecycle, the consenting process, and the interviews to be conducted with study participants.

At the time of study initiation, as part of the training process, the first week of testing was considered a trial period during which the nurses actively carried out consenting, HBV POC testing, and counselling in a controlled environment. After the trial week, they were fully trained and capable of performing all the steps of the HBV POCT unsupervised.

3.1.6 Study population

Study participants were recruited from the employees attending the OCSA OH clinics (Figure 3.1-1). Clinic attendees residing in the Western Cape, older than 18 years of age, and able to provide informed consent were enrolled.

3.1.7 Study flow

Study participants were enrolled in the study following the algorithm presented in Figure 3.1-2.

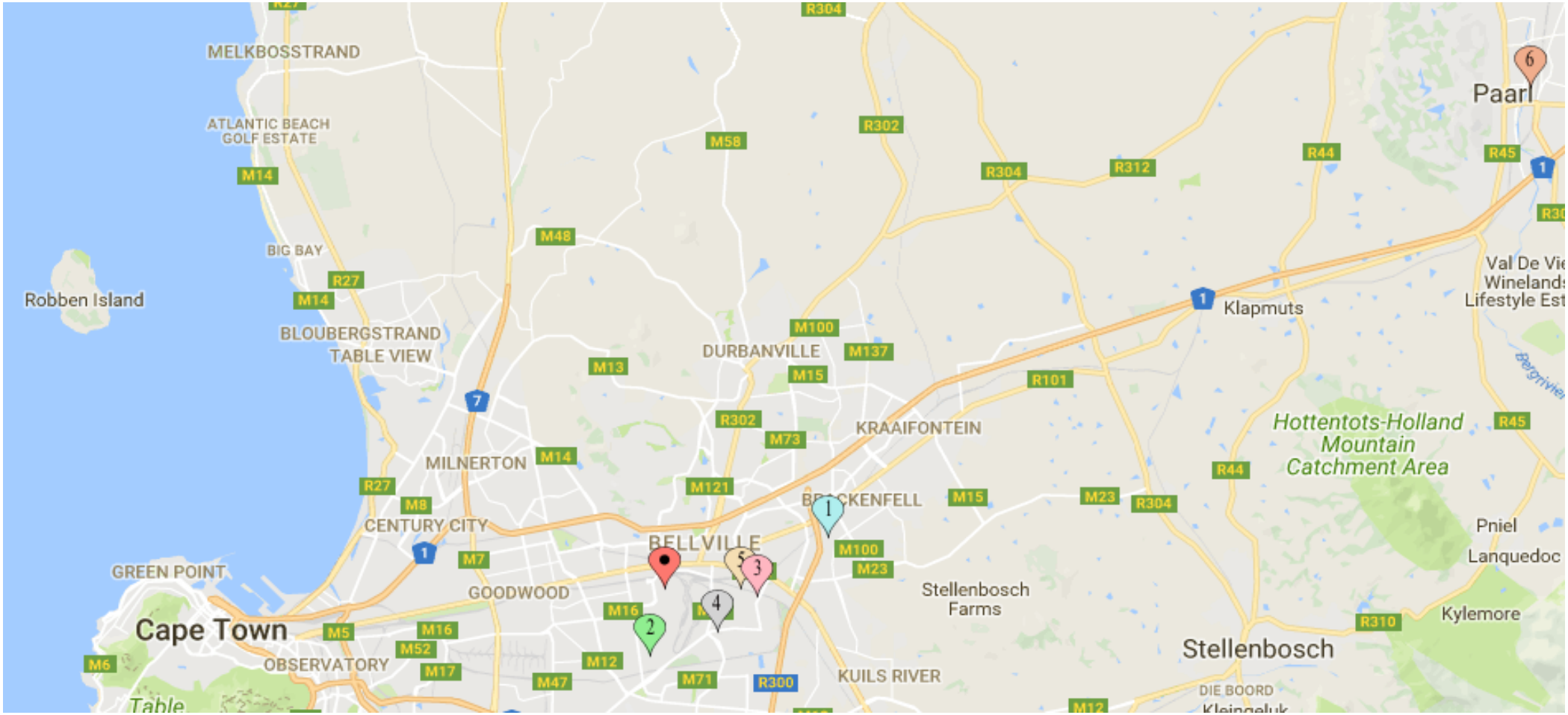
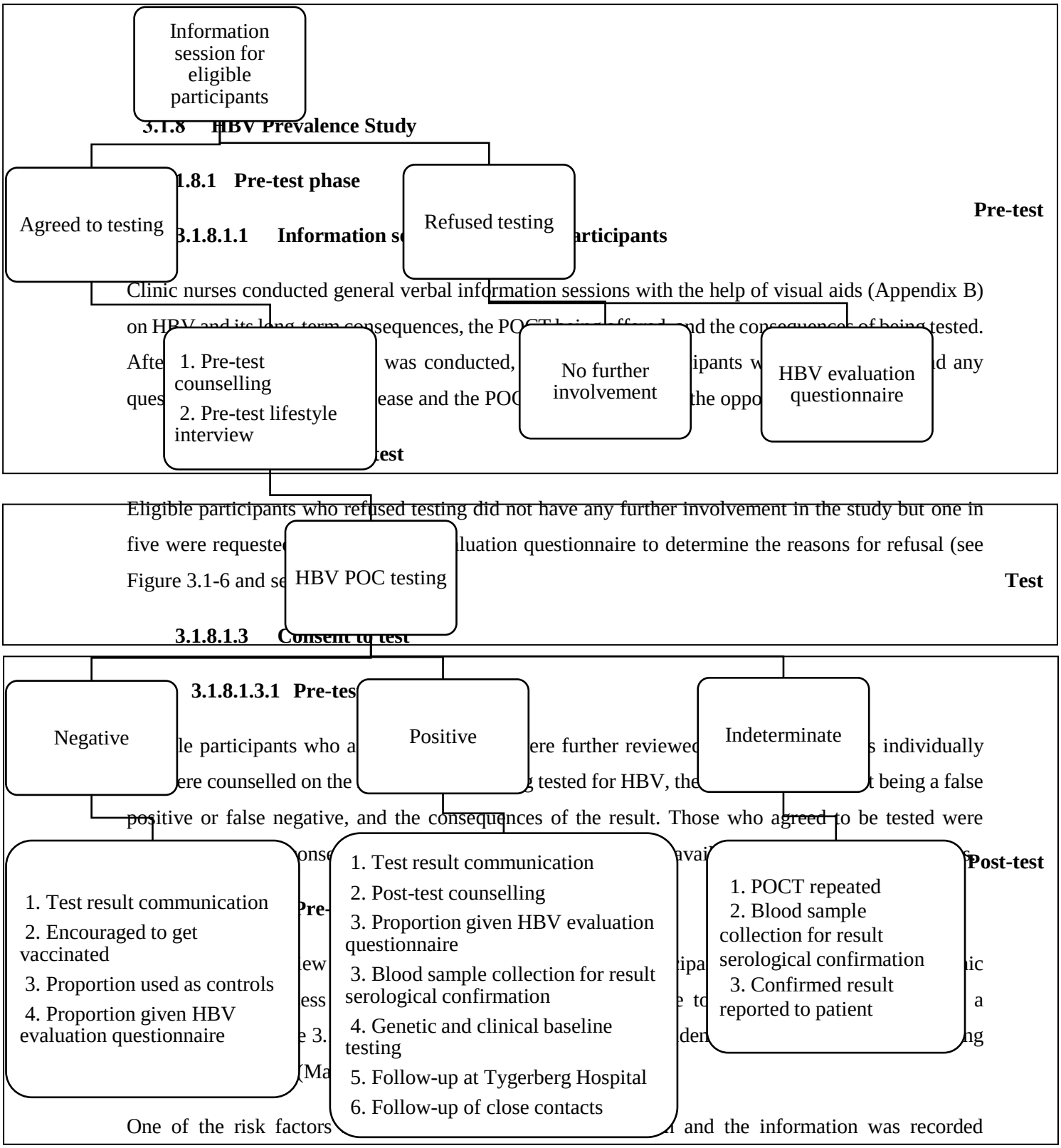


Figure 3.1-1 Map showing clinics where study participant recruitment was conducted. These clinics (1–6) were all managed by OCSA, with the head clinic (Clinic 1) in Brackenfell and the other clinics found on-site in Parow/Bellville (Clinics 2–5) and Paarl (Clinic 6). These clinics were specifically chosen because they were close to Tygerberg Hospital (red pointer with black dot) where HBV-positive patients newly diagnosed through the study would be reviewed by a specialist.



One of the risk factors and the information was recorded following WHO guidelines (Stockwell, Chikritzhs, Holder, Single, Elena, *et al.*, 2000). According to

Figure 3.1-2 Algorithm for study participant enrolment and management POCT: Point of care test; HBV: hepatitis B virus

medium risk, high risk, and very high risk (Table 3.1-1) depending on the amount of alcohol consumed on a single drinking day.

Table 3.1-1 WHO-Criteria for risk of consumption on a single drinking day in relation to acute problems, Stockwell *et al.*, 2000

	Male	Female
Low risk	1 to 40 g	1 to 20 g
Medium risk	41 to 60 g	21-40 g
High risk	61 to 100 g	41 to 60 g
Very high risk	101 g and above	61 g and above

g: gram

Self-reported alcohol misuse, defined as excessive alcohol use that affected their relationships and/or employment, and cigarette smoking habits (current, past, or never) were also recorded.

Herbal medicine was defined as any herbal medication that was not obtained over-the-counter or through prescriptions at the pharmacy. Responders were given the opportunity to elaborate on the herbal medication to ensure that it corresponded to the study's definition of herbal medicine and to find out what was being consumed.

This interview was conducted by the research team who asked the questions directly to the study participants and recorded the answers provided on the case record form. With this form of questioning study participants could be probed for more information where their answers were not specific enough for the study purposes so the information could be recorded in a consistent manner. On the other hand, however, some participants could withhold information if they felt uncomfortable sharing personal information with the clinic nurse. To minimise this risk, the interviewer ensured that the patient was aware that the information provided for study purposes would remain confidential and would not be recorded in their clinic files.

3.1.8.1.3.2.1 Statistical analyses

The frequencies of the questionnaire responses were analysed using SPSS Statistics for Windows, Version 25.0 (IBM Corp., New York, USA). Cross-tabulations were used to compare the categorical demographics and risk factors between the HBsAg positive and negative study participants. Any statistically significant differences between the two groups were determined using Fisher's exact test or Pearson's Chi-Squared test. Fisher's exact test was used where the sample size was less than 5 and Pearson's Chi-Squared test was used for large sample sizes. Statistical significance was set at $p < 0.05$.

OCSA HBV RAPID TEST STUDY CASE RECORD FORM

PATIENT NAME: _____

DETERMINE RAPID TEST RESULT: (CIRCLE) **CONTROL STRIP** **PRESENT** **ABSENT**

HBsAg **POSITIVE** **NEGATIVE**

DEMOGRAPHIC DATA

Date: _____ Age: _____ Date of birth: _____

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

Gender: Male ☐ Female ☐

Weight *(in kilograms)*:_____ height *(in metres)*:_____

Place of birth:_____

Area Born: Urban ☐ Rural ☐

RISK FACTORS		
1. Current alcohol consumption	YES	NO
2. How many UNITS of alcohol do you drink on a typical day when you are drinking?		
3. Past alcohol misuse (defined as alcohol affecting employment or relationships)	YES	NO
4. Smoking Current ☐ Past ☐ Never ☐		
5. Use of herbal medicine Current ☐ Past ☐ Never ☐ If yes, please explain _____		

Figure 3.1-3 Demographic information and lifestyle interview case record form During their interview, study participants were asked the above questions by the research team and their answers were recorded on the case record form. The outcome of each participant's test result was also recorded on the form.

3.1.8.2 HBV POC testing phase

The Determine™ Point-of-care HBsAg test (Alere, Oxfordshire, UK) was used to test study participants for active HBV infection as per manufacturer's instructions. This test has previously been validated for use in our setting (Kariem *et al.*, 2015). After study participants received a finger prick, a capillary tube lined with ethylenediaminetetraacetic acid (EDTA) was used to collect approximately 50 µl of their blood which was placed on the test strip. A drop of chase buffer was added to help the blood flow up the immunochromatographic strip and a timer set for 20 minutes was started. The result was then read after 20 minutes.

Figures 3.1-4 and 3.1-5 show possible valid results after a test was performed. If the control line alone was observed, the participant was deemed to be negative for active HBV infection. If both the control and test lines were present, the participant was deemed to be positive for active HBV infection. In the absence of a control line, the result was considered invalid, the POCT repeated, and a blood sample collected from the participant to confirm the result by enzyme-linked immunosorbent assay (ELISA)

Figure 3.1-5 HBV POCT positive result The control line is present confirming the test result is valid. A second line indicates the test is positive.

Figure 3.1-4 HBV POCT negative result The control line is present confirming the test result is valid. The absence of a second line indicates the test is negative.

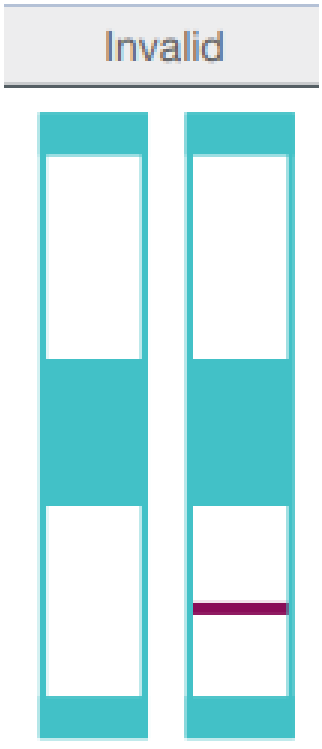


Figure 3.1-6 HBV POCT invalid results Representation of invalid results. In the absence of a control line, whether the patient line is present or absent, the results are considered to be invalid.

using the ARCHITECT® HBsAg Qualitative assay on the automated ARCHITECT® i2000SR (Abbott Laboratories, Illinois, USA) system in the Division of Medical Virology, Stellenbosch University. Nurses were requested to take pictures of every POCT they carried out with the relevant study participant name included so that an external quality control could be performed for every POCT conducted. If the picture was blurry or missing part of the test strip and the result could therefore not be read accurately, it was discarded and that particular participant excluded from the study.

3.1.8.3 Post-test phase

3.1.8.3.1 HBV POCT result communication

Each result was privately communicated to each study participant and the POCT strip shown to them with an explanation of the meaning of the results.

3.1.8.3.1.1 Negative result

If the result was negative, the study participant was advised to get vaccinated at their local hospital to prevent any future infection if they were exposed to the virus.

Moreover, each fifth study participant found to be negative on the HBV POCT was consented to provide 5 ml of blood to confirm the result by laboratory testing to determine the number of false negatives being potentially missed. These control blood samples were tested by ELISA using the ARCHITECT® HBsAg Qualitative assay on the automated ARCHITECT® i2000SR (Abbott Laboratories, Illinois, USA) system in the Division of Medical Virology, Stellenbosch University.

In the eventuality of a false negative result, the study participant was recalled, counselled, and referred to Tygerberg Hospital. False negative results were also reported to the POCT supplier. Nurses who performed false-negative tests were retrained and all tests they conducted from the false negative test onward were excluded from the study.

3.1.8.3.1.2 Positive result

Participants whose test results were positive received a post-test counselling session and were referred to a gastroenterologist at the Liver Clinic of the Division of Gastroenterology and Hepatology at Tygerberg Hospital usually on the following Wednesday. Every positive result was confirmed by ELISA using the ARCHITECT® HBsAg Qualitative assay on the automated ARCHITECT® i2000SR (Abbott Laboratories, Illinois, USA) system in the Division of Medical Virology, Stellenbosch University.

In case of a false positive result, the study participant was recalled and counselled. The supplier was also informed of the false positive result. The nurse responsible for the testing was then retrained and the tests they conducted from the previous control sample onward were excluded from the study.

3.1.8.3.1.3 Indeterminate results

Indeterminate results were repeated and a venous blood sample collected from the participant for confirmation by ELISA using the ARCHITECT® HBsAg Qualitative assay on the automated ARCHITECT® i2000SR (Abbott Laboratories, Illinois, USA) system in the Division of Medical Virology, Stellenbosch University. Study participants were informed telephonically of their result if it was negative. If they were found to be HBV-positive, they were requested to come to Tygerberg Hospital where they received counselling and were reviewed by a gastroenterologist.

3.1.8.3.2 HBV POCT result confirmation and baseline testing

3.1.8.3.2.1 HBV serological testing

Venous blood samples were collected from the HBsAg positive study participants for HBV serological confirmatory and additional tests (HBsAg, HBeAg, anti-HBe, anti-HBc [total and IgM], and anti-HBs) by ELISA on the automated ARCHITECT® i2000SR system. The corresponding ARCHITECT® immunoassays (ARCHITECT® HBsAg Qualitative, ARCHITECT® HBeAg, ARCHITECT® Anti-HBe, ARCHITECT® Anti-HBc II, ARCHITECT® Anti-HBc IgM, and ARCHITECT® Anti-HBs) were used following manufacturer's protocols (Abbott Laboratories, Illinois, USA) at the SANAS-accredited Division of Medical Virology of the National Health Laboratory Services of Tygerberg Hospital.

3.1.8.3.2.2 HB Viral load testing

HB viral load was also determined using the COBAS® AmpliPrep/COBAS® TaqMan® HBV Test on the automated COBAS® AmpliPrep Instrument (Roche Molecular Diagnostics, California, USA) at the SANAS-accredited Division of Medical Virology of the National Health Laboratory Services of Tygerberg Hospital.

3.1.8.3.2.3 HIV testing

The HIV status of study participants who consented to testing was determined by HIV-1 antigen/antibody testing using the Cobas® Elecsys® HIV combi PT 4th Gen (Roche Molecular Diagnostics, California, USA). Pre- and post-test counselling were provided by the HIV counsellors at Tygerberg Hospital.

3.1.8.3.2.4 HBV genotyping

HBV genotyping was performed using an in-house assay developed by Public Health England (Public Health England, 2007) and established at the South African National Accreditation System (SANAS)-accredited Division of Medical Virology of the National Health Laboratory Services of Tygerberg Hospital (Andersson *et al.*, 2013; Chotun *et al.*, 2017, in press; Maponga, 2016). This assay is described below.

3.1.8.3.2.4.1 DNA extraction

Venous blood samples were centrifuged at 700 RCF for 10 minutes and the serum or plasma collected and stored at -20°C until testing. DNA was extracted from the serum or plasma of the HBsAg positive study participants using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) following manufacturer's instructions. To 20 µl of proteinase-K, 200 µl of serum/plasma was added in a 1.5 ml microcentrifuge tube for tissue digestion followed by 200 µl of lysis buffer AL and the tubes mixed by pulse-vortexing for 15 s and incubated at 56°C for 10 min. The microcentrifuge tubes were briefly centrifuged and 200 µl of ethanol was added and the tubes mixed by pulse-vortexing for 15 s. The solution from each tube was then applied to a silica gel membrane spin column and centrifuged at maximum speed for one minute. The filtrate was discarded and the column placed back into the tube. The membrane-bound DNA was washed with 500 µl of two different buffers to remove leftover ethanol. The column was then placed in a new collection tube and centrifuged at full speed for one minute to eliminate possible wash buffer carry-over. Lastly, 50 µl of the elution buffer containing 10 mM Tris-Cl and 0.5 mM EDTA pH 9.0 was added and the column incubated at room temperature for five minutes and centrifuged at 5800 RCF for one minute.

3.1.8.3.2.4.2 PCR amplification

Two rounds of PCR were performed on the DNA extracts of samples from the HBsAg-positive study participants. The primers shown in Table 3.1-2 and the PCR master mix in Table 3.1-3 were used in the pre-nested PCR. The master mix was distributed in volumes of 20 µl in 0.2 ml PCR tubes to which 5 µl of DNA extract was added and the PCR reaction performed as per the thermocycling parameters given in Table 3.1-4.

Table 3.1-2 Primers used in pre-nested PCR of HBV genotyping Public Health England, 2007

Primer name	Primer sequence	Amplicon size
HBV Z_F	5′-AGC CCT CAG GCT CAG GGC ATA-3′	2008 bp
HBV 3_R	5′-CGT TGC CKD GCA ACS GGG TAA AGG-3′	

5′: 5 prime; 3′: 3 prime; bp: base pair

Table 3.1-3 Master mix used in pre-nested PCR of HBV genotyping, Public Health England, 2007

Components	Volume/µl	Starting concentration	Final concentration
PCR buffer	2.50	10X	1X
Magnesium chloride	0.75	50 mM	1.5 mM
Deoxynucleoside triphosphate	0.50	10 mM	0.2 mM
Taq Polymerase	0.10	5 U/µl	0.5 U/µl
HBV Z_F	0.50	20 µM	0.4 µM
HBV 3_R	0.50	20 µM	0.4 µM
Nuclease-free water	15.15	n/a	n/a
Total volume	**20.00**	n/a	n/a

µl: microlitre; x: times; mM: millimolar; U: unit; µM: micromolar; n/a: not applicable

Table 3.1-4 Thermocycling conditions of pre-nested PCR for HBV genotyping, Public Health England, 2007

Cycling parameter	Cycles	Temperature	Time
Initial denaturation	1 hold	95°C	5 min
Denaturation	34	94°C	30 s
Annealing		55°C	30 s
Extension		72°C	1 min
Final extension	1 hold	72°C	2 min

°C: degrees Celcius; min: minute; s: second

A second round of PCR amplification was performed on the pre-nested amplicons to increase reaction specificity and product yield. The primers shown in Table 3.1-5 and the master mix in Table 3.1-6 were used for the nested PCR. The master mix was aliquoted in volumes of 49 µl into 0.2 ml PCR tubes to which 1 µl of the pre-nested product was added and the PCR was performed following the thermocycling parameters given in Table 3.1-7.

Table 3.1-5 Primers used in nested PCR of HBV genotyping, Public Health England, 2007

Primer name	Primer sequence	Amplicon size
HBV P_F	5′-TCA TCC TCA GGC CAT GCA GT-3′	1014 bp
HBV M_R	5′-GAC ACA CTT TCC AAT CAA TNG G-3′	

5′: 5 prime; 3′: 3 prime; bp: base pair

Table 3.1-6 Master mix used in nested PCR of HBV genotyping, Public Health England, 2007

Components	Volume/µl	Starting concentration	Final concentration
PCR buffer	5.0	10X	1X
Magnesium chloride	1.5	50 mM	1.5 mM
Deoxynucleoside triphosphate	1.0	10 mM	0.2 mM
Taq Polymerase	0.2	5 U/µl	0.5 U/µl
HBV Z_F	1.0	20 µM	0.4 µM
HBV 3_R	1.0	20 µM	0.4 µM
Nuclease-free water	39.3		
Total volume	**49.0**		

µl: microlitre; X: times; mM: millimolar; U: unit; µM: micromolar

Table 3.1-7 Thermocycling conditions of nested PCR for HBV genotyping, Public Health England, 2007

Cycling parameter	Cycles	Temperature	Time
Initial denaturation	1 hold	95°C	5 min
Denaturation	34	94°C	30 s
Annealing		50°C	30 s
Extension		72°C	1 min
Final extension	1 hold	72°C	7 min

°C: degrees Celcius; min: minute; s: second

3.1.8.3.2.4.3 Gel electrophoresis

A 1% agarose gel was prepared by melting 0.75 g of agarose powder (SeaKem® LE Agarose, Maine, USA) in 75 ml of 1X Sodium Borate (SB) Buffer. Five microlitres of each PCR product was mixed with one microlitre of 6X Novel Juice (GeneDireX Inc., Taoyuan, Taiwan) and pipetted into wells on the agarose gel to separate any PCR product according to size. The samples were electrophoresed alongside a 1 Kb DNA ladder (GeneRuler™ 100 bp DNA Ladder, ThermoFisher Scientific, Massachusetts, USA) for 30 minutes at 100 V. The agarose gel was then visualised under ultraviolet light at a wavelength of 254 nm using the Platinum HD Gel Documentation System (UVItec Limited, Cambridge, UK) and the image acquired using the UVIband-1D gel analysis software. PCR products with visible bands of the correct size were selected for further testing downstream.

3.1.8.3.2.4.4 Purification of PCR products

PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN) by following manufacturer's instructions. A buffer was first added to the PCR products and the mixture applied to

silica gel membrane spin columns to allow efficient binding of the amplified DNA to the column membrane. This was followed by a wash step using an ethanol-containing buffer to ensure the removal of primers and other impurities that could inhibit sequencing downstream. A centrifugation step in an empty tube then ensured the removal of residual ethanol-containing buffer. The DNA was finally eluted in 30 µl of elution buffer containing 10 mM Tris-Cl, pH 8.5.

3.1.8.3.2.4.5 Sequencing PCR

Sequencing PCR reactions were carried out in a 96-well plate using four different primers (Table 3.1-8) to sequence the region of interest. A master mix consisting of the components listed in Table 3.1-9 was prepared and distributed in volumes of 8 µl into the appropriate number of wells. Next, 1 µl of each primer and 1 µl of purified template DNA (between 20–30 ng) from each sample was added to the appropriate wells. The cycling conditions shown in Table 3.1-10 were used.

Table 3.1-8 Sequencing primers for HBV genotyping, Public Health England, 2007

Primer name	Primer sequence
HBV P_F	5′-TCA TCC TCA GGC CAT GCA GT-3′
HBV M_R	5′-GAC ACA CTT TCC AAT CAA TNG G-3′
HBV H_F	5′-TAT CAA GGA ATT CTG CCC GTT TGT CCT-3′
HBV N_R	5′-ACT GAG CCA GGA GAA ACG GAC TGA GGC-3′

5′: 5 prime; 3′: 3 prime

Table 3.1-9 Sequencing reaction master mix, adapted from manufacturer's instructions (ThermoScientific)

Reagent	Volume/µl
ABI Sequencing Buffer	3
Big Dye terminator	1
Primer, 2 µM	1
Water	3
Total volume	**8**

µl: microlitre; µM: micromolar

Table 3.1-10 Cycling parameters for HBV genotyping sequencing reaction, adapted from manufacturer's instructions (ThermoScientific)

Cycling parameter	Cycles	Temperature	Time
Denaturation	30	96°C	20 s
Annealing		50°C	20 s
Extension		60°C	4 min

°C: degrees Celcius; min: minute; s: second

3.1.8.3.2.4.6 Sequencing PCR purification

The sequencing PCR reaction products were purified using the BigDye® Xterminator Purification Kit consisting of SAM™ and XTerminator® solutions as per manufacturer's instructions (ThermoScientific); 45 µl of SAM solution and 10 µl of Xterminator® solution were added to each well before the wells were sealed with adhesive film. The 96-well plate was vortexed for 30 minutes at 2000 RCF and centrifuged for 1 minute at 1000 RCF.

3.1.8.3.2.4.7 Capillary electrophoresis and sequencing data analysis

Capillary electrophoresis was performed on the samples using the ABI Prism 3130XL Genetic Analyzer (ThermoFisher Scientific). The information obtained was converted to raw data files using DNA sequencing analysis software (ThermoFisher Scientific). The length of the capillaries allowed sequence reads of approximately 1 Kb.

The raw trace files were further analysed using Geneious R11 (Biomatters Ltd., Auckland, New Zealand). The quality of each sequence was improved individually by looking at the chromatograms and trimming the ends where necessary. The forward and reverse sequences for each sample were aligned to create a consensus sequence. Mismatches or ambiguities were verified manually. A reference sequence was created using the corresponding primer sequences and was used to trim the sequences beyond the primer region.

The consensus sequence was saved in FASTA format before being aligned against the Stanford University HBVSeq sequence database to obtain the HBV genotype (Rhee, Margeridon-Thermet, Nguyen, Liu, Kagan, *et al.*, 2010). This allowed HBV genotype identification of study participant sequences.

3.1.8.3.2.5 Clinical biochemical and haematological testing

Venous blood specimens were also sent to the Division of Chemical Pathology, National Health Laboratory Services, Tygerberg Hospital, for liver function tests (bilirubin, albumin, ALT, AST, and AFP) and to the Division of Haematology, National Health Laboratory Services, Tygerberg Hospital, for platelet count testing to determine the baseline disease status. The biochemical tests were all performed on the automated Cobas® 6000 Chemistry Analyzer (Roche, Rotkreuz, Switzerland) using the Bilirubin Total Gen.3 kit (for total bilirubin levels), the Bilirubin direct Diazo Gen. 2 Jendrassik-Grof kit (for conjugated bilirubin levels), the Albumin Gen. 2 kit (for albumin levels), the Alanine Aminotransferase according to IFCC with pyridoxal phosphate activation kit (for ALT levels), the Aspartate Aminotransferase according to International Federation of Clinical Chemistry and Laboratory

Medicine (IFCC) with pyridoxal phosphate activation kit (for AST levels), and the AFP α1-fetoprotein kit (for AFP levels), using the manufacturer's recommended protocols.

The AST platelet ratio index (APRI) and FIB-4 tests were used to determine the presence of hepatic fibrosis as recommended by the WHO (World Health Organization, 2015). The APRI score is as follows: APRI = (AST/upper limit of normal) x 100/platelet count where a score < 0.5 excluded fibrosis and a score > 1.5 suggested significant fibrosis (World Health Organization, 2015). The FIB-4 calculation is as follows: (age (in years) x AST [IU/L])/(platelet count (10^9/L) x [ALT (IU/L)$^{1/2}$]) where a score of 3.25 and above indicated significant fibrosis (World Health Organization, 2015).

3.1.8.3.3 Study participant follow-up at Tygerberg Hospital

Confirmed HBV-positive study participants were referred to the Liver Clinic of the Division of Gastroenterology and Hepatology at Tygerberg Hospital where they were reviewed by a gastroenterologist collaborating on the present study. Laboratory results available for the study participants were used to determine if they required treatment. As per the AASLD guidelines (Terrault, Bzowej, Chang, Hwang, Jonas, *et al.*, 2016), treatment was discussed and offered to HBeAg-positive HBV monoinfected study participants with viral loads of greater than 20 000 IU/ml and abnormal liver function tests (ALT, AST, AFP) and to anti-HBe positive HBV monoinfected study participants with viral loads greater than 2000 IU/ml and abnormal liver function tests. Antiretroviral therapy including tenofovir was also initiated in HIV/HBV co-infected study participants who were not already on treatment. All HBV-positive participants received an abdominal ultrasound scan to determine the presence of cirrhosis or HCC.

3.1.8.3.4 Follow-up of contacts of HBV-positive study participants

The close contacts (children and sexual partners) of the HBV-positive study participants were offered free HBV testing at Tygerberg Hospital. They were tested for HBsAg, anti-HBc, and levels of anti-HBs as described in section 3.10.2.1. Any contact who was positive was treated the same way as previously described in section 3.10.1.2. Any contacts with negative anti-HBc results and levels of anti-HBs below 10 mIU/ml were strongly encouraged to get vaccinated to protect them from any future exposure and infection.

3.1.9 HBV POCT Implementation Study

3.1.9.1 HBV POCT Performance

Measures of sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) were used to assess the performance of the HBV POCT.

The sensitivity of a POCT is its ability to detect a true positive. The formula used to calculate the sensitivity of a POCT is as follows:

$$\text{Sensitivity} = \frac{\text{True positives} \times 100}{\text{True positives} + \text{False negatives}}$$

A POCT's specificity is its ability to exclude a true negative. The formula used to calculate the specificity of a POCT is as follows:

$$\text{Specificity} = \frac{\text{True negatives} \times 100}{\text{True negatives} + \text{False positives}}$$

The PPV is the probability that participants with a positive rapid test result truly have the disease. The formula used to calculate the PPV of a POCT is as follows:

$$\text{PPV} = \frac{\text{True positives}}{\text{True positives} + \text{False positives}}$$

The NPV is the probability that participants with a negative rapid test result truly do not have the disease. The formula used to calculate the NPV of a POCT is as follows:

$$\text{NPV} = \frac{\text{True negatives}}{\text{True negatives} + \text{False negatives}}$$

The results obtained from laboratory confirmation by ELISA of a proportion of HBsAg negatives (section 4.10.1.1) and all HBsAg positives (section 4.10.1.2) on the HBV POCT were used as reference (gold standard) to determine these values.

3.1.9.2 Test perception by study population

A questionnaire was administered to one in five study participant to determine their pre-test knowledge about HBV and perception of the test. The questionnaire was first piloted on 10 people and modified

based on the feedback given (Figure 3.1-7). Part of this questionnaire was also administered to one in five approached individuals who refused to get tested to determine the reasons for refusal.

3.1.9.2.1 Statistical analyses

The statistical analyses were performed using SPSS Statistics for Windows, Version 25.0 (IBM Corp., New York, USA). The median age of the questionnaire responders was calculated. The frequencies of the categorical gender data and questionnaire responses were also recorded.

HBV RAPID TEST-BASED SCREENING QUESTIONNAIRE

CLINIC NAME: **Date** : .....

DEMOGRAPHIC DATA:

Race: ☐ Black ☐ White ☐ Coloured ☐ Indian ☐ Other

Age: ..... Gender: ☐ Male ☐ Female

Please mark your answers with a cross (☒). Choose one answer only, unless otherwise specified.		
1. Had you ever heard of hepatitis B before today?	☐ Yes	☐ No
2. Have you previously had a rapid test (e.g. HIV fingerprick test)?	☐ Yes	☐ No
3. Was the explanation provided by the nurse clear?	☐ Yes	☐ No
4. Did you have the opportunity to ask questions?	☐ Yes	☐ No
5. Did you choose to get tested for hepatitis B?	☐ Yes	☐ No
6. If you said YES to question 5, why did you choose to get tested?	☐ Want to know my status ☐ Good opportunity to be tested ☐ Other:	
7. If you said NO to question 5, why did you choose NOT to get tested?	☐ Scared of needles ☐ Don't think I am at risk ☐ No time to be tested ☐ Not interested ☐ No particular reason ☐ Other:	
8. Was the test painful?	☐ Yes	☐ No
9. Were you satisfied with the test overall?	☐ Yes	☐ No
10. Do you have any complaints?	☐ Yes	☐ No
11. If you said yes to question 10, please justify your answer:		

Figure 3.1-7 Questionnaire to determine study population's perception to testing. Study participants answered all questions except question 7. Eligible individuals who refused testing answered questions 1-5, and 7.

3.1.9.3 Test perception by nursing staff

Another standardised questionnaire was administered to the study nurses involved in study participant recruitment to determine the ease of use of the test and their perception of the test. The questionnaire was first piloted on two clinic nurses who were not involved with study testing and modified based on the feedback given (Appendix D).

3.1.9.3.1 Statistical analyses

The frequencies of the responses to questions with discrete answers in the questionnaires were analysed using SPSS Statistics for Windows, Version 25.0 (IBM Corp., New York, USA). Qualitative analyses were conducted on the open-ended questions and common themes were drawn from those responses.

3.2 RESULTS

3.2.1 HBV Prevalence Study

3.2.1.1 Demographics of enrolled population

Between April 2016 and February 2017, 960 individuals were recruited for this study. The demographics of the study participants are provided in Table 3.2-1.

Table 3.2-1 Demographics of tested population

	Study population ***n*** **= 960**
Age, mean ± SD	37.19 ± 11.02
Sex	
Male	663 (69.1%)
Female	297 (30.9%)
Race	
Black	319 (33.2%)
Coloured	524 (54.6%)
Indian	5 (0.5%)
Caucasian	111 (11.6%)
Other	1 (0.1%)
Place of birth	
Urban	636 (66.3%)
Rural	324 (33.7%)

3.2.1.2 Prevalence of HBV

Of the 960 tested individuals, 21 were found to be positive on the HBsAg POCT. All 21 were confirmed positive for active HBV infection by ELISA, giving an HBsAg prevalence of 2.2% (95% CI: 1.4%–3.3%). The demographics and anthropometrics of the HBsAg-positive participants compared to the HBsAg-negative participants are given in Table 3.2-2.

3.2.1.3 Demographics and Body Mass Index of HBsAg positive and negative groups

Table 3.2-2 Comparison of demographics and Body Mass Index between HBsAg positive and HBsAg negative groups

	HBsAg positive group *n* = 21	HBsAg negative group *n* = 939	p-value
Age, mean ± SD	42.48 ± 8.66	37.07 ± 11.04	0.026*
Sex			
Male	17 (2.6%)	646 (97.4%)	0.233
Female	4 (1.3%)	293 (98.7%)	
Race			
Black	9 (2.8%)	310 (97.2%)	0.518
Coloured	12 (2.3%)	512 (97.7%)	
Indian	0 (0.0%)	5 (100.0%)	
Caucasian	0 (0.0%)	111 (100.0%)	
Other	0 (0.0%)	1 (100.0%)	
Place of birth			
Urban	15 (2.4%)	621 (97.6%)	0.816
Rural	6 (1.9%)	318 (98.1%)	
BMI			
Underweight < 18.5	0 (0.0%)	15 (100.0%)	0.569
Normal 18.5-24.9	4 (1.3%)	293 (98.7%)	
Overweight 25-29.9	9 (2.8%)	318 (97.2%)	
Obese ≥ 30	8 (2.6%)	300 (97.4%)	

Statistical analyses found that the mean age of HBsAg-positive individuals was significantly higher than the mean age of HBsAg-negative individuals ($p < 0.05$). Sex, race, place of birth, and BMI were not found to be significantly different between the HBsAg-positive and HBsAg-negative groups.

3.2.1.4 Comparison of lifestyle risk factors between HBsAg positive and negative groups

The pre-test lifestyle interview showed that more than half of study participants (57.1% 95% CI: 53.9%-60.2%) were alcohol consumers. Among those, 49.1% (95% CI: 44.9%-53.3%) were exposed to very high-risk health behaviours as per WHO guidelines.

Lifestyle risk factors for HCC were compared between the HBsAg positive and negative groups (Table 3.2-3). Past alcohol misuse was the only risk factor that was statistically significant between the two groups ($p = 0.049$), indicating that HBsAg-positive individuals were more likely to consume an excessive amount of alcohol regularly than HBsAg-negative individuals.

Table 3.2-3 Comparison of lifestyle risk factors between HBsAg positive and negative groups

	HBsAg positive group *n* = 21	HBsAg negative group *n* = 939	p-value
Alcohol consumption			
Current	14 (2.6%)	534 (97.4%)	0.669
Past	2 (1.7%)	114 (98.3%)	
Never	5 (1.7%)	291 (98.3%)	
Health risk related to alcohol consumption			
Low risk	2 (2.3%)	85 (97.7%)	0.352
Medium risk	1 (1.4%)	72 (98.6%)	
High risk	1 (0.9%)	112 (99.1%)	
Very high risk	10 (3.8%)	253 (96.2%)	
Past alcohol misuse			
Yes	3 (8.3%)	33 (91.7%)	0.049*
No	13 (2.0%)	623 (98.0%)	
Smoking habits			
Current	8 (2.5%)	317 (97.5%)	0.887
Past	3 (1.8%)	164 (98.2%)	
Never	10 (2.1%)	458 (97.9%)	
Exposure to herbal medicine			
Current	1 (1.4%)	71 (98.6%)	0.162
Past	4 (5.2%)	73 (94.8%)	
Never	16 (2.0%)	795 (98.0%)	

3.2.1.5 HBV-positive participant baseline testing and follow-up

Additional testing for serological markers of HBV infection, HIV co-infection, and HB viral load was performed (Table 3.2-4) to determine the baseline status of the 21 HBV-positive participants, coded OCSA 1 to OCSA 21.

Two study participants, OCSA 3 and OCSA 19 had viral loads too low to permit sequencing. In the 19 patient samples sequenced, there was an equal prevalence of HBV genotypes A (10/19) and D (9/19) that could be further subcategorised as genotypes A1 (n = 10) and D1 (n = 1), D2 (n = 1), D3 (n = 6), and D4 (n = 1).

Three study participants, OCSA 2, OCSA 3, and OCSA 20, were co-infected with HIV. OCSA 3 was on antiretroviral therapy with tenofovir and was negative for both HBeAg/anti-HBe with a viral load of < 20 IU/ml. OCSA 2 and 20 were newly diagnosed with HIV through the present study. OCSA 20 was positive for HBeAg, negative for anti-HBe and had a viral load of 47 600 IU/ml. OCSA 2 was positive for anti-HBe, negative for HBeAg and had a viral load of 555 IU/ml. Both were immediately started on antiretroviral therapy, including tenofovir, which is available free-of-charge to all HIV-positive South African residents at their local primary healthcare clinics.

In HBV mono-infected participants, the biochemical results (Table 3.2-5) were used in conjunction with the virological results and clinical presentation of the patients to make a decision as to whether treatment was required. OCSA 7 was positive for anti-HBe and negative for HBeAg but had a high viral load of 469 000 IU/ml. She was infected with HBV sub-genotype D4 with a W28* BCP/PC mutation and her ALT levels were also elevated (70 U/L). She was therefore considered to have reached treatment threshold and started on treatment with tenofovir. Treatment for OCSA 7 was not available free-of-charge and required a monthly visit to the tertiary hospital for collection of the tenofovir tablets.

All study participants were reviewed at least once by a gastroenterologist during the study. The abdominal ultrasound scans of 20 of the patients were normal with no evidence of HCC or signs of cirrhosis. OCSA 12 consistently missed his ultrasound appointments and was therefore considered to have been lost to follow-up.

Table 3.2-4 Baseline serological and virological testing for HBV-positive patients.

Patient	HIV-1 status	HBsAg	anti-HBs	HBeAg	anti-HBe	anti-HBc (total)	anti-HBc (IgM)	HB Viral load: IU/ml	HBV genotype
OCSA 1	Negative	Positive	Negative	Negative	Positive	Positive	Negative	3850	D3
OCSA 2	Positive	Positive	Negative	Negative	Positive	Positive	Negative	555	A1
OCSA 3	Positive	Positive	Negative	Negative	Negative	Positive	Negative	< 20	NA*
OCSA 4	Negative	Positive	Negative	Negative	Positive	Positive	Negative	137	A1
OCSA 5	Negative	Positive	Negative	Negative	Positive	Positive	Negative	462	A1
OCSA 6	Negative	Positive	Negative	Negative	Positive	Positive	Negative	769	D3
OCSA 7	Negative	Positive	Negative	Negative	Positive	Positive	Negative	469 000	D4
OCSA 8	Negative	Positive	Negative	Negative	Positive	Positive	Negative	4500	D3
OCSA 9	Negative	Positive	Negative	Negative	Positive	Positive	Negative	1990	A1
OCSA 10	Negative	Positive	Negative	Negative	Positive	Positive	Negative	254	D1
OCSA 11	Negative	Positive	Negative	Negative	Positive	Positive	Negative	569	D3
OCSA 12	Negative	Positive	Negative	Negative	Positive	Positive	Negative	6320	D2
OCSA 13	Negative	Positive	Negative	Negative	Positive	Positive	Negative	132	A1
OCSA 14	Negative	Positive	Negative	Negative	Positive	Positive	Negative	128	D3
OCSA 15	Negative	Positive	Negative	Negative	Positive	Positive	Negative	79	A1
OCSA 16	Negative	Positive	Negative	Negative	Positive	Positive	Negative	1240	A1
OCSA 17	Negative	Positive	Negative	Negative	Positive	Positive	Negative	169	A1
OCSA 18	Negative	Positive	Negative	Negative	Positive	Positive	Negative	57	A1
OCSA 19	Negative	Positive	Negative	Negative	Positive	Positive	Negative	< 20	NA*
OCSA 20	Positive	Positive	Positive	Positive	Negative	Positive	Negative	47 600	A1
OCSA 21	Negative	Positive	Negative	Negative	Positive	Positive	Negative	133	D3

OCSA 3 and 4 had viral loads too low for sequencing. NA*: Not applicable; HIV-1: human immunodeficiency virus-1; HBV: hepatitis B virus; HBsAg: hepatitis B surface antigen; anti-HBs: antibody to hepatitis B surface antigen; HBeAg: hepatitis B envelope antigen; anti-HBe: antibody to hepatitis B envelope antigen; anti-HBc: antibody to hepatitis B core antigen; IgM: Immunoglobulin M; IU/ml: International Units per millilitre; <: less than.

Table 3.2-5 Haematological and clinical chemical laboratory results of HBV-positive study participants

Tests / Patient (Normal range)	Albumin: g/L (35-52)	Total bil: µmol/L (5-21)	Conj bil: µmol/L (0-3)	ALT: U/L (10-40)	AST: U/L (15-40)	AFP: µl (0-8)	Platelet count: cells/L (171-388)	APRI score (> 1.5)	FIB-4 score (> 3.25)
OCSA 1	43	10	4	16	15	3.5	394	0.095	0.352
OCSA 2	37	5	2	39	65	8.1	285	0.570	1.242
OCSA 3	43	4	2	51	48	< 1.3	251	0.478	1.312
OCSA 4	44	4	< 2	23	70	1.3	228	0.768	2.177
OCSA 5	42	4	2	23	47	4.6	331	0.355	1.095
OCSA 6	46	6	2	17	20	2.4	370	0.135	0.682
OCSA 7	45	Unknown	Unknown	70	37	2.7	226	0.409	0.626
OCSA 8	44	8	3	34	26	2.1	225	0.289	1.050
OCSA 9	42	6	3	26	23	18.9	173	0.332	1.069
OCSA 10	45	5	< 2	45	35	2.4	202	0.433	1.395
OCSA 11	45	10	4	15	15	4.3	240	0.156	0.613
OCSA 12	46	6	< 2	19	18	2.6	319	0.141	0.544
OCSA 13	46	Unknown	2	22	85	2.5	219	0.970	4.634
OCSA 14	45	8	3	19	29	2.2	280	0.259	1.449
OCSA 15	47	4	2	24	15	2	226	0.166	0.596
OCSA 16	47	5	2	33	27	6.4	205	0.329	0.848
OCSA 17	49	8	3	21	23	4.4	203	0.283	1.063
OCSA 18	43	5	3	48	59	11.9	168	0.878	1.723
OCSA 19	42	5	3	22	22	0.7	208	0.264	0.947
OCSA 20	45	6	3	18	27	3.2	299	0.226	0.639
OCSA 21	45	7	2	26	24	2.6	369	0.163	0.536

bil: bilirubin; Conj bil: conjugated bilirubin; ALT: alanine aminotransferase; AST: aspartate transaminase; AFP: alpha-fetoprotein; APRI: Aminotransferase Platelet Ratio index; FIB-4: Fibrosis-4; g/L: grams per litre; µmol/L: micromoles per litre; U/L: units per litre; µl: microlitre; cells/L: cells per litre. An APRI score and FIB-4 score of greater than 1.5 and 3.25, respectively, suggested significant fibrosis.

3.2.1.6 Follow-up of contacts of HBV-positive participants

Two study participants (OCSA 2 and OCSA 16) did not have any sexual partners or children. The sexual contacts of 4 of the remaining 19 HBV-positive study participants (OCSA 1, OCSA 6, OCSA 17, and OCSA 20) were seen by a gastroenterologist and tested for anti-HBc, anti-HBs, and HBsAg. All were

negative for active HBV infection (HBsAg negative), were positive for anti-HBs, but showed evidence of past exposure to HBV (positive for total anti-HBc).

Similarly, the children of 4 of the 19 HBV-positive study participants (OCSA 7, OCSA 10, OCSA 13, OCSA 15) were seen by a gastroenterologist and tested for anti-HBc, anti-HBs, and HBsAg. All were negative for active HBV infection (HBsAg negative), showed no past exposure to HBV (negative for anti-HBc), and showed immunity to HBV infection (positive for anti-HBs).

3.2.2 HBV POCT Implementation Study

3.2.2.1 POCT performance

3.2.2.1.1 External validation of results

A total of 963 result strips were photographed by the study nurses and the results validated by visual confirmation by the researcher. Three reported negative results were excluded from the study because the poor quality of the pictures taken prevented external validation.

3.2.2.1.2 Indeterminate results

There were no indeterminate results that needed laboratory confirmation.

3.2.2.1.3 Test sensitivity and specificity

Blood samples were collected from 81 study participants who had tested negative on the HBV POCT and were re-tested for HBsAg by ELISA to ensure the validity of negative HBV POCT results. All were confirmed to be negative serologically (8.6%; 95% CI: 7.0%-10.6%). Likewise, all HBV POCT positive results were confirmed to be positive serologically (2.2%; 95% CI: 1.4%–3.3%).

Based on these results, the HBV POCT had 100% sensitivity and specificity (95% CI: 100%–100%) under clinic conditions in the present study (Table 3.2-6).

3.2.2.1.4 Positive and negative predictive values

In the present study, the PPV and NPV of the HBV POCT were both found to be 100% (95% CI: 100%–100%) (Table 3.2-6).

Table 3.2-6 2x2 contingency table showing calculations for sensitivity, specificity, positive and negative predictive values (PPV and NPV)

	Positive on ELISA (Gold Standard)	Negative on ELISA (Gold Standard)	
Positive on HBV POCT	21	0	PPV = [21/(21+0)] x 100 = 100
Negative on HBV POCT	0	81	NPV = [81/(0+81)] x 100 = 100
	Sensitivity = [21/(21+0)] x 100 = 100	Specificity = [81/(0+81)] x 100 = 100	

HBV POCT: hepatitis B virus Point of Care Test; ELISA: enzyme-linked immunosorbent assay; PPV: Positive Predictive Value; NPV: Negative Predictive Value

3.2.2.2 HBV POCT perception by study participants

3.2.2.2.1 Demographics of questionnaire responders

Evaluation questionnaires were completed by 195 study participants (38 females, 138 males, and 19 unreported). The median age of the responders was 29 years (range: 19-64 years).

3.2.2.2.2 Pre-study knowledge about HBV in tested population

Of the 195 responders, 144 had never heard of HBV previously (73.8%; 95% CI: 67.1%–79.9%) and 51 had prior knowledge of HBV (26.2%; 95% CI: 20.1%–32.9%).

3.2.2.2.3 Study participant understanding and perception of HBV POC testing

Of the 195 responders, 127 (65.1%; 95% CI: 58.2%-71.5%) had prior experience with receiving POC testing (such as HIV-1 POC testing). All found the explanation provided by the study nurses to be clear and that they had been given the opportunity to ask questions with regard to HBV and the POCT. Eleven responders (5.6%; 95% CI: 3.2%-9.8%) found the test to be painful. All were satisfied with the rapid test. One responder additionally suggested that the HBV vaccine be made available at the same clinic where the test was offered. In this way, individuals who tested negative on the POCT could immediately get vaccinated for protection from future exposure.

3.2.2.2.4 Study participants' reasons for taking the test

Study participants were asked their reasons for taking the test and could choose multiple options. Most responders (165/195) reported that they wanted to know their status. A smaller number (76/195) also indicated that it was a good opportunity to be tested. Some responders (4/195) provided more detailed responses and said that getting tested for HBV was a good health initiative.

3.2.2.3 Reasons for refusing HBV POC testing

3.2.2.3.1 Demographics

The HBV evaluation questionnaire was also administered to one in five individuals who were approached for testing and refused the test. Of the 86 people who refused testing, 14 (13 male and 1 female) completed the evaluation questionnaire. The median age was 30 (age range: 20-46).

3.2.2.3.2 Reasons for refusing testing

Most responders (6/14) chose "other" as their reason for refusing testing; one refused because they lived in a different province, another because they had previously been tested as a kidney transplant patient, and a third one because they were reluctant to sign the consent form. The remaining three reported they

were not ready to be tested and needed time to think about it. Responders also indicated that they did not have time to be tested (4/14), did not think they were at risk (3/14), were scared of needles (2/14), and did not have any particular reason why they did not want to be tested (1/14).

3.2.2.4 Test perception by nursing staff

A different questionnaire was also administered to eight nurses involved in participant enrolment at the five study clinics.

3.2.2.4.1 Issues with performing the HBV rapid test

When asked on a scale of 1-5 about the complexity of performing the HBV POCT, all nurses (8/8) selected 4 and above, indicating that they were found the POCT easy to perform. While none of the nurses (0/8) had problems performing the test itself, two of eight nurses reported that they found the blood collection difficult. The HBV POCT required 50 µl of blood and although lancets with a penetration depth of 1.6 mm were used to obtain the desired amount of blood, most study participants were manual workers with rough, callused hands that made it difficult to pierce the skin for adequate blood collection.

All nurses (8/8) were very confident in their ability to read the results correctly. All (8/8) reported they received sufficient training to perform the HBV POCT and all (8/8) trusted the results of the test. This correlates with the lack of false positives and false negatives as well as no reported indeterminate results.

3.2.2.4.2 Perception of the HBV rapid test

All nurses (8/8) were experienced with performing POC testing having previously conducted HIV-1 rapid tests. All (8/8) had access to sufficient resources to carry out the POCT in their respective clinics. Two of eight nurses reported that the test interfered with their routine clinic duties. When questioned on a scale of 1-5 on the importance of providing a patient with their result after only 20 minutes, all nurses (8/8) selected 4 and above, indicating that they were all inclined to agree that it was important to be able to report the result of the POCT quickly.

3.2.2.4.3 Adequacy of patient counselling before and after testing

All nurses (8/8) reported that they were able to provide adequate pre- and post-test information to the study participants. When asked to elaborate, they stated that they received proper documentation and training from the researcher that equipped them for the task.

Moreover, five of eight nurses found they were able to provide adequate counselling to the HBV-positive study participants, two did not encounter HBV-positive study participants and one was unable

to provide adequate counselling as there were too many clients being seen at the OH clinic and the participants therefore had to wait for a long time before receiving counselling.

When questioned on the level of knowledge of study participants on HBV, six of eight were inclined to describe the level of knowledge of study participants as low. Five of eight nurses were also uncertain about whether their patients understood the information they received on HBV.

3.2.2.4.4 Barriers to HBV POC testing

All nurses (8/8) reported that patients were not offended when offered HBV POC testing. All (8/8) were comfortable discussing HBV POC testing with their patients. None (0/8) thought that offering the test affected the patients' opinion of the clinic. In fact, seven of eight reported that patients were inclined to be interested when offered the HBV rapid test.

The main barrier encountered when offering HBV POC testing was lack of patient awareness (6/8) followed by time taken for counselling (1/8), language barriers (1/8), and the fact that the test interrupted the routine workflow (1/8). None of the nurses (0/8) reported potential problems with performing the test or lack of patient interest as barriers to testing.

3.2.2.4.5 Concerns with confidentiality of HBV POC testing

Five of eight nurses observed that patients were concerned with whether their results would remain confidential. All (8/8) were able to convey each patient's result in a confidential manner.

3.2.2.4.6 Recommendations of nurses on implementation of HBV POCT

Six of eight nurses agreed that HBV testing was an important part of healthcare while the remaining two were unsure of its importance. When asked if they would recommend the test at the clinic, six of eight responded affirmatively while two did not know whether to recommend it or not. One nurse was unable to recommend the test's implementation, although they mentioned that the test was an important one to provide, as it would be difficult to include it in the routine OH services because of lack of staff and time. The other one thought that as an OH clinic, their main focus should be on education and prevention and not on active diagnosis.

3.3 DISCUSSION

The aim of the present study was to evaluate the need for and feasibility of HBV POC testing in a South African community setting. The main finding of this study was that, in a community-based sample of 960 patients, the prevalence of HBV was 2.2%. Further considerations of the test revealed excellent performance regarding sensitivity, specificity, NPV, and PPV, which were all 100% (95% CI: 100%–100%). Moreover, study participants who were confirmed to be HBsAg-positive were successfully linked to standard care and reviewed by a gastroenterologist. Lastly, the HBV POC test was well-perceived by the study population and by the nursing staff administering the test.

3.3.1 HBV prevalence

The HBV prevalence of 2.2% observed in the present study is lower than that reported previously in two studies conducted in the Western Cape, where prevalences of 3.1% and 4.5% were observed respectively (Andersson *et al.*, 2013; Chotun *et al.*, 2017). Although these two studies provided HBV prevalence data on an important cohort, namely pregnant women, their results could not be extrapolated to the prevalence in the general population. In contrast, the present study was conducted in a community-based setting of generally healthy working individuals attending OH clinics and provides a more accurate estimate of the real HBV prevalence in the Western Cape Province.

3.3.2 Performance of HBsAg POCT

The HBV POCT performed very well with sensitivity, specificity, NPV, and PPV of 100% (95% CI: 100%–100%) demonstrating that in the present study, the Determine™ HBsAg test fulfilled the ASSURED criterion on sensitivity and specificity for POCTs. It also met the WHO procurement eligibility for HBsAg POCTs requiring that such tests have a sensitivity of ≥ 99% and specificity of ≥ 98%. This finding stands in contrast to what has been reported in field testing of the same rapid test in Malawi where the sensitivity and specificity were lower (56% and 69% respectively) due to operator-dependent variables (Nyirenda, Beadsworth, Stephany, Hart, Hart, *et al.*, 2008). A lower sensitivity (88.5%) was also reported in a more recent study in The Gambia (Njai *et al.*, 2015). These discrepancies may be explained by rigorous training and dedication to rapid testing as well as better logistics (timers were provided to the nurses to ensure that the results were read timeously) that enabled the study nurses in the present study to perform the test to the desired standard.

3.3.3 Management of HBsAg positive study participants

The 21 HBsAg positive participants were successfully followed up using routine management protocols in place at the Liver Clinic of the Division of Gastroenterology and Hepatology at Tygerberg Hospital, a tertiary health care institution in Cape Town, South Africa. This process included baseline assessment

of HIV status, biochemical, serological, and virological testing as well as an abdominal ultrasound scan. However, in the present study, additional follow-up components were added which included HBV genotyping, an assessment and comparison of lifestyle risk factors between HBsAg positive and negative study participants, and follow-up of the family and close contacts of HBsAg positive individuals.

3.3.3.1 Routine management

Routine follow-up of all HBsAg positive study participants showed that most had low viral loads and were anti-HBe positive. In addition, HBV-positive study participants also had normal liver enzyme levels and normal ultrasound scans. These results suggest that most HBV-positive study participants were in the inactive chronic hepatitis phase characterised by the presence of anti-HBe, low viral loads (< 2000 IU/ml), and normal ALT levels, although this diagnosis should only be formally made after at least three testing events within a one-year follow-up period, according to international guidelines (Sarin *et al.*, 2016; Terrault *et al.*, 2016). These findings are important because there is currently little known about the natural history of CHB in SSA (Lemoine & Thursz, 2017). However, it has been shown elsewhere that HBeAg seroconversion occurs in the second and third decade of life of CHB patients and that HBeAg clearance increases with age (Liaw, Chu, Lin, Sheen, Yang, *et al.*, 1984) which corresponds with the fact that the HBV-positive cohort in this study was on average in their fourth decade of life.

In this cohort, 3/21 participants (14.3%) were HIV-positive. Only one male HBV-positive study participant was positive for HBeAg and co-infected with HIV and was immediately started on ART containing tenofovir. The proportion of HBeAg positive individuals in this study was much lower than that observed in other studies conducted in pregnant women from the Western Cape (4.8% *vs* 16.7%-18.1%) (Andersson *et al.*, 2013; Chotun *et al.*, 2017). Combined with the relatively low viral loads observed in the present study, the small proportion of HBeAg positives further suggests that the study participants were at low risk of transmitting the virus to their sexual partners and other close contacts.

There were difficulties encountered by HBV monoinfected study participants in accessing treatment compared to HIV/HBV co-infected study participants. The same treatment had to be paid for by HBV monoinfected patients but was available free-of-charge to the HBV/HIV co-infected patients as part of the national ART programme (Meintjes *et al.*, 2017). Moreover, monoinfected patients had to collect monthly prescriptions from the tertiary health care facility compared to co-infected participants who could collect their prescriptions from the PHC facility closest to their place of residence. These differences are likely to impede adherence to treatment in the monoinfected patients, especially because CHB is asymptomatic, and therefore patients are likely to feel less motivated to receive treatment.

3.3.3.2 Additional follow-up procedures

3.3.3.2.1 HBV genotyping

In this cohort of 21 HBV-positive study participants, the HBV strains in 19 of them were successfully sequenced and were equally distributed as either HBV genotype A (subgenotype 1) or D (subgenotypes 1-4). Although genotype A and D are the two major circulating genotypes in South Africa, genotype A is considered to be the predominant one in circulation (Kramvis & Kew, 2007a). However, a recent study from the Western Cape showed that 4/5 (80%) of HBV monoinfected mothers were carrying genotype D strains (Chotun *et al.*, 2017).

This finding indicates that genotype D could be increasing in circulation in the Western Cape, which is of clinical relevance as genotype D can form precore mutants. In the present study, 1/19 patients (5.3%) was infected with a precore mutant that did not express HBeAg and exponentially increased viral replication (Jammeh *et al.*, 2008; Tong *et al.*, 1990) despite a positive anti-HBe status (Friedt, Gerner, Lausch, Trübel, Zabel, *et al.*, 1999). In fact, her viral load was higher than that of the patient who was HBeAg positive. These results suggest that this patient could be in the immune-active HBeAg-negative phase of CHB, putting her at risk of HCC despite treatment (Papatheodoridis, Manolakopoulos, Touloumi, Nikolopoulou, Raptopoulou-Gigi, *et al.*, 2015). Moreover, these precore mutants can cause fulminant hepatitis B if passed on from mother-to-child at birth (Chotun *et al.*, in press). This finding highlights the importance of genotyping and viral load testing when managing such patients since serological testing alone gives an incomplete picture of the CHB patient's health status.

3.3.3.2.2 Follow-up of HBsAg positive participants' contacts

None of the followed-up close contacts of the HBV-positive study participants had an active HBV infection. However, their sexual partners showed evidence of past exposure to HBV (positive for total anti-HBc), although it was not possible to determine the origin of their past infection. Although it is possible that the close contacts were exposed to HBV elsewhere, it was anticipated that repeated sexual contact with their HBsAg positive partners caused infection with HBV and subsequent clearance, as only 5% of adults will develop chronic infections if exposed to HBV in adulthood (Tassopoulos *et al.*, 1987). Although the HB viral loads at time of diagnosis was low for most of the HBV-positive study participants, it is possible that they could have experienced flares in HBV replication when they were in the immune-active phase of the HBV life-cycle that would have increased their viral loads (Liaw *et al.*, 1987) and, therefore, risks of transmission to their sexual partners.

3.3.4 Comparison of risk factors between HBsAg-positive and negative cohorts

HBsAg positive participants were compared to HBsAg negative participants who were not subjected to further follow-up with regard to demographics and lifestyle risk factors. In the present study, the only variables that showed a significant difference between the two groups were mean age ($p = 0.026$) and past alcohol misuse ($p = 0.049$) although current alcohol use was not a significant factor ($p = 0.669$).

3.3.4.1 Comparison of sociodemographic risk factors

When comparing the demographics of the HBsAg positive and negative groups, the mean age of the HBsAg positive group was significantly higher than the mean age of the HBsAg negative group. The age range of the HBsAg positives was 30–61 and that of the HBsAg negatives was 19–63. As the HBV vaccine was introduced to the South African EPI in 1995, anyone below the age of 22 at time of testing would have been vaccinated. In the HBsAg-negative cohort, 7% of study participants were in that age category and would have been expected to be vaccinated which could explain why the HBV-infected cohort was older.

Although 81% of the HBV-positive study participants were male, sex was not a significant risk factor for being infected with CHB ($p = 0.233$) although this could be caused by the underrepresentation of females in the study population. Race was also not a significant risk factor ($p = 0.518$) although this could also potentially be an artefact caused by the underrepresentation of Caucasian, Indian, and "Other" ethnicities in the present study. Place of birth (either rural or urban) was not considered to be a risk factor for HBV infection in this cohort ($p = 0.816$), suggesting that with urban expansion and development, the differences between urban and rural areas are less significant, making it less likely that the place of birth would contribute to CHB development as has been previously reported (Kew, 1996).

3.3.4.2 Comparison of clinical and lifestyle risk factors

Comparisons of clinical and lifestyle risk factors that could contribute to liver disease independently of HBV infection were performed between the HBsAg positive and negative groups. This analysis showed that only past alcohol misuse was significantly higher in the HBV-positive group ($p = 0.049$), indicating that HBV-positive individuals were more likely to have experienced past excessive alcohol consumption. This finding is concerning because of the high reported rates of "binge-drinking" in South Africa, South Africans are already significantly exposed to liver damage including cirrhosis and HCC (Mohamed *et al.*, 1992) and in the context of an HBV infection, the risk of cancer development is significantly increased (Lin *et al.*, 2013; Mohamed *et al.*, 1992). Alcohol misuse was self-reported in the present study so there is a possibility of recall and reporting bias in the HBV-positive group. However, this risk was minimised by having the participants complete the questionnaires prior to knowing their test result.

BMI was also not significantly different between the two groups suggesting that HBV-positive individuals were not more likely to be overweight or obese than the rest of the South African general population. It was concerning to note that 67.1% of the tested population were either overweight or obese, both independent risk factors of HCC (Regimbeau *et al.*, 2004).

Exposure to herbal medicine was not significantly different between the two groups but there was a trend in that HBV-positive individuals were more likely to have been exposed to herbal medicine. Herbal medicine in South Africa is widely used, from the Aloe vera that is grown in the garden to traditional medicine or *muti* such as the African potato that is given for certain ailments. While some of these plants may appear innocuous, they can potentially interact with other medication and reduce their efficacy or potency (Fasinu, Gurley & Walker, 2015; Mills, Foster, van Heeswijk, Phillips, Wilson, *et al.*, 2005). Moreover, several studies have reported an association between herbal medicine and hepatocellular carcinoma because some components of the ingested herbs are metabolised in the liver (Ma, Peng & Hu, 2014; Ng, Poon, Huang, Lim, Boot, *et al.*, 2017).

3.3.5 Perception of the tested population and nursing staff to the HBV POCT

In addition to the HBV POCT performance, qualitative aspects of testing were also assessed that could impede or advance the application of the test in clinical practice. Towards this objective, perception to clinical application of the HBV POCT was assessed in the study population and the study nurses.

There were no complaints from study participants on the HBV POCT although 5.6% found the test to be painful. Once the patients were informed about HBV and its consequences, they were keen on getting tested and, in fact, those getting tested reported wanting to know their health status. In addition, study nurses had a positive attitude toward the test and the majority agreed that the test should be implemented in a health care setting. Moreover, they reported that the test itself was easy to perform, although the volume of blood required was prohibitive to the test in instances where the patients had rough callused hands. An insufficient amount of blood can lead to a false negative result which is potentially a way in which other studies have experienced a decrease in sensitivity with this test (Njai *et al.*, 2015; Nyirenda *et al.*, 2008). None of the nurses experienced any trouble with reading the test results once the test had been performed, suggesting that with sufficient training and the appropriate resources the nurses involved with HIV-1 testing could perform HBV rapid testing.

The nurses also reported that individuals approached for testing responded positively to being offered the HBV POCT. They were not offended when offered the test and were inclined to be interested in getting tested. The nurses also reported that study participants were concerned with the confidentiality of their test result, which was also mirrored in one of the responses offered by an approached study participant who refused testing because he had to sign the consent form before getting tested. This issue may be limited to the setting where the study was conducted; the study was carried out in an OH clinical

setting where the outcome of the health check performed on the study participants would determine whether they could keep their jobs or not. Therefore, this may not be an issue that would be relevant to a PHC setting that individuals would attend voluntarily.

3.3.5.1 Barriers to implementing HBV POC testing

Knowledge of HBV in this setting was low (only 26.2% of questionnaire responders had any prior knowledge of HBV), mirroring what has been reported elsewhere in Africa (Malungu Ngaira *et al.*, 2016). This lack of knowledge on HBV, in turn, increased the time taken for counselling prior to administering the HBV POCT and both factors were considered to be a barrier by 75% of the nurses involved in the present study. Similarly, a previous study investigating barriers to carrying out POCTs for STIs in general (excluding the test used in the present study) reported that the major issues were "time-frame required" (40%), interruption of workflow (30%), and perceived waiting time for patients (30%).

These issues could potentially be resolved if HBV POC testing was offered in a clinical setting, in concomitance with HIV-1 testing, where dedicated counsellors would be available for HBV counselling and testing. Another potential solution could be the use of multiplex tests that allow simultaneous testing for HIV-1, HIV-2, anti-HCV, and HBsAg, once they are validated in this setting (Robin *et al.*, 2018).

Moreover, two main reasons given for refusing HBV testing were individuals not thinking they were at risk and not being ready to get tested, indicating that the reluctance to getting tested could potentially decrease as knowledge of HBV and its consequences become more widespread. Language was also reported to be a barrier by one nurse and was compounded by the fact that if a translator was not present at the time of testing, they could not consent and test the potential participant for HBV.

The majority of the nurses agreed that the test should be implemented in a health care setting. One expressed reservations about its introduction given the lack of staff and the time taken from pre-test counselling to post-test counselling. These concerns reflect the need for dedicated staff to perform this test as is currently done for HIV-1 testing.

3.3.6 Study strengths and limitations

The strengths of this study lie in that this is the first cross-sectional South African study seeking to determine the prevalence of active HBV infection in a community setting. This study also demonstrated successful linkage to care of HBV-positive individuals and importantly followed up their close contacts to determine if horizontal transmission had occurred. It is also the first study looking at perception of the HBV POCT from both the patient's and provider's perspectives. Moreover, it is the first to utilise the HBV POCT in addition to HBV genotyping and lifestyle assessment to complement clinical assessment of HBsAg positive individuals. It made use of a comprehensive framework to better understand the risk factors that may be predisposing HBsAg positive individuals to developing HCC.

However, there were some limitations that should also be considered. Firstly, cross-sectional studies conducted in HIV-infected and uninfected pregnant women in other provinces of South Africa have shown significant differences between the HBV prevalence observed in those provinces (Diale, Pattinson, Chokoe, Masenyetse & Mayaphi, 2015; Thumbiran, Moodley, Parboosing & Moodley, 2014) and that encountered in the Western Cape (Andersson *et al.*, 2013; Chotun *et al.*, 2017). This would indicate that the results from the present study cannot be extrapolated to the general South African population. Secondly, the present study was conducted at an OH clinic and not a PHC facility, which means that the feasibility of implementation of HBV POC testing into routine clinical duties could not be studied. However, primary OHCs were targeted so that a healthy population representative of the Western Cape could be tested. Thirdly, study participants were not tested for so-called occult HBV infections (negative for HBsAg but positive for anti-HBc and levels of HBV DNA $\leq$ 200 IU/ml) because it was beyond the scope of the study objectives. It is therefore possible that the observed HBV prevalence has been underestimated. Lastly, although alcohol misuse was the only significant risk factor between the HBsAg positive and negative groups, no objective test was used such as the Alcohol Use Disorders Identification Test (AUDIT). Future studies should include the use of this test or similar objective tests to determine the proportion of patients who misused alcohol.

3.4 CONCLUSION

The present study has added to the current body of evidence that supports the call for the implementation of HBV POC testing in South Africa by demonstrating the feasibility of HBV POC testing implementation in a community setting. The HBV point-of-care testing study found the general prevalence of HBsAg in a South African community setting to be 2.2%. Moreover, in this study, the Determine™ HBsAg test fulfilled the ASSURED criteria, as it was reported to be user-friendly, it was inexpensive, rapid and could be stored at room temperature (robust), did not require technical equipment to be utilised in a clinical setting, and showed excellent performance in the field with sensitivity, specificity, NPV, and PPV of 100%. The test was well-received by study participants and by the nursing

staff responsible for administering the test and HBV-positive individuals were successfully linked to long-term care and reviewed by a gastroenterologist at a local tertiary health care facility.

A recent WHO report on HBV surveillance and elimination strongly recommends that HBV screening be implemented, at the very least in pregnant women, in regions where the prevalence of HBV in the general population is higher than 2% (World Health Organization, 2017c). Importantly, the prevalence observed in the general population in the present study shows that South Africa meets this requirement. The implementation of HBV POC testing in pregnant women would, at the very least, break the cycle of mother-to-child transmission in this setting and move South Africa closer to the possibility of eliminating HBV as a major public health issue. Moreover, a recent South African study has shown that the implementation of a screen-and-treat HBV intervention for pregnant women alone within the framework of a Hepatitis Action Plan had an ICER of $5021 per additional DALY averted was less than one times of South Africa's gross domestic product per capita ($7620) and, therefore, highly cost-effective (Hecht *et al.*, 2018).

Most barriers to testing (such as time taken for counselling) that were encountered in the present study, were linked to a lack of knowledge of HBV (73.8% of study participants had no prior knowledge of HBV). It is therefore recommended that HBV POC testing implementation in South Africa be preceded by health campaigns providing information on HBV and its long-term consequences and management. It is expected that with increased knowledge of the disease, uptake of the test would be high. Moreover, so as to maximise the use of resources, it would be helpful to introduce the HBV POCT to specific clinics already involved with STD testing, such as HIV clinics. Although this could potentially introduce an additional layer of stigmatisation by association with HIV, this strategy would allow counsellors with specialised knowledge in HBV counselling to carry out the test and effectively address patient concerns. This is especially important in cases where positive results are obtained and where post-test counselling needs to be performed on patients newly diagnosed with HBV.

As this study was conducted in the Western Cape, it is also recommended that similar studies be carried out in community settings in other South African provinces to confirm the results seen in the present study.

4. STUDY II: BIOMARKER STUDY

4.1 MATERIALS AND METHODS

4.1.1 Study design

4.1.1.1 Choice of study design

In this retrospective case-control study, molecular and histological tests were performed on formalin fixed paraffin embedded (FFPE) liver tissue samples previously collected from individuals with and

without HCC to identify potential biomarkers that could be used for the early detection of malignancy. Toward this aim, FFPE liver tissue samples from patients with a confirmed histological diagnosis of HCC irrespective of aetiology were compared to FFPE liver tissue samples from controls without HCC for risk factors of HCC development. Molecular testing was performed on the liver tissue samples to test for HBV status, HIV status, methylation levels in tumour promoter genes *RASSF1A*, *LINE-1*, and *p16*, and presence of the R249S mutation in the human genome within the *TP53* gene which is indicative of aflatoxin exposure, all well-known risk factors for HCC (see sections 2.5.3.2 and 2.5.3.6). In particular, *RASSF1A* and *p16* were selected because of their described involvement in multistep hepatocarcinogenesis in the published literature (Um, Kim, Oh, Kim, Kim, *et al.*, 2011; Zhang *et al.*, 2002) and potential for use as screening markers in HBV-related HCC (Lambert *et al.*, 2011; Lee *et al.*, 2003). *LINE*-1 was included as there are 0.5 million long interspersed nucleotide elements (LINE-1 elements) that are normally heavily methylated, and it is estimated that more than one-third of DNA methylation occurs in repetitive elements. Thus, the analysis of the *LINE-1* methylation would act as a surrogate marker of global hypomethylation (Yang, Estécio, Doshi, Kondo, Tajara, *et al.*, 2004) that has been reported to contribute to carcinogenesis through its ability to promote genomic instability (Ehrlich, 2002), including hepatocarcinogenesis (Lin *et al.*, 2001). Histological testing was also performed to test for cirrhosis.

4.1.1.2 Rationale for study design

HCC is rarely diagnosed histologically in SSA (Maponga, 2016) because HCC patients present too late to necessitate a biopsy for diagnosis (Kew, 2012). Imaging studies (such as ultrasound and CT scans) and biochemical testing (such as serum ALT and AFP levels) are often deemed sufficient to confirm the presumptive clinical diagnosis and disease management is often palliative (Bruix & Sherman, 2005; Kew, 2012). Therefore, the retrospective nature of this study design enabled the identification of multiple cases in a short period of time. Costs associated with recruitment were also avoided as sampling (in the form of liver biopsies) was already done and existing hospital records could be used to retrieve demographic and medical information on selected samples. However, these data could not be verified and in certain instances, were missing which made case-control matching difficult (Lewallen & Courtright, 1998; Singh & Mahmud, 2009).

Samples were divided into two groups, one with HCC (HCC group) and one without HCC (non-HCC group), to allow comparison of neoplastic liver tissue to non-neoplastic liver tissue for the identification of potential biomarkers that could be used to screen for HCC.

4.1.2 Ethical approval and considerations

This study was first granted ethical approval by the Health Research Ethics Committee (HREC) of Stellenbosch University in 2013 (S13/04/084) and by the HREC of the University of Cape Town in

2014 (057/2014) (Appendix E). This approval was renewed on a yearly basis after submission of a progress report.

Respect for persons: The hospital records of the patients were reviewed and only their demographical information (age, sex, and race) and medical history were retrieved. Since the additional testing performed on the retrieved samples was expected to generate information that would be of no clinical relevance, either to the patient or any family members of the patient, a waiver of consent was obtained from the HREC of Stellenbosch University and all samples were de-identified.

Autonomy: There was no direct contact between patients and the researcher as this was a retrospective study.

Beneficence: Although there was no benefit for patients from whom these samples were previously collected, the results of the study are relevant to a better understanding of HCC in South Africa.

Non-maleficence: This was a minimal-risk study as retrospective samples in storage were accessed for testing.

Justice: Understanding HCC in the South African context is a prerequisite for developing reliable diagnostic tools for its early detection in a setting where HCC affects primarily men in their early forties (Yang *et al.*, 2017) and is often diagnosed too late for treatment to be possible (Kew, 2012).

4.1.3 Sample size

This was an exploratory study and therefore all accessible HCC cases matching the study inclusion criteria (Figure 4.1-1) were reviewed for potential inclusion in the study. Non-HCC controls matching the study inclusion criteria were also reviewed and at least one control per case was included in the present study.

4.1.4 Study flow

Stringent search and selection criteria were applied to the study and selected samples were then tested as outlined in Figure 4.1-1.

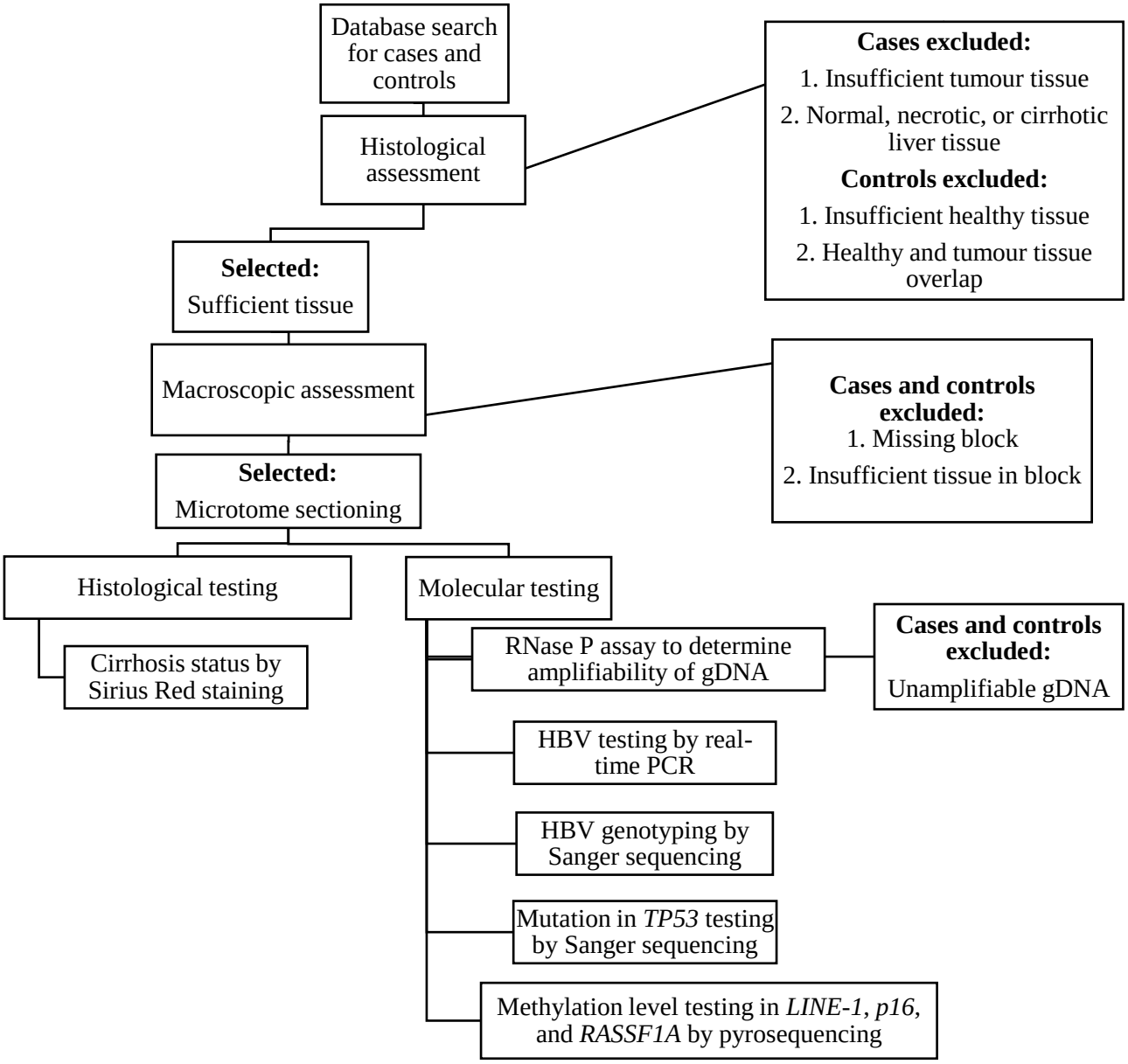


Figure 4.1-1 Algorithm for sample selection and study flow
RNase P: Ribonuclease P; gDNA: genomic DNA; HBV: hepatitis B virus; PCR: polymerase chain reaction; *TP53*: Tumour protein 53; *LINE-1*: Long interspersed nuclear elements-1; *p16*: Cyclin-dependent kinase inhibitor 2A (CDKN2A)/inhibitor of CDK4; *RASSF1A*: Ras association domain family 1 isoform A.

4.1.5 Pre-testing phase

4.1.5.1 Database search for cases and controls

The Disa*Lab and TrakCare Patient Databases of the National Health Laboratory Services at Tygerberg Hospital and Groote Schuur Hospital, two tertiary hospitals in Cape Town, South Africa, as well as the National Health Laboratory Services Western Cape Database were searched to identify the case numbers of histologically diagnosed HCC and metastatic liver cancer from January 1995 to August 2017.

Non-neoplastic (healthy) liver tissue excised from liver biopsies obtained from patients with histologically confirmed metastatic liver cancers were used as controls in the present study. In those individuals, the tumour did not originate in the liver (in most cases, the tumour originated in the colon and metastasised to the liver) and would therefore not have the characteristics of the organ where it originated and therefore not have the characteristics of a primary liver cancer such as HCC (Lambert *et al.*, 2011). In the biopsies that were selected for the present study, there was sufficient non-neoplastic (healthy) liver tissue, adjacent to the metastatic tumour tissue, that could be isolated for DNA extraction and testing to be possible.

Although ideally, liver tissue from healthy individuals should have been used as controls in the present study, it is difficult to obtain because liver biopsies are not typically performed on individuals with healthy livers. Other options for obtaining control liver tissue included the use of non-neoplastic liver tissue from each HCC case (so each HCC case would act as its own control) or cadaverous liver tissues from otherwise healthy individuals who had died from trauma or other non-liver related liver diseases. The former option was excluded as most liver biopsies in this setting target the neoplastic tissue with very little non-neoplastic background liver tissue that could have been used as control. Moreover, although a proportion of HCC samples had background tissue that appeared to be non-neoplastic, genetic studies would be performed on the samples downstream and it was not possible to ascertain that the genome of the non-neoplastic cells of a patient with HCC had not undergone pre-malignant change (Sherman, 2008). The second option was excluded after discussion with collaborators on the study because most individuals' livers would have started autolysing at the time of autopsy, making it difficult to obtain sufficiently undamaged samples for the present study.

4.1.5.2 Selection of cases and controls

4.1.5.2.1 Histological assessment

Once potential cases and controls were identified, the corresponding H&E slides were re-assessed histologically to confirm the initial diagnosis by a consultant anatomical pathologist with 10 years of experience in the field and with a special interest in gastrointestinal and hepatobiliary pathologies (Figure 4.1-2 A). Tissue staining and morphology were assessed to determine whether the cases had sufficient neoplastic tissue and the controls sufficient non-neoplastic tissue (Figure 4.1-2 B) to be included in the present study.

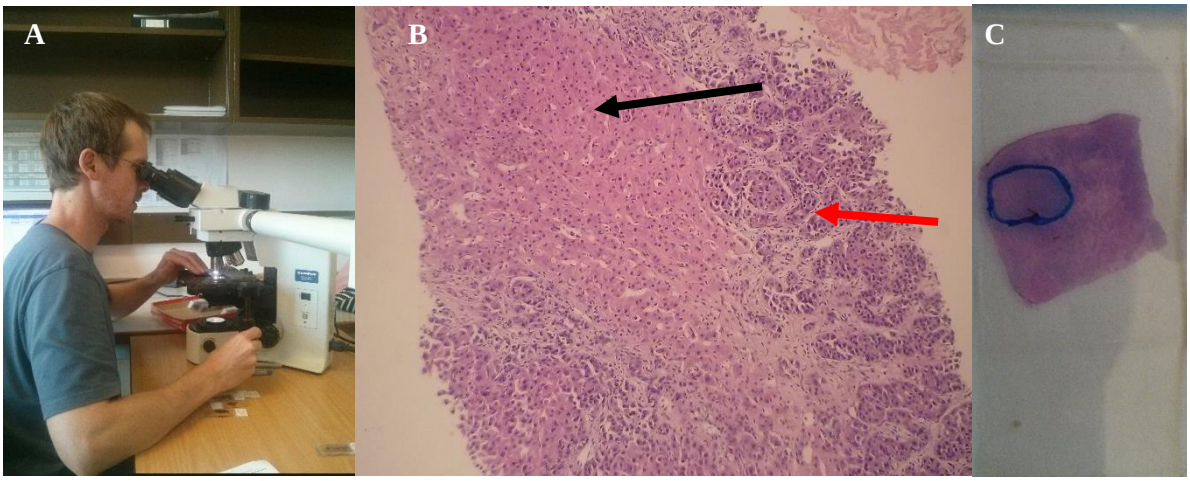


Figure 4.1-2 Histological assessment of HCC cases and non-HCC controls Haemotoxylin & Eosin stained slides. A The consultant anatomical pathologist assessed the slides to determine whether the corresponding cases/controls were suitable for inclusion in the study. **B** The black arrow shows a microscopic view of non-neoplastic (healthy) liver parenchyma. The hepatocytes are arranged in plates that are one to two cells thick separated by sinusoids and have a maintained portal tract-central vein relationship. The red arrow shows a microscopic view of neoplastic tissue. Here, atypical hepatocytes are arranged in plates that are three or more cells thick and have lost portal tract-central vein relationship. The hepatocyte nuclei are larger, more hyperchromatic, and have less abundant cytoplasm. **C** The neoplastic areas were manually outlined on the hepatocellular carcinoma case slides with a fine point permanent marker to aid with manual macrodissection during the subsequent DNA extraction step. Similarly, the non-neoplastic liver areas were manually outlined on the metastatic liver cancer control slides.

The consultant anatomical pathologist manually marked neoplastic areas on the HCC H&E slides to aid subsequent manual macrodissection (Figure 4.1-2 C) and ensure that DNA could be extracted from neoplastic tissue and minimise contamination with non-neoplastic liver tissue DNA. Potential cases were discarded if the corresponding H&E-stained sections showed only normal, necrotic, or cirrhotic liver tissue or if the neoplastic area was too small to permit DNA extraction. Similarly, when the non-HCC slides were examined, the consultant anatomical pathologist outlined non-neoplastic liver tissue areas to minimise contamination with neoplastic liver tissue DNA in the subsequent DNA extraction step. Potential controls were discarded if the corresponding H&E-stained sections showed insufficient

non-neoplastic liver tissue or if there were patches of neoplastic tissue within the non-neoplastic regions.

4.1.5.2.2 Macroscopic assessment of FFPE tissue blocks

Formalin-fixed paraffin-embedded tissue blocks corresponding to cases and controls were thus selected and retrieved from storage. Blocks that were missing or were found to have insufficient liver tissue after macroscopic examination were excluded from the study.

4.1.5.3 FFPE tissue sectioning

FFPE tissue blocks that were included in the study were sectioned using a microtome by a laboratory technologist who was experienced in sectioning slides for molecular testing. Since the samples were used downstream for molecular applications, several steps were taken to minimise potential nucleic acid contamination. Before sectioning any samples, the microtome blade and microtome surfaces were wiped down first with 0.35% bleach followed by 70% ethanol. The first few sections of each block were discarded as they would have been exposed to the atmosphere and contaminants during storage. A new microtome blade was used to section each tissue block to prevent cross-contamination. Each section was cut to a thickness of 8 µM and floated on a water bath at 45°C. A non-polarised slide was used to capture a maximum of three sections per slide, depending on the surface area of the section and air-dried.

4.1.6 Testing phase

4.1.6.1 Molecular testing of cases and controls

4.1.6.1.1 Nucleic acid extraction from FFPE liver tissue

A slide extraction protocol previously described by Baker *et al.* (2013) was modified for the extraction process. The slides were incubated overnight at 60°C to melt the wax after which they were deparaffinised using three xylene washes. This was followed by two wash steps with 100% ethanol and 95% ethanol to remove the xylene and two final wash steps with distilled water to remove the ethanol. The wash steps were all performed in separate Coplin staining dishes on a shaking tray for five minutes at room temperature under a fume hood. The deparaffinised slides were wiped to remove excess water (without touching the tissue) and left to air dry. Three sections of appendix tissue were included with each batch of extractions to act as controls for the extraction process.

Subsequently, DNA extraction was carried out on the deparaffinised tissue using either the DNA FFPE Tissue Kit or DNeasy Mini Tissue Kit (QIAGEN). The DNA FFPE Tissue Kit (QIAGEN) was initially used following manufacturer's instructions but gave low yields of DNA of poor quality. The slide

extraction method was subsequently found to produce the best DNA yield and quality. Therefore, DNA was extracted from a first batch of 10 samples using the DNA FFPE Tissue Kit and the remaining samples were extracted with the DNeasy Mini Tissue Kit (QIAGEN) which was less expensive than the DNA FFPE Tissue Kit and used the same reagents, with the exception of the elution buffer.

Firstly, 180 µl of lysis buffer from the kits were aliquoted into 1.5 ml microcentrifuge tubes corresponding to each sample or control. Manual macrodissection was performed using scalpels on all FFPE tissue sections using the marked H&E slides to guide the dissection. Next, tissue digestion was achieved by adding 20 µl proteinase-K to each tube and incubating at 56°C. Where the overnight incubation was insufficient for digestion, 2 µl proteinase-K solution was added to the tubes and incubated for a further hour until the solution was clear. FFPE samples often present with degraded DNA because of crosslink formation between formaldehyde and DNA. In order to remove the crosslinks, the samples were further incubated at 90°C for one hour. After incubation, the tubes were vortexed for 15 s and centrifuged briefly to remove drops from the lids. Then, 200 µl of lysis buffer and 100% ethanol were added to the tubes and the solutions transferred to silica gel membrane spin columns and centrifuged at 5800 RCF for two minutes. The membrane-bound DNA was washed with 500 µl of two different buffers to remove all ethanol. The columns were then placed in new collection tubes and centrifuged at full speed for three minutes to eliminate wash buffer carry-over. Finally, the DNA was eluted twice in 30µl of elution buffer in two separate tubes. The FFPE kit used elution buffer ATE (10 mM Tris-Cl pH 8.3, 0.1 mM EDTA, and 0.04% sodium-azide) that was meant to help preserve sensitive DNA. The DNeasy kit, on the other hand, used buffer AE (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0). The use of either buffer did not compromise the extracted DNA or the molecular applications conducted downstream.

4.1.6.1.2 RNase-P assay to assess amplifiability of genomic DNA from tissue samples

A commercially available human Ribonuclease (RNase) P assay was used to determine whether the genomic DNA extracted was amplifiable as described previously (Life Technologies, 2013). This housekeeping gene was chosen as it is present in all cells. In addition, the assay produced an amplicon of 87 bp that was small enough to be detectable by real-time PCR on degraded FFPE DNA. This real-time assay used a ready-made primer-probe master mix and followed a demonstrated protocol on the ABI 9700 (ThermoFisher Scientific) platform (Life Technologies, 2013).

A 1:10 and 1:20 dilution of each DNA extract was prepared using the same elution buffer provided with the kit. A PCR master mix was then prepared for 20 µl reactions using the components given in table 4.1-1.

Table 4.1-1 RNase-P assay master mix components according to manufacturer's instructions (ThermoFisher Scientific)

Component	Volume/µl
Taqman® Universal PCR Master Mix (2X)	10.0
20X RNase P primer-Probe mix	1.0
Nuclease-free water	6.5

µl: microlitre; X: times; RNase: ribonuclease

A 96-well plate was used for real-time PCR and 17.5 µl of master mix was added to each well to be used. This was followed by 2.5 µl of each sample DNA in their 1:10 and 1:20 dilutions in triplicate. Lastly, 2.5 µl of nuclease-free water was added to the plate in triplicate. The plate was then sealed securely with adhesive film, vortexed briefly to mix the reagents and template DNA in each well, and centrifuged at 100 RCF for five minutes to collect the contents at the bottom of the plate. The plate was then loaded onto the Applied Biosystems® 7500 Real-Time PCR instrument (ThermoFisher Scientific) and the real-time PCR performed at the cycling parameters in Table 4.1-2.

Table 4.1-2 Real-time PCR cycling parameters, according to manufacturer's instructions (ThermoFisher Scientific)

Cycling parameter	Cycles	Temperature	Time
UNG Incubation	Hold	50°C	2 min
Enzyme activation	Hold	95°C	10 min
PCR denaturation	40 cycles	95°C	15 s
PCR annealing/extension		60°C	1 min

°C: degrees Celsius; min: minute; s: second

4.1.6.1.3 Determination of HBV status by real-time PCR

Two methods were used to determine the HBV status of samples tested in the present study. Firstly, the hospital records of the patients were reviewed to determine whether the HBV status of the patient was known. Secondly, irrespective of whether the HBV status was known or unknown, the samples were all subjected to a monoplex, hydrolysis probe-based, real-time PCR assay modified from Garson *et al*. 2005 to determine their HBV status.

The HBV primers and probe used in this assay target a highly conserved 97 base-pair area in the pre-S2 region of the surface gene (Table 4.1-3). The probe used a tetramethyl-6-Carboxyrhodamine (TAMRA) quencher and FAM as the reporter dye. 6-carboxyfluorescein (FAM) has an emission wavelength of 517 nm which can be detected by the green channel of the real-time PCR instrument.

A master mix was prepared for the desired number of samples and controls using the appropriate volumes and concentrations for each reagent (Table 4.1-4). The QuantiTect Probe PCR Kit (QIAGEN) was used for this reaction because of its high sensitivity in detecting low-copy numbers.

Table 4.1-3 Primer sequences for HBV real-time PCR and amplicon size, Garson *et al.* 2005

Name of primer	Sequence	Amplicon size
HBV_Forward	5′-GTG TCT GCG GCG TTT TAT CA-3′	97 bp
HBV_Reverse	5′- GAC AAA CGG GCA ACA TAC CTT-3′	
HBV_Probe	5′ FAM-CCT CT(T/G) CAT CCT GCT GCT ATG CCT CAT C-3′-TAMRA	

5′: 5 prime; 3′: 3 prime; bp: base pair; FAM: 6-carboxyfluorescein; TAMRA: tetramethyl-6-Carboxyrhodamine

Table 4.1-4 Master mix composition for HBV real-time PCR, Garson *et al.* 2005

	Volume/µl	Starting Concentration	Final Concentration
QuantiTect Probe PCR Buffer	12.50	2X	1X
HBV_Forward Primer	0.10	100 µM	400 nM
HBV_Reverse Primer	0.10	100 µM	400 nM
HBV_Probe	0.05	100 µM	200 nM
Water	2.25	n/a	n/a
Total volume	**15.00**	n/a	n/a

X: times; mM: millimolar; U: unit; µM: micromolar; n/a: not applicable

Fifteen microlitres of the master mix were distributed in individual 0.2 ml PCR tubes and 10 µl of DNA extract from each sample or nuclease-free water as control was added to their respective tubes for a final reaction volume of 25 µl. The real-time PCR was then performed using the cycling parameters in Table 4.1-5.

Table 4.1-5 Cycling parameters of HBV real-time PCR, Garson *et al.* 2005

Cycling parameter	Cycles	Temperature	Time
Initial denaturation	1 hold	95°C	15 min
Denaturation	45 cycles	95°C	15 s
Annealing/Extension		60°C	60 s

°C: degrees Celsius; min: minute; s: second

4.1.6.1.4 HBV genotyping

All cases and controls found to be positive by real-time PCR were also subjected to an HBV genotyping assay previously described (Ruiz-Tachiquín, Valdez-Salazar, Juárez-Barreto, Dehesa-Violante, Torres, *et al.*, 2007) with some modifications to the PCR master-mix (4.1-7).

This assay targeted the "a-determinant" region of the HB viral genome and amplified a fragment of 279 bp that was short enough to be amplified from DNA extracted from FFPE tissue samples and large enough for accurate genotyping.

4.1.6.1.4.1 Amplification using PCR

Two rounds of PCR were carried out using primer sets described in Table 4.1-6. The pre-nested PCR master mix (Table 4.1-7) was aliquoted in volumes of 20 µl to which 5 µl of sample DNA was added. The nested PCR master mix (Table 4.1-8) was aliquoted in volumes of 49 µl to which 1 µl of the pre-nested product was added. The thermocycling parameters for the pre-nested and nested PCRs are given in Tables 4.1-9 and 4.1-10.

Table 4.1-6 Pre-nested and nested primers used for HBV genotyping, Ruiz-Tachiquín *et al.* 2007

Name of primer	Sequence	Amplicon size
HBV-1 sense	5'-CGCTGGATGTGTCTGCGGCGT-3'	334 bp
HBV-2 antisense	5'-CGAACCACTGAACAAATGGCA CT-3'	
HBV-3 sense	5'-CATCCTGCTGCTATGCCTCATCT-3'	279 bp
HBV-4 antisense	5'-GGCACTAGTAAACTGAGCCA-3'	

5′: 5 prime; 3′: 3 prime; bp: base pair

Table 4.1-7 Pre-nested PCR master mix for HBV genotyping, Ruiz-Tachiquín *et al.* 2007 (adapted)

Components	Volume/µl	Starting concentration	Final concentration
QIAGEN Taq Buffer	2.5	10X	1X
HBV 1-sense	0.4	25 µM	0.4 µM
HBV 2-antisense	0.4	25 µM	0.4 µM
Deoxynucleoside triphosphate mix	1	10 mM each	0.32 mM each
Hot Star DNA polymerase	0.1	5 U/µl	0.5 U/µl
Nuclease-free water	15.6	n/a	n/a
Total (excluding DNA)	**20**	n/a	n/a

X: times; mM: millimolar; U/µl: unit per microlitre; µM: micromolar; n/a: not applicable

Table 4.1-8 PCR cycling parameters for pre-nested PCR, Ruiz-Tachiquín *et al.* 2007

Cycling parameter	Cycles	Temperature	Time
Initial denaturation	1 hold	95°C	15 min
Denaturation	45	94°C	30 s
Annealing		55°C	90 s
Extension		72°C	60 s
Final extension	1 hold	72°C	15 min

°C: degrees Celsius; min: minute; s: second

Table 4.1-9 Nested PCR master mix for HBV genotyping, Ruiz-Tachiquín *et al.* 2007

Components	Volume/µl	Starting concentration	Final concentration
QIAGEN Taq Buffer	5	10X	1X
HBV 1-sense	0.8	25 µM	0.4 µM
HBV 2-antisense	0.8	25 µM	0.4 µM
Deoxynucleoside triphosphate mix	2	10 mM each	0.32 mM each
Hot Star DNA polymerase	0.2	5 U/µl	0.5 U/µl
Nuclease-free water	40.2	n/a	n/a
Total (excluding DNA)	**49**	n/a	n/a

X: times; mM: millimolar; U/µl: unit per microlitre; µM: micromolar; n/a: not applicable

Table 4.1-10 PCR cycling parameters for nested PCR, Ruiz-Tachiquín *et al.* 2007

Cycling parameter	Cycles	Temperature	Time
Initial denaturation	1 hold	95°C	15 min
Denaturation	40	94°C	30 s
Annealing		55°C	90 s
Extension		72°C	60 s
Final extension	1 hold	72°C	15 min

°C: degrees Celsius; min: minute; s: second

4.1.6.1.4.2 Gel electrophoresis

A 2% agarose gel was prepared by melting 1.5 g of agarose powder (SeaKem® LE Agarose, Maine, USA) in 75 ml of 1X SB Buffer. Five microlitres of each PCR product was mixed with one microlitre of Novel Juice (GeneDireX Inc., Taoyuan, Taiwan) and pipetted into wells on the agarose gel to separate any PCR product according to size. The samples were electrophoresed alongside a 100 bp DNA ladder (GeneRuler™ 100 bp DNA Ladder, ThermoFisher Scientific) for 30 minutes at 100 V. The agarose gel was then visualised under ultraviolet light at a wavelength of 254 nm using the Platinum HD Gel

Documentation System (UVItec Limited, Cambridge, UK) and the image acquired using the UVIband-1D gel analysis software. PCR products with visible bands of the correct size were selected for further testing downstream.

4.1.6.1.4.3 Purification of PCR products

PCR products were purified using the QIAamp PCR Purification Kit (QIAGEN) by following manufacturer's instructions. Five volumes of binding buffer were first added to one volume of PCR product and the mixture applied to silica gel membrane spin columns to allow efficient binding of the amplified DNA to the column membrane. This was followed by a wash step using 750 µl of an ethanol-containing buffer to ensure the removal of primers and other impurities that could inhibit sequencing downstream. A centrifugation step in an empty tube then ensured the removal of residual ethanol-containing buffer. The DNA was finally eluted in 30 µl of elution buffer containing 10 mM Tris-Cl, pH 8.5.

4.1.6.1.4.4 Sequencing PCR

Sequencing PCR reactions were carried out in a 96-well plate using the two nested PCR primers (Table 4.1-6) to sequence the region of interest, as per manufacturer's instructions. A master mix consisting of the components listed in Table 4.1-11 was prepared and distributed in volumes of 8 µl into the appropriate number of wells. Next, 1 µl of each primer and 1 µl of purified template DNA (20–30 ng) from each sample was added to the appropriate wells. The cycling conditions shown in Table 4.1-12 were used.

Table 4.1-11 Sequencing reaction master mix, adapted from manufacturer's instructions (ThermoFisher Scientific)

Reagent	Volume/µl
ABI Sequencing Buffer	3
Big Dye Terminator	1
Primer, 2 µM	1
Water	3
Total volume	**8**

µl: microliter; µM: micromolar

Table 4.1-12 Cycling parameters for HBV genotyping sequencing reaction, adapted from manufacturer's instructions (ThermoFisher Scientific)

Cycling parameter	Cycles	Temperature	Time
Denaturation	30	96°C	20 s
Annealing		50°C	20 s
Extension		60°C	4 min

°C: degrees Celsius; min: minute; s: second

4.1.6.1.4.5 Sequencing PCR purification

The sequencing PCR reaction products were purified using the BigDye® Xterminator Purification Kit consisting of SAM™ and XTerminator® solutions as per manufacturer's instructions (ThermoFisher Scientific); 45 µl of SAM solution and 10 µl of Xterminator® solution were added to each well before the wells were sealed with adhesive film. The 96-well plate was vortexed for 30 minutes at 2000 RCF and centrifuged for 1 minute at 1000 RCF.

4.1.6.1.4.6 Capillary electrophoresis and sequencing analysis

Capillary electrophoresis was performed on the samples using the ABI Prism 3130XL Genetic Analyzer (ThermoFisher Scientific). The information obtained was converted to raw data files using DNA sequencing analysis software (ThermoFisher Scientific). The length of the capillaries allowed sequence reads of approximately 1 Kb.

The raw trace files were further analysed using Geneious R11 (Biomatters Ltd., Auckland, New Zealand). The quality of each sequence was improved individually by looking at the chromatograms and trimming the ends where necessary. The forward and reverse sequences for each sample were aligned to create a consensus sequence. Mismatches or ambiguities were verified manually. A reference sequence was created using the corresponding primer sequences and used to trim the sequences beyond the primer region.

The consensus sequence was saved in FASTA format before being aligned against the Stanford University HBVSeq sequence database to obtain the HBV genotype (Rhee *et al.*, 2010).

The HBV genotype was further confirmed by phylogenetic analysis and comparing the sample sequences with reference sequences obtained from GenBank.

4.1.6.1.5 Testing for past exposure to aflatoxin

All cases and control liver samples were also tested for past exposure to aflatoxin by testing for the presence of the R249S mutation in exon 7 of the human gene *TP53*, which codes for the tumour

suppressor protein p53, using an established Sanger sequencing protocol developed by the International Agency for Research on Cancer (IARC) (International Agency for Research on Cancer, 2010).

4.1.6.1.5.1 Amplification using PCR

Two separate PCR reactions were carried out using two different sets of primers covering exon 7 (Table 4.1-13).

Table 4.1-13 Primer sequences and amplicon size, International Agency for Research on Cancer, 2010

Name of primer	Sequence	Amplicon size
P-237 F	5′-aggcgcactggcctcatctt-3′	177 bp
P-238 R	5′-tgtgcagggtggcaagtggc-3′	
P-333 F	5′-cttgccacaggtctccccaa-3′	237 bp
P-313 R	5′-aggggtcagaggcaagcaga-3′	

5′: 5 prime; 3′: 3 prime; bp: base pair

PCR master mixes were prepared for the corresponding number of samples and controls according to the primer set being used (Tables 4.1-14 and 4.1-15) using a minimum of 3 ng of DNA. The PCR was carried out using the parameters given in Table 4.1-16 and 4.1-17.

Table 4.1-14 PCR Master mix used with primers P-237 F and P-238 R, International Agency for Research on Cancer, 2010

Components	Volume/µl	Starting concentration	Final concentration
PCR buffer without magnesium chloride	4	5X	1X
Magnesium chloride	1.2	25 mM	1.5 mM
P-237 F	0.8	10 µM	0.4 µM
P-238 R	0.8	10 µM	0.4 µM
Deoxynucleoside triphosphate mix	0.8	5 mM each	0.2 mM each
GoTaq Hot Start DNA polymerase	0.1	5 U/µl	0.5 U/µl
Nuclease-free water	12.3	n/a	n/a
DNA		~ 3 ng	n/a
Total	**20**	n/a	n/a

µl: microlitre; X: times; mM: millimolar; U/µl: unit per microliter; µM: micromolar; ng: nanogram; n/a: not applicable

Table 4.1-15 PCR Master mix used with primers P-333 F and P-313 R, International Agency for Research on Cancer, 2010

Components	Volume/µl	Starting concentration	Final concentration
PCR buffer with 15 mM magnesium chloride	2	10X	1X
Q-Solution	4	5X	1X
Deoxynucleoside triphosphate mix	0.8	5 mM each	0.2 mM each
P-333 F	0.8	10 µM	0.4 µM
P-313 R	0.8	10 µM	0.4 µM
Hot StartTaq DNA polymerase	0.1	5 U/µl	0.5 U/µl
Nuclease-free water	11.5	n/a	n/a
DNA		3 ng	n/a
Total	**20**	n/a	n/a

µl: microlitre; X: times; mM: millimolar; U/µl: unit per microlitre; µM: micromolar; ng: nanogram; n/a: not applicable

Table 4.1-16 PCR cycling parameters with primers P-237 F and P-238 R, International Agency for Research on Cancer, 2010

Cycling parameter	Cycles	Temperature	Time	Special parameters
Initial denaturation	1 hold	94°C	2 min	
Denaturation	20	94°C	30 s	- 0.5°C every 3 cycles
Annealing		63°C	45 s	
Extension		72°C	1 min	
Denaturation	30	94°C	30 s	None
Annealing		60°C	45 s	
Extension		72°C	1 min	
Final extension	1 hold	72°C	10 min	

°C: degrees Celsius; min: minute; s: second

Table 4.1-17 PCR cycling parameters with primers P-333 F and P-313 R, International Agency for Research on Cancer, 2010

Cycling parameter	Cycles	Temperature	Time
Initial denaturation	1 hold	95°C	15 min
Denaturation	50	94°C	30 s
Annealing		60°C	30 s
Extension		72°C	30 s
Final extension	1 hold	72°C	10 min

°C: degrees Celsius; min: minute; s: second

4.1.6.1.5.2 Agarose gel electrophoresis

A 2% agarose gel was prepared by melting 1.5 g of agarose powder (SeaKem® LE Agarose, Maine, USA) in 75 ml of 1X SB Buffer. Five microlitres of each PCR product was mixed with one microlitre of 6X Novel Juice (GeneDireX Inc., Taoyuan, Taiwan) and pipetted into wells on the agarose gel to separate any PCR product according to size. The samples were electrophoresed alongside a 100 bp DNA ladder (GeneRuler™ 100 bp DNA Ladder, ThermoFisher Scientific) for 30 minutes at 100 V. The agarose gel was then visualised under ultraviolet light at a wavelength of 254 nm using the Platinum HD Gel Documentation System (UVItec Limited, Cambridge, UK) and the image acquired using the UVIband-1D gel analysis software. PCR products with visible bands of the correct size were selected for further testing downstream.

4.1.6.1.5.3 Purification of PCR products

The PCR products were purified using the MinElute PCR Purification Kit (QIAGEN) by following manufacturer's instructions. A buffer was first added to the PCR products and the mixture applied to silica gel membrane spin columns to allow efficient binding of the amplified DNA to the column membrane. This was followed by a wash step using an ethanol-containing buffer to ensure the removal of primers and other impurities that could inhibit sequencing downstream. A centrifugation step in an empty tube then ensured the removal of residual ethanol-containing buffer. The DNA was finally eluted in 10 µl of elution buffer containing 10 mM Tris-Cl, pH 8.5.

4.1.6.1.5.4 Sequencing PCR

Sequencing PCRs were carried out in a 96-well plate using the same primers used for PCR amplification reactions (Table 4.1-14). A master mix consisting of the components listed in Table 4.1-19 was prepared and distributed in volumes of 8 µl into the appropriate number of wells. Next, 1 µl of each primer and 1 µl of purified template DNA from each sample was added to the appropriate wells. The cycling conditions shown in Table 4.1-20 were used.

Table 4.1-18 Sequencing PCR master mix, adapted from manufacturer's instructions (ThermoScientific)

Reagent	Volume/µl
ABI Sequencing Buffer	3
Big Dye Terminator	1
Primer, 2 µM	1
Water	3
Total volume	**8**

µl: microlitre; µM: micromolar

Table 4.1-19 Cycling parameters for *TP53* sequencing reaction, adapted from manufacturer's instructions (ThermoScientific)

Cycling parameter	Cycles	Temperature	Time
Denaturation	30	96°C	10 s
Annealing		58°C	5 s
Extension		60°C	2.5 min

°C: degrees Celsius; min: minute; s: second

4.1.6.1.5.5 Sequencing PCR purification

The sequencing PCRs were purified using the BigDye® Xterminator Purification Kit consisting of SAM™ and XTerminator® solutions as per manufacturer's instructions (ThermoScientific); 45 µl of SAM solution and 10 µl of Xterminator® solution were added to each well before the wells were sealed with adhesive film. The 96-well plate was vortexed for 30 minutes at 2000 RCF and centrifuged for 1 minute at 1000 RCF.

4.1.6.1.5.6 Capillary electrophoresis and sequencing data analysis

Capillary electrophoresis was performed on the samples using the ABI Prism 3130XL Genetic Analyzer (ThermoFisher Scientific). The information obtained was converted to raw data files using DNA sequencing analysis software (ThermoFisher Scientific). The length of the capillaries allowed sequence reads of approximately 1 Kb.

The raw trace files were further analysed using Geneious R11 (Biomatters Ltd., Auckland, New Zealand). The quality of each sequence was improved individually by looking at the chromatograms and trimming the ends where necessary. The forward and reverse sequences for each sample were aligned to create a consensus sequence. Mismatches or ambiguities were verified manually. A reference sequence was created using the corresponding primer sequences and was used to trim the sequences beyond the primer region.

The consensus sequence was renamed and saved in FASTA format before being aligned against the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) Human Genome GRCh37.p13. Aflatoxin exposure would cause a non-synonymous G>C transversion that would cause an amino acid change at codon 747 (R249S) on chromosome 17.

4.1.6.1.6 Determination of methylation levels in tumour promoter genes

Pyrosequencing on the Pyromark Q96 ID (QIAGEN) was used to quantitatively analyse the level of DNA methylation in CpG islands in the promoter regions of the following human genes of interest:

RASSF1A, *LINE-1*, and *p16*. This assay was applied to all HCC cases and non-HCC controls included in the present study.

The seven *p16* deoxycytidylyl-deoxyguanosine dinucleotides (CpG) sites targeted in this study were located in the CpG island of the promoter region of exon 1 of the cyclin-dependent kinase Inhibitor 2A (*CDKN2A*) gene located in chromosome 9 of the human genome (Huang, Krocker, Kirk, Merwat, Ju, *et al.*, 2014). The six *RASSF1A* CpG sites targeted in the present study were located in the CpG island of the promoter region of exon 6 of the *RASSF1A* gene located in chromosome 3 of the human genome (Vaissière, Cuenin, Paliwal, Vineis, Hainaut, *et al.*, 2009). Altered CpG island methylation in those genes has been associated with their silencing in cancers (Matsuda, Ichida, Matsuzawa, Sugimura & Asakura, 1999; Merlo, Herman, Mao, Lee, Gabrielson, *et al.*, 1995). The *LINE-1* primers amplified repetitive elements of 146-148 bp present on all chromosomes and contained three CpG sites (Yang *et al.*, 2004). *LINE*-1 was included as there are 0.5 million long interspersed nucleotide elements (LINE-1 elements) that are normally heavily methylated, and it is estimated that more than one-third of DNA methylation occurs in repetitive elements. Thus, the analysis of the *LINE-1* methylation would act as a surrogate marker of global hypomethylation (Yang *et al.*, 2004) that has been reported to contribute to carcinogenesis through its ability to promote genomic instability (Ehrlich, 2002), including hepatocarcinogenesis (Lin *et al.*, 2001).

The gold standard for DNA methylation analysis is bisulfite sequencing where bisulfite treatment of DNA converts unmethylated cytosines to uracil, which is subsequently read as thymine, whereas methylated cytosines resist this conversion and the CG sequence is preserved (Frommer, McDonald, Millar, Collis, Watt, *et al.*, 1992). Several technologies can be used to distinguish between methylated and unmethylated DNA, such as methylation-specific PCR, microarray, or COLD-PCR. However, these methods are expensive, time-consuming, and are limited because they are not quantitative or can only quantify a few CpG sites at a time. Pyrosequencing overcomes these constraints by allowing the simultaneous analysis of multiple CpG sites within a target sequence of up to 200 bp (Kurdyukov & Bullock, 2016). Pyrosequencing also requires a low amount of input DNA (10 ng) for accurate and reproducible analysis. These features make pyrosequencing ideal for FFPE DNA methylation analysis.

The following steps were followed for DNA methylation analysis in *RASSF1A*, *LINE-1*, and *p16*:

1. Bisulfite conversion of sample DNA
2. PCR amplification using primers targeting the region of interest
3. Pyrosequencing

4.1.6.1.6.1 Bisulfite conversion of DNA

Bisulfite conversion was performed on DNA extracted from all cases and controls using the EpiTect® Fast Bisulfite Conversion Kit (QIAGEN) according to the manufacturer's instructions. A master mix was prepared using the components and volumes provided in Table 4.1-20.

Table 4.1-20 Bisulfite reaction master mix according to manufacturer's instructions (QIAGEN)

Reagent	Volume per reaction, µl
Bisulfite Solution	85
DNA Protect Buffer	35
Total volume	**120**

µl: microliter

The amount of input DNA was between 1 ng and 2 µg and the final combined volume of DNA and RNase-free water added to each PCR tube was 20 µl. After DNA addition, the master mix was aliquoted in volumes of 120 µl to each PCR tube. One additional tube was used as a negative control and contained 20 µl of RNase-free water and the master mix. Each PCR tube was closed and vortexed thoroughly to ensure proper mixing and centrifuged briefly to bring the solution to the bottom of the tube. The bisulfite conversion was carried out in a thermocycler using the cycling conditions in Table 4.1-21 with incubation times of 20 minutes to ensure complete bisulfite conversion.

Table 4.1-21 Bisulfite conversion thermal cycling conditions, according to manufacturer's instructions (QIAGEN)

Cycling parameter	Time	Temperature
Denaturation	5 min	95°C
Incubation	20 min	60°C
Denaturation	5 min	95°C
Incubation	20 min	60°C
Hold	Indefinite	20°C

°C: degrees Celsius; min: minute; s: second

4.1.6.1.6.2 Purification of bisulfite-converted DNA

The purification of bisulfite-converted DNA was carried out according to manufacturer's instructions (QIAGEN). The PCR tubes were removed from the thermocycler and centrifuged before their contents were transferred to sterile 1.5 ml microcentrifuge tubes. Carrier-RNA at a final concentration of 10 µg/ml was first added, followed by a buffer promoting binding of the converted single-stranded DNA to the silica gel membrane spin columns. The tubes were mixed by vortexing and centrifuged briefly; 100% ethanol was added and the tubes mixed and vortexed for 15 s and centrifuged briefly to collect the contents at the bottom of the tubes. The contents of each PCR tube were transferred to silica gel membrane spin columns with collection tubes provided with the kit. These columns were then

centrifuged at 15 500 RCF for one minute and the flow-through discarded. The membrane-bound DNA was washed with a buffer to remove residual bisulfite solution and the columns were centrifuged at 15 500 RCF for one minute. A desulfonation buffer was then added and the columns were incubated at room temperature (15–25°C) for 15 minutes. After incubation, the columns were centrifuged at 15 500 RCF for one minute and the flow-through discarded. Two consecutive wash steps were performed each followed by a centrifugation step at 15 500 RCF for one minute. After a final wash step with 100% ethanol and centrifugation at 15 500 RCF for one minute, the columns were placed in new 2 ml collection tubes and centrifuged for a further minute at maximum speed to remove any residual ethanol. Columns were then placed into sterile 1.5 ml microcentrifuge tubes and incubated, with the lids open, at 60°C for five minutes to ensure evaporation of any remaining ethanol. Lastly, the columns were placed in 1.5 ml microcentrifuge tubes and 15 µl of an elution buffer was added to the centre of each column and incubated for one minute before being centrifuged at 15 500 RCF for one minute to elute the DNA. Bisulfite-converted DNA was stored at 2–8°C if it was used within 24 hours or at -20°C if it needed to be stored for longer.

4.1.6.1.6.3 PCR amplification

A master-mix containing HotStarTaq DNA polymerase (QIAGEN), PCR buffer (QIAGEN) with 1.5 mM $MgCl_2$, dNTPs, and two primers was prepared for this PCR. One of the two primers used was biotinylated at its 5′ end in order to prepare a single-stranded PCR product for use in the subsequent pyrosequencing procedure. The amplicon produced was short in length and was less than 200 bp long which is an optimal length for pyrosequencing.

The Pyromark PCR Master Mix (QIAGEN) containing the above-listed components, except for the primers, was recommended for PCR amplification of bisulfite-converted DNA which is a difficult PCR template to use as all unmethylated cytosines would have been converted to uracils, resulting in a less complex DNA template consisting of adenines, guanines, and thymidines/uracils only. The components of this kit ensured that the PCR retained its specificity and sensitivity while preventing the accumulation of biotinylated PCR primer and artefacts that could interfere with the subsequent pyrosequencing step (QIAGEN).

For each primer pair used in this study (Table 4.1-22), trial PCR reactions were first conducted to ensure that the primers were working optimally. Reactions were performed in parallel with and without Q-solution (Table 4.1-23) provided with the kit to determine the effect the Q-solution would have on PCR yield (amplification of a reaction that previously failed, increased efficiency, no effect, or reduced efficiency).

Table 4.1-22 Primers used in PCR reaction prior to pyrosequencing. **p16* primers were obtained from Huang *et al.* 2014, #*RASSF1A* primers from Vaissière *et al.* 2009, and †*LINE-1* primers from Yang *et al.*, 2004

Name of primer	Sequence	Amplicon size
p16-F*	5′- aggggttggttggttattag-3′	75 bp
p16-R*	5′- Biotin-ctacctactctccccctctc-3′	
RASSF1A-F#	5′-agtttggattttgggggagg-3′	136 bp
RASSF1A-R#	5′- Biotin-caactcaataaactcaaactcccc-3′	
LINE-1 F†	5′-ttttgagttaggtgtgggatata-3′	148 bp
LINE-1 R†	5′-aaaatcaaaaaattccctttc-3′	

5′: 5 prime; 3′: 3 prime; bp: base pair

Table 4.1-23 PCR reaction prior to pyrosequencing, according to manufacturer's instructions (QIAGEN)

Reagent	Volume/reaction with Q-solution, µl	Volume/reaction without Q-solution, µl	Final concentration
Pyromark PCR Master Mix 2X	12.5	12.5	1X
Coral load concentrate 10X	2.5	2.5	1X
Q-solution 5X	5.0	0.0	1X
Primer A, 50 µM	0.1	0.1	0.2 µM
Primer B, 50 µM	0.1	0.1	0.2 µM
Total volume	**20.2**	**15.2**	n/a

µl: microlitre; X: times; µM: micromolar; n/a: not applicable

The additional components of the reaction were RNase-free water and the DNA template. These were added separately according to the amount of input bisulfite-converted DNA. RNase-free water was first added to each corresponding well in a PCR plate, followed by 20 ng of bisulfite-converted DNA for a total volume of 4.8 µl for the reactions with Q-solution and 9.8 µl for the reactions without Q-solution. The master mixes were then gently mixed by pipetting up and down before being aliquoted into the PCR wells. An adhesive film cover was then used to cover the wells of the plate. The plate was centrifuged at 1000 RCF for one minute and placed in the thermocycler. The PCR reactions were then performed according to the thermal cycling conditions in Table 4.1-24 to Table 4.1-26. Two-fold serial dilutions were also performed on a sample of known high DNA concentration to determine the minimum amount of DNA required for successful PCR reactions for each primer pair.

Table 4.1-24 Thermal conditions for *p16* PCR reaction prior to pyrosequencing, Huang *et al.* 2014 (adapted)

Step		Time	Temperature
Enzyme activation step		15 min	95°C
Denaturation	45 cycles	30 s	94°C
Annealing*		30 s	56°C
Extension		30 s	72°C
Final extension		10 min	72°C

°C: degrees Celsius; min: minute; s: second; *p16:* cyclin-dependent kinase inhibitor 2A/inhibitor of CDK4

Table 4.1-25 Thermal conditions for *RASSF1A* PCR reaction prior to pyrosequencing, Vaissière *et al.* 2009 (adapted)

Step		Time	Temperature
Enzyme activation step		15 min	95°C
Denaturation	45 cycles	30 s	94°C
Annealing*		30 s	60°C
Extension		30 s	72°C
Final extension		10 min	72°C

°C: degrees Celsius; min: minute; s: second

Table 4.1-26 Thermal conditions for *LINE-1* PCR reaction prior to pyrosequencing adapted from Yang *et al.*, 2004

Step		Time	Temperature
Enzyme activation step		15 min	95°C
Denaturation	45 cycles	30 s	94°C
Annealing*		30 s	50°C
Extension		30 s	72°C
Final extension		10 min	72°C

°C: degrees Celsius; min: minute; s: second

4.1.6.1.6.4 Pyrosequencing of PCR products

Pyrosequencing uses sequencing-by-synthesis and detection of visible light by a charged coupled device camera attached to the pyrosequencer. After PCR amplification of the region of interest, the amplicon was denatured into single-stranded form to which a sequencing primer was annealed. Nucleotides were added in a predetermined order in each pyrosequencing cycle. Complementary nucleotides were incorporated into the DNA strand releasing pyrophosphate which was converted to ATP by ATP

sulfurylase in the presence of adenosine 5' phosphosulfate. The ATP produced was then used to convert luciferin to oxyluciferin by luciferase, generating visible light. The amount of light generated was proportional to the number of nucleotides incorporated. The results were then displayed in the form of peaks in a pyrogram with an average methylation level for each CpG site being analysed (Choudhuri, 2014). The methylation detection limit at individual CpG sites was approximately 5% (Mikeska, Felsberg, Hewitt & Dobrovic, 2011; Wu, Yang, Wang, Chen & Santella, 2017).

The first time each assay was performed, the following controls were included to ensure that none of the reagents and primers being used would create background amplification and signals that would compromise the results obtained during pyrosequencing of the samples included in the present study:

1. A non-template PCR control to verify that the PCR primers were not interacting and to verify there was no DNA contamination from an external source.

2. A PCR control with template DNA but no sequencing primer to verify that the template was not looping back on itself.

3. Sequencing primer without any PCR product to verify that the sequencing primer was not forming duplexes or hairpins.

4. Biotinylated primer without any PCR product to verify that the biotinylated primer was not forming duplexes or hairpins.

5. Sequencing primer and biotinylated primer together without PCR product to verify that the sequencing primer and the biotinylated primer were not forming.

4.1.6.1.6.4.1 Pyrosequencing assay setup

The pyrosequencing assay was first set up on the computer linked to the Pyromark Q96 ID using the Pyromark Q96 ID Version 2.5.10 software (QIAGEN). Each assay had a “Sequence to Analyse” which corresponded to the CpG sites being analysed after bisulfite treatment within the gene of interest (Table 4.1-27) and which was kindly provided by collaborators at the Mailman School of Public Health, Department of Environmental Health Sciences, Columbia University in the City of New York (Prof Regina Santella, personal communication). Based on this sequence, a nucleotide dispensation order was automatically generated by the software. A bisulfite conversion control was also inserted within the Sequence to Analyse to ensure completeness of the bisulfite conversion process performed prior to the PCR reaction. Three different assays were prepared for *p16*, *RASSF1A*, and *LINE-1*, respectively. A virtual plate corresponding to the wells to be analysed was created for each reaction.

Table 4.1-27 "Sequence to Analyse" for genes of interest, Prof Regina Santella (personal communication)

Gene	Sequence to Analyse
RASSF1A	YGTTYGGTTYGYGTTTGTTAGYGTTTAAAGTTAGYG
P16	GGGGYGGATYGYGTGYGTTYGGYGGTTGYGGAGA
LINE-1	TTYGTGGTGYGTYGTTT

P16: cyclin-dependent kinase inhibitor 2A/inhibitor of CDK4; *LINE-1:* Long Interspersed Nuclear Elements-1; *RASSF1A*: Ras association domain family 1 isoform A; YG: CpG site after bisulfite conversion, Y could be a T or a C

4.1.6.1.6.4.2 Immobilisation of PCR product to sepharose beads

Biotinylated PCR products were immobilized on streptavidin-coated Sepharose beads (GE Healthcare, Chicago, Illinois) as per manufacturer's instructions (QIAGEN). A master mix of streptavidin-coated Sepharose beads, binding buffer, and high-purity water was prepared (Table 4.1-28) for use with 20 µl of biotinylated PCR product and aliquoted in volumes of 60 µl into the wells of a PCR plate (QIAGEN).

Table 4.1-28 Master mix for immobilisation of PCR product to sepharose beads, as per manufacturer's instructions (QIAGEN)

Reagent	**Volume per reaction, µl**
Streptavidin-coated Sepharose beads	1.5 µl
PyroMark Binding Buffer	40 µl
High-purity water	Variable

µl: microlitre

The PCR plate was then sealed with an adhesive film and agitated for at least 10 minutes at 1400 rpm using a mixer.

4.1.6.1.6.4.3 PCR product strand separation

During the shaking step, a master mix with sequencing primer (Table 4.1-29) in annealing buffer was prepared (Table 4.1-30) and aliquoted in volumes of 40 µl into wells of a PyroMark Q96 Plate Low (QIAGEN) corresponding to the PCR plate, all as per manufacturer's instructions (QIAGEN).

Table 4.1-29 Pyrosequencing primer sequence, *p16* sequencing primer from Huang *et al.* 2014, *RASSF1A* sequencing primer from Vaissière *et al.* 2009, and *LINE-1* sequencing primer from Yang *et al.*, 2004

Name of primer	**Sequence**
p16-S	5′-ggttggttattagagggt-3′
RASSF1A-S	5′-gggttagttttgtggttt-3′

LINE-1 S	5′-agttaggtgtgggatatagt-3′

5′: 5 prime; 3′: 3 prime; bp: base pair; *p16:* cyclin-dependent kinase inhibitor 2A/inhibitor of CDK4; *RASSF1A*: Ras association domain family 1 isoform A; *LINE-1:* Long Interspersed Nuclear Elements-1

Table 4.1-30 Master mix for annealing primer, as per manufacturer's instructions (QIAGEN)

Reagent	Initial concentration	Final Concentration	Volume per reaction, µl
Annealing Buffer			38.4 µl
Sequencing primer	10 µM	0.4 µM	1.6 µl
Total volume			**40** µl

µl: microlitre; µM: micromolar

Following manufacturer's instructions, the vacuum workstation was prepared for the next step, strand separation. The five different troughs of the vacuum workstation (Figure 4.1-3) were filled with the following different solutions:

1. Approximately 110 ml of 70% ethanol (Position 1)
2. Approximately 90 ml Denaturation Solution (Position 2)
3. Approximately 110 ml Wash Buffer (Position 3)
4. Approximately 110 ml high-purity water (Position 4)
5. Approximately 180 ml high-purity water (Parking Position, "P")

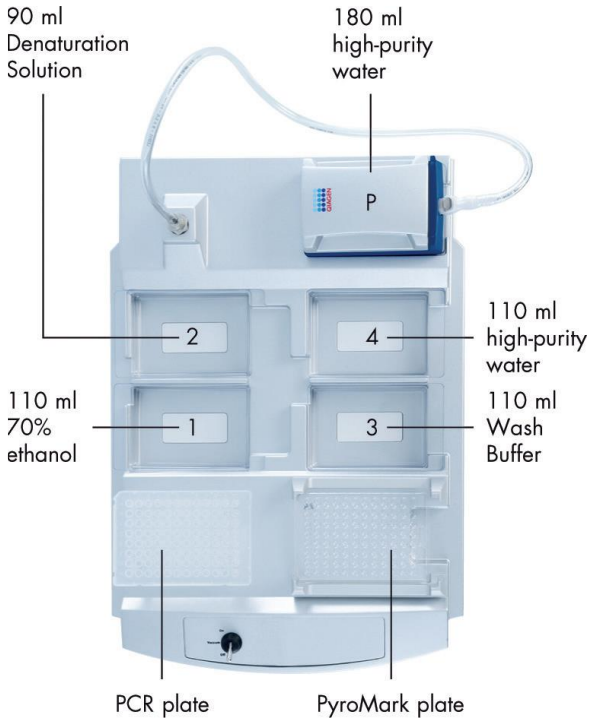


Figure 4.1-3 Vacuum workstation with different working solutions for strand separation
ml: millilitre; %: per cent

The vacuum pump was switched on and the filter probes flushed with 180 ml Milli-Q water. After shaking, the PCR plate was placed onto the workstation (Figure 4.1-3). The tool with the filter probes was lowered into the PCR plate to capture the beads with the immobilized template. The tool was then transferred to trough 1 with 70% ethanol to wash residual salts and unlabelled DNA, then to trough 2 with denaturing solution to denature the DNA to single-stranded form and finally to trough 3 with wash buffer to neutralise the denaturing solution and allow pyrosequencing to proceed at the right conditions. The tool was then raised beyond 90° vertical for 5 s to drain all the liquid from the filter probes. The probes were aligned to the Pyromark Q96 Plate Low (QIAGEN), the vacuum switched off and the probes lowered into the wells and shaken vigorously to transfer the samples.

4.1.6.1.6.4.4 Annealing of sequencing primer

The Pyromark Q96 Plate Low (QIAGEN) was then placed onto a heating block at 80°C for two minutes to eliminate any secondary structure in the single-stranded template that could interfere with primer annealing or enzymatic addition of nucleotides. It was then allowed to cool to room temperature (15–25°C) for at least 5 min.

4.1.6.1.6.4.5 Preparing the pyrosequencer

The appropriate volumes of enzyme, substrate, and nucleotides were obtained from the Pyromark Q96 ID Version 2.5.10 software (QIAGEN). These reagents were pipetted into a cartridge using filter-free tips. The cartridge was loaded onto the Pyromark Q96 ID instrument.

4.1.6.1.6.4.6 Performing pyrosequencing reaction

After the cool-down period, the Pyromark Q96 Plate Low (QIAGEN) was placed onto the Pyromark Q96 ID instrument (QIAGEN) and the pyrosequencing reaction was started.

4.1.6.1.6.4.7 Pyrosequencing results analysis

The results of the pyrosequencing reaction were analysed in CpG mode using the PyroMark Q96 Version 2.5.10 software (QIAGEN). A pyrogram with peaks corresponding to the sequence of interest was generated. The methylation levels for each targeted CpG site were evaluated by converting the peak heights of the pyrograms to numerical values and expressed either as the percentage of methylation for individual CpG sites or as the mean of all CpG sites analysed in the region of interest. Quality control verifications were performed by the Pyromark software to determine the reliability of the results. Non-CpG sites were used as reference peaks and compared to the expected peak heights generated from the original Sequence to Analyse input into the assay file. Typically, CpG sites that passed the quality control stage were marked in blue, those that deviated from expected peak heights were indicated in yellow, and CpG sites that failed were assigned red. Pyrograms that failed the quality control checks were verified individually to determine if the sample needed to be retested.

4.1.6.2 Histological testing

4.1.6.2.1 Determination of cirrhosis status

Two methods were used to determine if cirrhosis was observed on the tissue sections. Firstly, histopathological reports associated with each liver sample were reviewed to determine if a diagnosis of cirrhosis was recorded. Secondly, sample sections were stained with Sirius Red and were reviewed histologically by an experienced consultant anatomical pathologist to determine the level of fibrosis (Sweat, Puchtler & Rosenthal, 1964).

4.1.7 Post-testing phase – statistical analyses

Statistical analyses were performed using SPSS Statistics for Windows, Version 25.0 (IBM Corp., New York, USA). A p value of less than 0.05 was considered statistically significant. Ages were reported as mean ± SD. The methylation data were analysed as reported previously (Lambert *et al.*, 2011). The hypermethylation frequency in the HCC group was calculated based on the number of HCC samples with methylation levels above the 95th percentile observed in the non-HCC group.

Cross-tabulations with frequency tables were first used to determine significance of all variables tested in the present study. Subsequently, the Mann-Whitney U test was used to determine the significance of the differences between cases and controls for non-parametric data. Logistic regression analysis was performed to verify whether any of the risk factors (HBsAg positivity, HBV genotype, aflatoxin exposure, promoter hypermethylation levels in *p16, RASSF1A,* and *LINE-1*) or clinical characteristics (sex, age, race, and cirrhosis status) could be predictors of HCC. Univariate logistic regressions were first performed to identify potential confounding variables that were then included in a multivariate model. A backward stepwise approach was used to build a parsimonious multivariate model; independent variables identified from the univariate model were removed in a stepwise manner from the full model and the fit of the new model tested at each step. The best predictive variables and their corresponding predicted probabilities were used to calculate sensitivity, specificity, NPV, and PPV. No corrections for multiple testing were performed in the present study because it was considered to be too conservative for two reasons. First, all the comparisons that were performed in the present study were pre-specified in the protocol. Second, this was an exploratory study and did not have sufficient power to apply correction for multiple testing.

4.2 RESULTS

4.2.1 Selection of cases and controls

From a pool of 87 potential cases and 119 potential controls histologically diagnosed from January 1995 to August 2017, 34 cases and 33 controls fitted the inclusion criteria and were included in the present study. After DNA extraction from all FFPE liver tissues, one case and one control were further excluded from the study as the RNase P assay showed that they had unamplifiable DNA.

4.2.2 Demographics of cases and controls

A comparison of the demographics of unmatched cases and controls is given in Table 4.2-1. Mean age was found to be different between the case and control groups and this was statistically significant.

Table 4.2-1 Comparison of demographics of cases and controls included in study

	Cases ***n* = 33**	**Controls** ***n* = 32**	**p-value**
Age, Mean ± SD	42.09 ± 15.37	54.53 ± 13.01	0.001*
Sex			
Female	10 (30.3%)	16 (50%)	0.105
Male	23 (69.7%)	16 (50%)	
Race			
Black	8 (24.2%)	7 (21.9)	0.896
Coloured	5 (15.2%)	5 (15.6)	
Caucasian	6 (18.2%)	6 (18.7)	
Indian	1 (3.0%)	0 (0.0)	
Unknown	13 (39.4%)	14 (43.8)	

*: level of significance set at $p < 0.05$; SD: Standard deviation; %: per cent; n: number

4.2.3 HBV status retrieved from medical records

Of the 33 HCC cases selected for the present study, only two had records of prior HBV testing. One was positive for HBV infection and the other was negative for HBV infection. Of the 32 controls, none had a medical record of previous HBV testing.

All cases and controls were subjected to HBsAg testing by real-time PCR. Of the 33 HCC cases, 25 (75.8%) were positive for HBsAg (including the case that was known to be HBV-positive) and of the 32 non-HCC controls, 10 (31.3%) were positive for HBsAg.

4.2.4 Results from molecular testing of cases and controls

Table 4.2.2 shows the hypermethylation cut-offs calculated for each CpG site in *RASSF1A* and *p16*.

Table 4.2-2 95th percentile hypermethylation cut-off

***p16* CpG site**	**95th percentile (hypermethylation cut-off)**
CpG site 1	6.8%
CpG site 2	6.0%
CpG site 3	2.4%
CpG site 4	0.0%
CpG site 5	6.0%
CpG site 6	6.0%
CpG site 7	9.8%
Overall % methylation	2.8%
***RASSF1A* CpG site**	**95th percentile (hypermethylation cut-off)**
CpG site 1	64.4%
CpG site 2	68.4%
CpG site 3	60.2%
CpG site 4	47.4%
CpG site 5	51.8%
CpG site 6	62.6%
Overall % methylation	51.8%

p16: cyclin-dependent kinase inhibitor 2A/inhibitor of CDK4; *RASSF1A*: Ras association domain family 1 isoform A; CpG: deoxycytidylyl-deoxyguanosine dinucleotides

Molecular testing showed that HBsAg status, *p16* hypermethylation levels at CpG sites 2, 4-6, and *RASSF1A* overall hypermethylation levels as well as hypermethylation levels at CpG sites 1, 3-6, were significantly different between the case and control groups (Table 4.2-3). The distribution of the methylation percentages observed in all cases and controls is shown using the box-whisker plots (Figures 4.2-1 and 4.2-2).

Table 4.2-3 Comparison of results from molecular testing between the HCC and non-HCC groups

	HCC group *n* = 33	Non-HCC group *n* = 32	p-value
HBsAg status			
Positive	25 (75.8%)	10 (31.3%)	0.0005*
Negative	8 (24.2%)	22 (68.8%)	
Aflatoxin exposure	***n* = 33**	***n* = 32**	
Yes	1 (3.0%)	0 (0.0%)	0.297
No	30 (90.9%)	27 (84.4%)	
Unknown	2 (6.1%)	5 (15.6%)	
***p16* hypermethylation**	***n* = 33**	***n* = 31**	
CpG site 1	6 (18.2%)	1 (3.2%)	0.105
CpG site 2	6 (18.2%)	0 (0.0%)	0.025*
CpG site 3	7 (21.2%)	1 (3.2%)	0.054
CpG site 4	8 (24.2%)	0 (0.0%)	0.005*
CpG site 5	6 (18.2%)	0 (0.0%)	0.025*
CpG site 6	6 (18.2%)	0 (0.0%)	0.025*
CpG site 7	6 (18.2%)	1 (3.2%)	0.105
Overall % methylation	6 (18.2%)	1 (3.2%)	0.105
***RASSF1A* hypermethylation**	***n* = 31**	***n* = 31**	
CpG site 1	8 (25.8%)	1 (3.2%)	0.026*
CpG site 2	6 (19.4%)	1 (3.2%)	0.104
CpG site 3	11 (35.5%)	1 (3.2%)	0.003*
CpG site 4	14 (45.2%)	1 (3.2%)	< 0.01*
CpG site 5	11 (35.5%)	1 (3.2%)	0.003*
CpG site 6	10 (32.3%)	1 (3.2%)	0.006*
Overall % methylation	14 (45.2%)	1 (3.2%)	< 0.01*

*: level of significance set at $p < 0.05$; CpG: deoxycytidylyl-deoxyguanosine dinucleotides; %: per cent; *p16:* cyclin-dependent kinase inhibitor 2A/inhibitor of CDK4; *RASSF1A*: Ras association domain family 1 isoform A

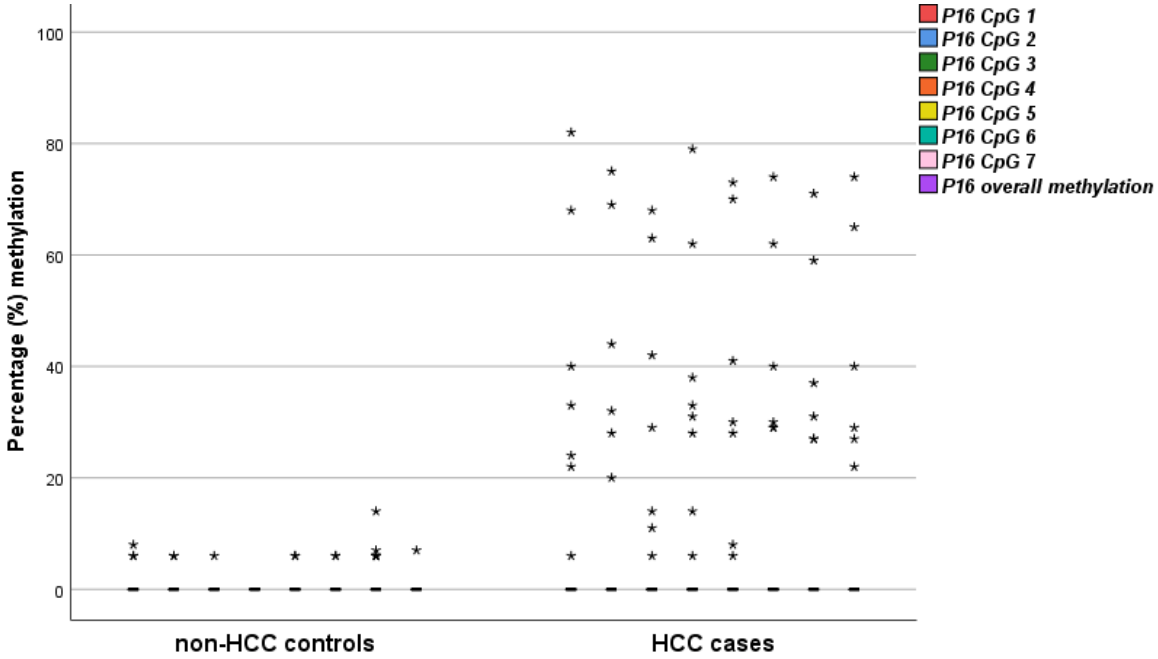


Figure 4.2-1 Box-whisker plot demonstrating distribution of percentage (%) methylation at the different *p16* CpG sites in cases and controls The median, 25th percentile, and 75th percentile were all zero. *p16*: Cyclin-dependent kinase inhibitor 2A (CDKN2A)/inhibitor of CDK4

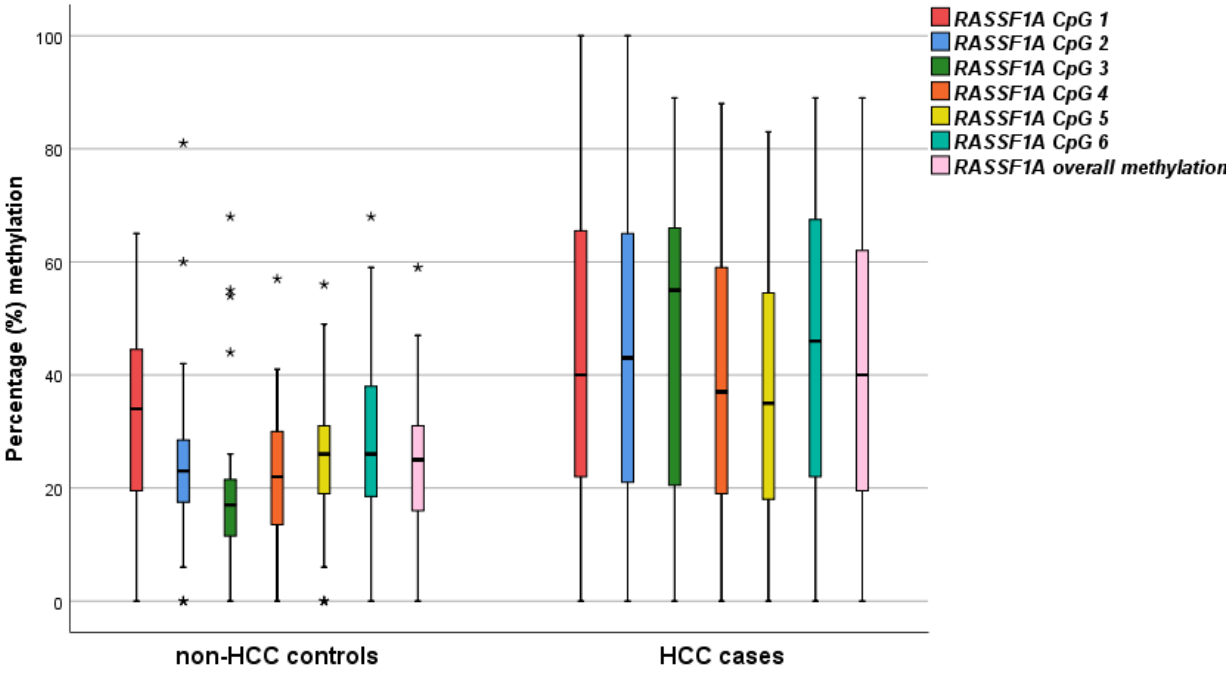


Figure 4.2-2 Box-whisker plot demonstrating distribution of percentage (%) methylation at the different *RASSF1A* CpG sites in cases and controls *RASSF1A*: Ras association domain family 1 isoform A

4.2.5 Results from comparisons of HBsAg positive cases and controls

Sub-group analyses were conducted between HBsAg positive cases and controls. As none of the HBsAg positive controls belonged to genotype A, the odds ratio could not be calculated. Hence, one control of unknown HBV genotype was reclassified as belonging to genotype A to remove the zero frequency.

Significant differences in age, HBV genotype, and overall promoter methylation in *RASSF1A* as well as at CpG sites 3–5 were observed between the two groups (Tables 4.2-4 and 4.2-5).

Table 4.2-4 Comparison of demographics of HBsAg positive cases and controls included in study

	HBsAg +ve cases *n* = 25	HBsAg +ve controls *n* = 10	p-value
Age, Mean ± SD	41.40 ± 15.17	55.60 ± 16.02	0.019*
Sex			
Female	7 (28.0%)	4 (40.0%)	0.689
Male	18 (72.0%)	6 (60.0%)	
Race			
Black	5 (20.0%)	4 (40.0%)	0.636
Coloured	4 (16.0%)	1 (10.0%)	
Caucasian	3 (12.0%)	2 (20.0%)	
Indian	1 (4.0%)	0 (0.0%)	
Unknown	12 (48.0%)	3 (30.0%)	

*: level of significance set at $p < 0.05$; SD: Standard deviation; %: per cent; +ve: positive; -ve: negative

Table 4.2-5 Comparison of molecular testing results of HBsAg positive cases and controls included in study

	HBsAg +ve cases *n* = 25	HBsAg +ve controls *n* = 10	p-value
HBV genotype			
A	9 (36%)	1 (10%)	0.012*
D/E	3 (12%)	6 (60%)	
Unknown	13 (52%)	3 (30%)	
Aflatoxin exposure	***n* = 25**	***n* = 10**	
Yes	1 (4%)	0 (0%)	0.804
No	22 (88%)	9 (90%)	
Unknown	2 (8%)	1 (10%)	
***p16* hypermethylation**	***n* = 25**	***n* = 10**	
CpG site 1	4 (16%)	1 (10%)	1.000
CpG site 2	4 (16%)	1 (10%)	1.000
CpG site 3	5 (20%)	0 (0%)	0.292
CpG site 4	5 (20%)	1 (10%)	0.649
CpG site 5	4 (16%)	1 (10%)	1.000
CpG site 6	4 (16%)	1 (10%)	1.000
CpG site 7	4 (16%)	0 (0%)	0.303
Overall % methylation	4 (16%)	1 (10%)	1.000
***RASSF1A* hypermethylation**	***n* = 23**	***n* = 10**	
CpG site 1	6 (26.1%)	0 (0.0%)	0.145
CpG site 2	5 (21.7%)	0 (0.0%)	0.291
CpG site 3	9 (39.1%)	0 (0.0%)	0.032*
CpG site 4	11 (47.8%)	0 (0.0%)	0.013*
CpG site 5	9 (39.1%)	0 (0.0%)	0.032*
CpG site 6	8 (34.8%)	0 (0.0%)	0.071
Overall % methylation	11 (47.8%)	0 (0.0%)	0.013*

*: level of significance set at $p < 0.05$; %: per cent; +ve: positive; HBV: hepatitis B virus; *p16:* cyclin-dependent kinase inhibitor 2A/inhibitor of CDK4; *RASSF1A*: Ras association domain family 1 isoform A; CpG: CpG: deoxycytidylyl-deoxyguanosine dinucleotides

4.2.6 Results from logistic regression analyses

Univariate analyses showed that none of the *p16* CpG sites were significant predictors of HCC (Table 4.2-6). However, when adjusted for HBV status and age, *p16* CpG 4 was a significant predictor of HCC (OR: 10.905, 95% CI: 1.113–106.821) and had an improved sensitivity of 84.8%, specificity of 67.7%, PPV of 73.7%, and NPV of 80.8% (Table 4.2-8).

Univariate analyses showed that *RASSF1A* CpG sites 1, 3-6 and overall percentage methylation of the promoter region of *RASSF1A* were significant predictors of HCC ($p < 0.05$ for all) (Table 4.2-7), although they had poor sensitivities as HCC predictors (19.4%–45.2%) (Table 4.2-8). After controlling for HBV status, these variables were still significant predictors of HCC and had improved sensitivities (77.4%–83.9%) (Table 4.2-8).

The sub-group univariate analyses in HBsAg positive cases and controls showed that age and HBV genotype, in particular, HBV genotype A, were significant predictors for HCC (Table 4.2-9). Logistic regression analyses could not be performed on the *RASSF1A* methylation data as there was a zero frequency in the control group (none of the HBsAg positive controls were hypermethylated) and the sample size was too small ($n = 10$) to move one of the non-hypermethylated controls to the hypermethylated group to remove this zero frequency (Appendix F).

Table 4.2-6 Univariate and multivariate logistic regression analyses for demographics and *p16* hypermethylation

Variable	Cases *n* = 33	Controls *n* = 31	Crude OR (95% CI)	Significance	OR (95% CI)*	Significance
Age	-	-	0.941 (0.905–0.979)	0.002*	0.942 (0.903–0.983)	0.006*
HBsAg status	-	-	6.875 (2.307–20.490)	0.001*	-	-
Hypermethylation status						
***p16* CpG site 1**						
Negative	27 (81.8%)	30 (96.8%)	1.0	0.088	1.0	0.119
Positive	6 (18.2%)	1 (3.2%)	6.667 (0.754–58.970)		6.332 (0.621–64.525)	
***p16* CpG site 2**						
Negative	27 (81.8%)	30 (96.8%)	1.0	0.088	1.0	0.119
Positive	6 (18.2%)	1 (3.2%)	6.667 (0.754–58.970)		6.332 (0.621–64.525)	
***p16* CpG site 3**						
Negative	26 (78.8%)	30 (96.8%)	1.0	0.058	1.0	0.050
Positive	7 (21.2%)	1 (3.2%)	8.077 (0.931–70.043)		10.113 (1.002–102.023)	
***p16* CpG site 4**						
Negative	25 (75.8%)	30 (96.8%)	1.0	0.039	1.0	0.040*
Positive	8 (24.2%)	1 (3.2%)	9.600 (1.123–82.048)		10.905 (1.113–106.821)	
***p16* CpG site 5**						
Negative	27 (81.8%)	30 (96.8%)	1.0	0.088	1.0	0.119
Positive	6 (18.2%)	1 (3.2%)	6.667 (0.754–58.970)		6.332 (0.621–64.525)	
***p16* CpG site 6**						
Negative	27 (81.8%)	30 (96.8%)	1.0	0.088	1.0	0.119
Positive	6 (18.2%)	1 (3.2%)	6.667 (0.754–58.970)		6.332 (0.621–64.525)	
***p16* CpG site 7**						
Negative	27 (81.8%)	30 (96.8%)	1.0	0.088	1.0	0.062
Positive	6 (18.2%)	1 (3.2%)	6.667 (0.754–58.970)		9.367 (0.897–97.787)	
***p16* overall methylation**						
Negative	27 (81.8%)	30 (96.8%)	1.0		1.0	0.062
Positive	6 (18.2%)	1 (3.2%)	3.222 (0.598–17.358)	0.173	9.367 (0.897–97.787)	

p16: cyclin-dependent kinase inhibitor 2A/inhibitor of CDK4; OR: odds ratio; CI: confidence interval *adjusted for HBsAg status

Table 4.2-7 Univariate and multivariate logistic regression analyses for *RASSF1A* hypermethylation status

Hypermethylation status	Cases *n* = 31	Controls *n* = 31	Crude OR (95% CI)	Significance	OR (95% CI)*	Significance
***RASSF1A* CpG site 1**						
Negative	23 (74.2%)	30 (96.8%)	1.0	0.032*	1.0	0.036*
Positive	8 (25.8%)	1 (3.2%)	10.435 (1.217–89.461)		11.318 (1.168–109.636)	
***RASSF1A* CpG site 2**						
Negative	25 (80.6%)	30 (96.8%)	1.0	0.076	1.0	0.108
Positive	6 (19.4%)	1 (3.2%)	7.200 (0.812–63.854)		6.627 (0.659–66.663)	
***RASSF1A* CpG site 3**						
Negative	20 (64.5%)	30 (96.8%)	1.0	0.010*	1.0	0.018*
Positive	11 (35.5%)	1 (3.2%)	16.500 (1.973–137.996)		14.463 (1.595–131.173)	
***RASSF1A* CpG site 4**						
Negative	17 (54.8%)	30 (96.8%)	1.0	0.003*	1.0	0.005*
Positive	14 (45.2%)	1 (3.2%)	24.706 (2.983–204.639)		23.043 (2.568–206.766)	
***RASSF1A* CpG site 5**						
Negative	20 (64.5%)	30 (96.8%)	1.0	0.010*	1.0	0.018*
Positive	11 (35.5%)	1 (3.2%)	16.500 (1.973–137.996)		14.463 (1.595–131.173)	
***RASSF1A* CpG site 6**						
Negative	21 (67.7%)	30 (96.8%)	1.0	0.014*	1.0	0.022*
Positive	10 (32.3%)	1 (3.2%)	14.286 (1.698–120.203)		13.315 (1.441–123.037)	
***RASSF1A* overall** methylation						
Negative	17 (54.8%)	30 (96.8%)	1.0	0.003*	1.0	0.005*
Positive	14 (45.2%)	1 (3.2%)	24.706 (2.983–204.639)		23.043 (2.568–206.766)	

RASSF1A: Ras association domain family 1 isoform A; OR: odds ratio; CI: confidence interval *adjusted for HBsAg status

Table 4.2-8 Sensitivity and specificity of variables in discriminating between HCC and non-HCC cases

Variable	Sensitivity	Specificity	PPV	NPV	Sensitivity*	Specificity*	PPV*	NPV*
HBsAg status	75.8%	68.8%	71.4%	73.3%	-	-	-	-
p16								
CpG site 1	18.2%	96.8%	85.7%	52.6%	81.8%	67.7%	73.0%	77.8%
CpG site 2	18.2%	100.0%	100.0%	53.4%	81.8%	67.7%	73.0%	77.8%
CpG site 3	21.2%	96.8%	87.5%	53.6%	81.8%	64.5%	71.1%	77.0%
CpG site 4	24.2%	100.0%	100.0%	55.4%	84.8%	67.7%	73.7%	80.8%
CpG site 5	18.2%	100.0%	100.0%	53.4%	81.8%	67.7%	73.0%	77.8%
CpG site 6	18.2%	100.0%	100.0%	53.4%	81.8%	67.7%	73.0%	77.8%
CpG site 7	18.2%	96.8%	85.7%	52.6%	81.8%	64.5%	71.1%	77.0%
Overall methylation	18.2%	96.8%	85.7%	52.6%	81.8%	64.5%	71.1%	77.0%
RASSF1A								
CpG site 1	25.8%	96.8%	88.9%	56.6%	80.6%	64.5%	69.4%	77.0%
CpG site 2	19.4%	96.8%	85.7%	54.5%	77.4%	64.5%	68.6%	74.1%
CpG site 3	35.5%	96.8%	91.7%	60.0%	80.6%	64.5%	69.4%	77.0%
CpG site 4	45.2%	96.8%	93.3%	63.8%	83.9%	64.5%	74.3%	80.0%
CpG site 5	35.5%	96.8%	91.7%	60.0%	80.6%	64.5%	69.4%	77.0%
CpG site 6	32.3%	96.8%	90.9%	58.8%	80.6%	64.5%	69.4%	77.0%
Overall methylation	45.2%	96.8%	93.3%	63.8%	83.9%	64.5%	74.3%	80.0%

PPV: positive predictive value; NPV: negative predictive value; HBsAg: hepatitis B surface antigen; *p16:* cyclin-dependent kinase inhibitor 2A/inhibitor of CDK4; *RASSF1A*: Ras association domain family 1 isoform A; CpG: CpG: deoxycytidylyl-deoxyguanosine dinucleotides. *adjusted for HBsAg status

Table 4.2-9 Univariate and multivariate logistic regression analyses for age, genotype, and *p16* hypermethylation status in HBsAg positive cases and controls

	Cases *n* = 25	Controls *n* = 10	Crude OR (95% CI)	Significance	OR (95% CI)*	Significance
HBV genotype						
A	9	1	18.000 (1.496–216.620)	0.023*	-	-
Age	25	10	0.943 (0.895–0.994)	0.030*	0.963 (0.904–1.025)	0.236
Hypermethylation status						
***p16* CpG site 1**						
Negative	21 (84%)	9 (90%)	1.0	0.650	1.0	0.374
Positive	4 (16%)	1 (10%)	1.714 (0.167–17.554)		3.601 (0.214–60.588)	
***p16* CpG site 2**						
Negative	21 (84%)	9 (90%)	1.0	0.650	1.0	0.374
Positive	4 (16%)	1 (10%)	1.714 (0.167–17.554)		3.601 (0.214–60.588)	
***p16* CpG site 3**						
Negative	20 (80%)	10 (100%)	1.0	-	1.0	-
Positive	5 (20%)	0 (0%)	-		-	
***p16* CpG site 4**						
Negative	20 (80%)	9 (90%)	1.0	0.487	1.0	0.309
Positive	5 (20%)	1 (10%)	2.250 (0.229–22.144)		4.110 (0.270–62.477)	
***p16* CpG site 5**						
Negative	21 (84%)	9 (90%)	1.0	0.650	1.0	0.374
Positive	4 (16%)	1 (10%)	1.714 (0.167–17.554)		3.601 (0.214–60.588)	
***p16* CpG site 6**						
Negative	21 (84%)	9 (90%)	1.0	0.650	1.0	0.374
Positive	4 (16%)	1 (10%)	1.714 (0.167–17.554)		3.601 (0.214–60.588)	
***p16* CpG site 7**						
Negative	21 (84%)	10 (100%)	1.0	-	1.0	-
Positive	4 (16%)	0 (0%)	-		-	
***p16* overall methylation**						
Negative	21 (84%)	9 (90%)	1.0	0.650	1.0	0.374
Positive	4 (16%)	1 (10%)	1.714 (0.167–17.554)		3.601 (0.214–60.588)	

p16: Cyclin-dependent kinase inhibitor 2A (CDKN2A)/inhibitor of CDK4; *RASSF1A*: Ras association domain family 1 isoform A; HBV: hepatitis B virus; OR: odds ratio *adjusted for HBV genotype

4.3 DISCUSSION

The present case-control study sought to identify biological predictors of HCC, including viral (HBsAg and HBV genotype), genetic (methylation levels of promoter regions of *p16* and *RASSF1A*), and environmental (aflatoxin exposure) biomarkers. The most important study findings were that HBsAg, methylation levels in *RASSF1A*, and HBV genotype A were significant predictors of HCC that could potentially be used to develop prediction models for predicting HCC risk, as has been done previously in Asia (Hsu *et al.*, 2018; Wong *et al.*, 2010; Yang *et al.*, 2011; Yuen *et al.*, 2009).

4.3.1 Variables with diagnostic potential

Univariate logistic regression analysis identified HBsAg status, age, hypermethylation of *RASSF1A* CpG sites 1, 2–4 and overall hypermethylation of *RASSF1A* as significant predictors of HCC ($p < 0.05$). *RASSF1A* hypermethylation at all CpG sites as well as overall *RASSF1A* hypermethylation were still significant after adjusting for HBsAg status.

These variables (except age) were then used in multivariate logistic regression models to identify a model with the best sensitivity, specificity, NPV, and PPV for HCC diagnosis. Age was excluded from the model because control tissues were obtained from older patients with primary colon cancer which would have already skewed the age of the control population included in the present study. The best predictive model was obtained using the variables overall promoter hypermethylation of *RASSF1A* and HBsAg status (sensitivity: 83.9%; specificity: 64.5%; PPV: 74.3%; NPV: 80.0%). These values demonstrate the improved overall diagnostic potential of combining both variables, although further work is needed to verify this finding.

While early detection of HCC is desirable to improve survival outcomes, this is currently hampered by the lack of available screening biomarkers (Bruix & Sherman, 2005). In the present study, the use of methylation as an additional biomarker to HBsAg status improved the ability to discriminate between HCC and non-HCC cases. Although the methylation data were collected in HCC tissue samples, it has been shown that hypermethylation of tumour promoter genes occurs early in HCC development (Araújo, Rosa, Fernandes, Niel, Villela-Nogueira, *et al.*, 2016; Shen, Wang, Zhang, Kappil, Wu, *et al.*, 2012), further suggesting that it should be possible to use this biomarker for the early diagnosis of HCC in this setting.

Predictive models used in non-SubSaharan African populations have shown the value of using multiple variables including methylation data to increase model sensitivity and specificity (Dong, He, Zhang, Yu, Wang, *et al.*, 2015; Wu *et al.*, 2017). Dong *et al.* 2015 showed that in a Chinese population, combining AFP levels and the hypermethylation levels of *RASSF1A* improved the sensitivity of their diagnostic model from 64.2% to 80.9%. Wu *et al.* 2017 reported that incorporating promoter

methylation levels of *TBX2* increased the Area Under the Curve of the Wen-HCC risk score from 68% to 71%.

In addition, in the HBsAg positive sub-group analysis, HBV genotype, and specifically, HBV genotype A was found to be a significant predictor of HCC, independent of methylation level. Previous HCC risk prediction scores have only incorporated genotypes B and C within their models as the latter are the predominant genotypes in Asia, where the scores were developed (Lee, Yang, Liu, Batrla-Utermann, Jen, *et al.*, 2013; Yang *et al.*, 2011). As these scores were not externally validated in different populations, the present results suggest they would not be applicable in a SubSaharan African setting.

4.3.2 Viral biomarkers

In this study, as expected from previous published studies (Beasley *et al.*, 1981), the proportion of HBsAg-positive individuals was higher in the HCC group compared to the control group and this was statistically significant ($p = 0.0005$). Moreover, HBV genotype A was the most dominant genotype in the HCC group (75% [9/12]) compared to HBV genotype D/E in the non-HCC group (25% [3/12]). The differences in proportions of the A compared to non-A genotypes was statistically significant ($p = 0.009$), which is consistent with a previous report that HBV genotype A was 4.5 times more likely to cause HCC in Southern Africans compared to other genotypes in circulation in South Africa (Kew *et al.*, 2005). Moreover, a study from The Gambia found an association between genotype A and risk of fibrosis (crude OR: 21.0 (95% CI:1.7–266.1) suggesting that individuals infected with HBV genotype A were at higher risk of disease progression when compared to those infected with HBV genotype E (Shimakawa *et al.*, 2016).

4.3.3 Epigenetic biomarkers

The HCC group had significantly higher levels of *RASSF1A* promoter hypermethylation than the non-HCC group and this was statistically different ($p < 0.05$). This finding is similar to what has been reported elsewhere in non-African populations as *RASSF1A* is one of the most frequently inactivated genes in cancer development (Pfeifer & Dammann, 2005), including HCC (Hu *et al.*, 2010), and hypermethylation of the promoter region of this gene is suspected to be a major inactivation pathway (Dammann *et al.*, 2003).

The frequency of hypermethylation observed in the present study in *RASSF1A* (45.2%) and *p16* (18.2%) were lower than those reported in previous studies conducted in HCC samples from Taiwan (*RASSF1A*: 85% and *p16*: 47%) (Zhang *et al.*, 2002) and China (*RASSF1A*: 88.6% and *p16*: 54.3%) (QU, JIANG, LI, YU & DING, 2015). As ethnicity, geographic location, and environmental exposures are known to affect the epigenome (Galanter *et al.*, 2017), it is possible that the differences observed between those studies and the present one were due to the populations being tested. These studies used a semi-

quantitative methylation-specific PCR to assess methylation levels in the study population. In contrast, the present study used a robust, quantitative assay to determine methylation levels for each CpG site and used specific cut-offs that were CpG site-specific. It is therefore also possible that the previous studies overestimated the levels of hypermethylation observed. However, a study on Thai and French HCC cases where the authors used the same pyrosequencing techniques and methylation analysis as in the present study found that *RASSF1A* was hypermethylated in 76% of all tumours (Lambert *et al.*, 2011). They also found that the Thai HCCs showed higher DNA methylation levels than the French HCCs in multiple genes, including *RASSF1A*, making it more likely that the differences observed in the present study are due to ethnic differences. Previous studies have also shown a statistically significant lower prevalence of *p16* hypermethylation in the South African HCCs when compared to Australian HCCs further supporting this theory (Herath, Kew, Walsh, Young, Powell, *et al.*, 2002b; Herath *et al.*, 2009). These findings would suggest that in a South African population, either non-epigenetic pathways contribute significantly to deactivation of *RASSF1A* during HCC development or that other pathways are preferentially targeted in hepatocarcinogenesis such as loss of heterozygosity (Herath *et al.*, 2002b, 2009).

4.3.4 Environmental biomarkers

Aflatoxin was not found to be a significant risk factor ($p > 0.05$) and was of minimal prevalence (1.5%; 95% CI: 0.0%-4.5%) in this cohort of cases and controls, demonstrating that aflatoxin exposure is not a major issue in the Western Cape. The observed aflatoxin prevalence is much lower than what has been reported in a previous study from South Africa (Kimbi *et al.*, 2005). In that study, however, they targeted South African Black individuals from rural areas who regularly consumed grains as part of their daily meals only, whereas the present study comprised of samples taken from individuals from multiple races. Moreover, as previous studies have also shown, rural inhabitants tend to be affected by aflatoxin exposure when compared to urban dwellers due to subsistence farming and improper storage of grains (Ncube, Flett, Waalwijk & Viljoen, 2010).

4.3.5 Study strengths and limitations

The strengths of the present study include the fact that this is the first study in SSA to assess the use of methylation levels in tumour suppressor genes as potential predictors of HCC. Although the sample size was small, it was sufficiently powered to show statistically significant significance. Stringent selection criteria were also applied so as to select the best possible tissue samples for testing.

However, the study must be interpreted within the context of its limitations. First, DNA was extracted from FFPE liver tissue samples in the present study. Formalin-fixation causes DNA damage in several ways such as the formation of crosslinks between DNA and formaldehyde and DNA fragmentation and affected the quality of DNA extracted which limited the testing that could be performed downstream.

Second, healthy liver tissue was isolated from secondary liver cancer tissue samples and used as controls in the present study and it is possible that the methylation levels observed in the controls do not reflect the methylation levels that would be normally observed in completely healthy liver tissue samples. However, microscopic examination and macrodissection were used to minimise contamination with neoplastic liver tissue and the methylation levels observed in the present study reflect what has been previously observed in other studies. Third, the sample size resulted in large confidence intervals for the odds ratios observed, thus limiting the generalizability of the results obtained in the present study. However, the sample size was influenced by the small number of cases and controls histologically diagnosed in the Western Cape (Maponga, 2016). Moreover, there were geographic restrictions on where sampling for cases and controls could be performed for the present study so that methylation levels, that are influenced by environmental exposures and geography, could be accurately quantified. Fourth, *LINE-1* methylation levels could not be analysed in the present study because of the low yields of DNA extracted from FFPE liver samples. It is expected that *LINE-1* would have been significantly hypomethylated in HCC cases compared to the controls as has been previously described elsewhere (Gao *et al.*, 2014; Lin *et al.*, 2001). Fifth, HIV status could not be included in the analysis because HIV testing could not be reliably performed on FFPE liver tissue. The tissue samples were collected over a 23-year period when it was not common practice to test for HIV status. Moreover, serum samples were unavailable that could have enabled the determination of HIV status by ELISA. Although attempts to determine HIV status were conducted using PCR protocols, none could reliably amplify the HIV proviral DNA that could have been present in FFPE liver tissue. Further studies in patients with known HIV status or in whom HIV status can be confirmed serologically should be performed to investigate the influence of HIV status on HCC risk. Sixth, the effect of race on the methylation levels observed could not be assessed because of the unavailability of this information for most samples tested in the present study. As race is known to influence DNA methylation levels in individuals of different ethnicities (Adkins, Krushkal, Tylavsky & Thomas, 2011), including in HCC cases (Ally, Balasundaram, Carlsen, Chuah, Clarke, *et al.*, 2017; Cheng, Wei, Ji, Chen, Yang, *et al.*, 2018), future studies should assess whether race is a potential risk factor associated with HCC. Last, the influence of cirrhosis on methylation levels could not be ascertained as most HCC cases did not have sufficient non-neoplastic liver tissue for a diagnosis of cirrhosis to be made.

4.4 CONCLUSION

The present biomarker study found that 73.5% of HCC cases were positive for HBsAg. Moreover, there were statistically significant differences observed in methylation levels in *RASSF1A* and *p16* between the HCC and non-HCC groups. Logistic regression analyses found HBsAg status and overall methylation levels of the promoter region of *RASSF1A* to be predictors of HCC. In addition, in this study, patients with HBV genotype A were shown to be at higher risk of developing HCC compared to those infected with non-A genotypes. Lastly, the use of a combination of these biomarkers had a higher sensitivity and specificity than the individual biomarkers, indicating that they could potentially be used to develop HCC risk prediction scores for use in HCC screening.

Typically, in SSA, HCC is diagnosed at a late stage, past the point of cure. In Asia, where HBV is endemic, HCC risk scores have been developed using biomarkers specific for their populations that can be used to screen for HCC. However, those scores were based on patient populations with different risk profiles to SubSaharan Africans and were also not externally validated, making them unreliable for use in SSA. The results of the present study have further demonstrated that the HCC predictors, including methylation levels, in South Africa are different from those found in Asia.

While the present study has added to the body of knowledge on the utility of biomarkers, such as *RASSF1A*, in an African setting, as this was an exploratory study, these biomarkers need to be further validated before they can be used in a clinical setting. In addition, similar studies should also be conducted in other areas of South Africa to determine whether the results observed in the present study are generalizable to the South African population. Future studies should also focus on addressing the limitations of the present study and investigate the effect of the missing risk factors such as HIV status, presence of cirrhosis, and *LINE-1* hypomethylation. They should also consider further utilising the HCC biomarkers identified in the present study to develop HCC risk prediction scores that could be used clinically to identify those at highest risk of HCC. Investigating methylation levels in non-invasive specimens such as serum or plasma is therefore also recommended to make such tests more accessible.

5. STUDY III: EXOME SEQUENCING STUDY

5.1 MATERIALS AND METHODS

5.1.1 Study design

5.1.1.1 Choice of study design

A genetic case-sibling study using next-generation sequencing was conducted in a group of patients with HBV-related HCC, comparing them to their HBV-positive siblings not suffering from HCC. In this context, whole exome sequencing was selected as the preferred method for the identification of novel driver mutations that could potentially contribute to early-onset HCC in HBV-positive individuals, in accordance with the study aim.

5.1.1.2 Rationale for study design

As comparisons would be made between HBV-positive affected cases and HBV-positive unaffected controls, the case-sibling study design was selected because familial clustering of HBV infection has previously been reported (Chen, Huang, Lee, Yang, Chen, *et al.*, 1998). Moreover, siblings would have had the same environmental exposures as the cases.

Parental controls were not used in the present study as it was expected that the potential cases would have at least one missing parent because, in 2016, the life expectancy of South Africans living in the Western Cape was only 64.2 years for males and 69.0 years for females (Statistics South Africa, 2016).

5.1.2 Ethics approval and considerations

The cases for this study were selected from a parent project ongoing at the Division of Medical Virology, with prior ethics approval from the Stellenbosch University HREC (N11-09-284). The cases were consented for genetic studies on their PBMC samples already in storage at the Division of Medical Virology, Stellenbosch University (N11-09-284). The inclusion of controls for the present study was added as a genomic sub-study to the main study and received ethics approval from the Stellenbosch University HREC (N11-09-284a-Appendix G).

Respect for persons: Hospital and laboratory records of cases were reviewed and their demographical information (age, sex, and race) retrieved. This information was stored in an Excel sheet and de-identified by record suppression (removal of identifying information such as name, surname, date of birth, and hospital numbers) and introducing codes for sample identification before genetic testing to protect the identities of the patients. DNA samples sent for sequencing to Otogenetics Corp. (Atlanta, USA) were destroyed by the company after completion of testing.

Autonomy: The cases in the present study had already provided consent for future genetic studies on their stored PBMC samples. The sibling controls approached for enrolment in the present study were informed that their participation was entirely voluntary.

Beneficence: There was a direct benefit for the cases' family members as they were tested for HBV and those found to be actively infected were referred to the Liver Clinic of the Division of Gastroenterology and Hepatology at Tygerberg Hospital for further follow-up and long-term management. The de-identified genetic sequencing results were not expected to benefit the patients or their families as the functional effects, if any, of the novel variants identified were not determined. Moreover, as the study was only investigating novel mutations of unknown penetrance and significance, the risk of incidental findings was considered minimal.

Non-maleficence: This was a minimal-risk study with no risk for the cases and only some discomfort and minor bruising from the phlebotomy for the sibling controls enrolled in the study.

Justice: Although there was no benefit for the cases involved in the study, the results of the present study have provided insight on a disease that affects young South Africans. Few genetic studies and no whole exome sequencing studies have been carried out on HCC in an African setting.

5.1.3 Algorithm for selection of cases and controls

Figure 5.1-1 shows the algorithm that was used to select the cases and the controls.

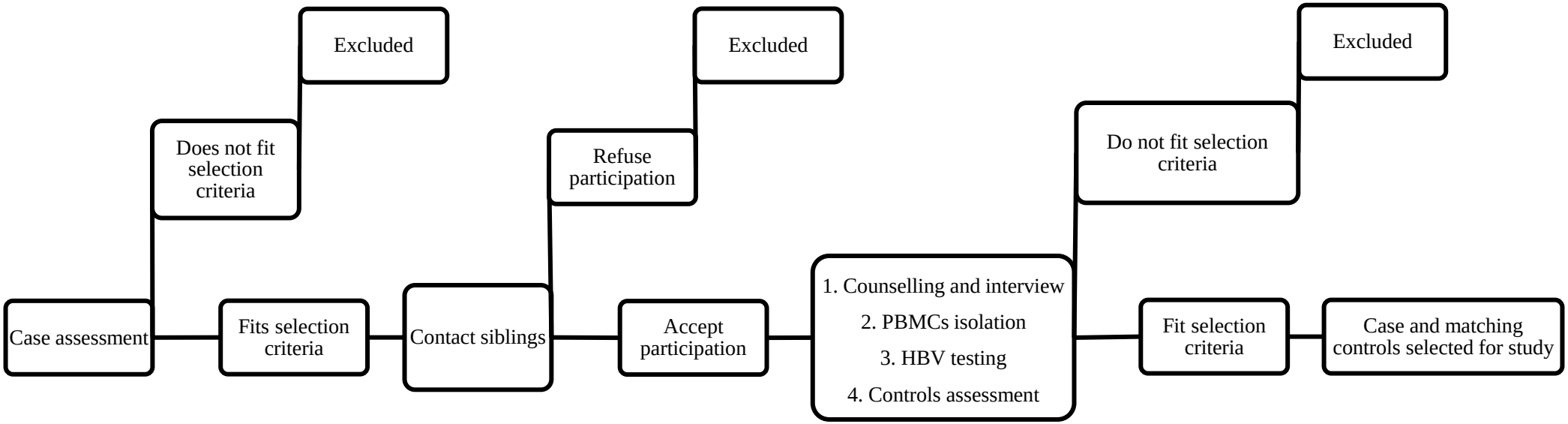


Figure 5.1-1 Algorithm showing the selection process of study cases and controls. PBMCs: Peripheral Blood Mononuclear Cells; HBV: hepatitis B virus.

5.1.4 Pre-testing phase

5.1.4.1 Assessment of available cases

The cases for the present study were selected from an ongoing parent project at the Division of Medical Virology that enrolled HCC patients at the time of diagnosis and collected PBMC, sera, and plasma samples from them for HBV, HCV, and HIV-1 marker testing. Structured interviews were also conducted on the study participants where their demographics, lifestyle habits, and medical history that could have contributed to hepatocarcinogenesis were recorded (Appendix H).

This information was then used to select cases that fit the selection criteria listed below:

1. Male sex. Male cases were chosen as early-onset HBV-related HCC affects men predominantly (Ervik *et al.*, 2016).
2. Early-onset HCC (younger than 30 years of age at time of HCC diagnosis). This was an important criterion because early-onset HCC was more likely to be triggered by underlying host genetic factors (Brandt, Bermejo, Sundquist & Hemminki, 2008b; Goldgar, Easton, Cannon-Albright & Skolnick, 1994b).
3. Diagnosed with HBV-related HCC. The focus of the present study was on HBV-related HCC more than 75% of HCC cases in SSA are associated with HBV infection.
4. No other risk factors for HCC such as HCV, HIV co-infection, or alcohol misuse.
5. Optionally, a family history of HCC (self-reported if known). Although family history is usually considered an important risk factor for familial HCC (Hassan, Spitz, Thomas, Curley, Patt, *et al.*, 2009b), patients are typically unaware of the medical histories of their relatives, which is why this criterion was optional.

5.1.4.2 Assessment of study controls

The selection criteria for the controls were as follows:

1. Siblings older than case at time of testing, who were unlikely to develop HCC. Older siblings were chosen, where possible, as they would have very similar genetic backgrounds, ethnicities, and environmental exposures that are known to influence genetic expression.
2. Male sex. The risk of HCC development is 6-8 times higher in HBV-positive males compared to HBV-positive females (Ervik *et al.*, 2016). The reason for this disparity is poorly understood and it has been suggested that estrogen could play a protective role in women (Naugler *et al.*, 2007). Therefore, in the present study, male controls were selected to control for any confounders that could arise from utilising female controls. It was expected that some genomic variants from the unaffected siblings would overlap with genomic variants from the cases.

Therefore, variants which were common to both case and control could be eliminated as they would be unlikely to have a cancer-causing potential.

3. Past or current HBV infection. This increased the chances of identifying variants which could potentially predispose an individual to developing HBV-related HCC.
4. No past or current history of liver disease besides HBV infection.
5. No other risk factors for HCC such as HCV, HIV co-infection, and alcohol misuse that could act as potential confounders in the present study.

5.1.4.3 Sample size

Previous published reports have shown that whole exome sequencing studies require as few as three or four individuals to be sufficiently powered to detect novel potentially pathogenic driver mutations (Bilgüvar, Öztürk, Louvi, Kwan, Choi, *et al.*, 2010; Esser, Holze, Haag, Schreiber, Krüger, *et al.*, 2017; Miller, Garcia, Pressey, Beierle, Kelly, *et al.*, 2017; Wang, Wang, Yang, Xia, Hu, *et al.*, 2010). Moreover, the present study did not aim to identify variants that were statistically significantly associated with HBV-related HCC development but rather those that had the highest biological plausibility of contributing to the hepatocarcinogenic process. Therefore, a sample size of two cases and two controls per case were selected for the present study.

5.1.4.4 Contacting potential controls

The siblings of the potential cases were contacted and invited to join the study. If they accepted, they were consented during a face-to-face interview and given further information on the study.

5.1.4.5 Pre-selection interview

A structured interview was conducted with each potential control to record their demographic information (age, sex, and race), exposures to HCC risk factors (Appendix H), and HIV status.

5.1.4.6 Liver fibrosis screening

Transient elastographic scans (Fibroscan, Echosens) were performed as described previously (Castera, Forns & Alberti, 2008) to ensure that there was no evidence of hepatic fibrosis in the potential controls. This scan measures shear wave velocity and converting those values into liver stiffness expressed in kilopascal (kPa). The harder the tissue the faster the shear wave propagation. A cut-off of 7.2 kPa was used for significant fibrosis (Marcellin, Ziol, Bedossa, Douvin, Poupon, *et al.*, 2009).

5.1.4.7 Clinical biochemical screening

Venous blood samples were collected from the potential controls for further testing and processing. Blood specimens were sent to the Division of Chemical Pathology, National Health Laboratory

Services, Tygerberg Hospital, for liver function tests (bilirubin, albumin, ALT, AST, and AFP). These tests were all performed on the automated Cobas® 6000 Chemistry Analyzer (Roche, Rotkreuz, Switzerland) using the Bilirubin Total Gen.3 kit (for total bilirubin levels), the Bilirubin direct Diazo Gen. 2 Jendrassik-Grof kit (for conjugated bilirubin levels), the Albumin Gen. 2 kit (for albumin levels), the Alanine Aminotransferase according to IFCC with pyridoxal phosphate activation kit (for ALT levels), the Aspartate Aminotransferase according to International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) with pyridoxal phosphate activation kit (for AST levels), and the AFP α1-fetoprotein kit (for AFP levels), using the manufacturer's recommended protocols. The results from these tests were communicated telephonically to the patients.

5.1.4.8 Serological and molecular screening

A blood sample from each potential control was also processed for markers of HBV (HBsAg, anti-HBc [total and IgM], anti-HBs, and if positive for HBsAg, HBeAg and anti-HBe testing were also performed) by ELISA on the automated ARCHITECT® i2000SR system. The corresponding ARCHITECT® immunoassays (ARCHITECT® HBsAg Qualitative, ARCHITECT® HBeAg, ARCHITECT® Anti-HBe, ARCHITECT® Anti-HBc II, and ARCHITECT® Anti-HBc IgM) were used following manufacturer's protocols (Abbott Laboratories, Illinois, USA). HB viral load was also determined using the COBAS® AmpliPrep/COBAS® TaqMan® HBV Test on the automated COBAS® AmpliPrep Instrument (Roche Molecular Diagnostics, California, USA). They were also tested for HCV (anti-HCV) using an in-house ELISA assay, and HIV (HIV-1 Ag/Ab) using the Cobas® Elecsys® HIV combi PT 4th Gen (Roche Molecular Diagnostics, California, USA). All serological assays were conducted at the SANAS-accredited Division of Medical Virology, National Health Laboratory Services, Tygerberg Hospital. These results were conveyed to the potential controls telephonically if they were negative. If any result was positive they were requested to attend a follow-up appointment and were counselled after being given their results (also see section 5.1.4.9.1).

5.1.4.9 PBMC isolation

One EDTA blood sample was processed within two hours of collection for PBMC isolation. The whole blood was poured into a 50 ml sterile conical centrifuge tube and an equal volume of Roswell Park Memorial Institute (RPMI) 1640 medium added. Twenty-five millilitres of Histopaque was added to a new sterile 50 ml centrifuge tube and the diluted blood carefully layered on top of the Histopaque using a seropipette. The centrifuge tube was then centrifuged at 400 RCF for 30 minutes with an acceleration and brake of zero. In the interim, freezing medium made of 2.4 ml RPMI 1640 medium, 0.6 ml dimethyl-sulfoxide (DMSO), and 3 ml fetal calf serum (FCS) per sample was prepared and placed on ice for a minimum of 20 minutes. After the centrifugation step, the plasma layer above the PBMC layer was carefully discarded without aspirating any cells. The PBMC layer was carefully aspirated using a

Pasteur pipette and placed in a fresh 50 ml centrifuge tube containing 10 ml of RPMI 1640. The centrifuge tube was centrifuged for 12 minutes at 320 RCF at an acceleration of 9 and brake of 5. After the centrifugation step, the supernatant was discarded and the centrifuge tube tapped firmly to dislodge the pellet formed. This pellet was washed with 10 ml of RPMI 1640 containing 10% FCS and centrifuged at 260 RCF for 10 minutes at an acceleration and brake of 9. After centrifugation, the supernatant was discarded and the pellet was dislodged by flicking the tube firmly. Then, 4 ml of freezing medium was added to the pellet in a dropwise manner while the centrifuge tube was shaken simultaneously.

Once the PBMCs were isolated and distributed into cryovials, they were transferred to a Mr. Frosty™ freezing container containing 100% isopropyl alcohol and placed at -80°C. The Mr. Frosty™ system ensured that the cells would cool at a rate of approximately -1°C/minute, which is optimal for cell preservation. After staying overnight in the Mr. Frosty™ freezer container, the cryovials with the PBMCs were transferred to a liquid nitrogen storage tank for long-term storage at -196°C until further processing.

5.1.4.9.1 Management of newly diagnosed HBV-positive individuals and close contacts

If any potential control was newly diagnosed with an active HBV infection through the study, they were reviewed by a gastroenterologist at the Liver Clinic of the Division of Gastroenterology and Hepatology at Tygerberg Hospital and underwent an abdominal ultrasound scan to ensure that there was no evidence of active liver disease (cirrhosis or space-occupying lesions). Their immediate contacts (children and sexual partners) were invited to come to Tygerberg Hospital for HBV testing and if found to be positive were offered the same treatment options. If the close contacts were negative for HBsAg, anti-HBc, and had levels of anti-HBs below 10 IU/ml, vaccination was recommended.

5.1.5 Testing phase

5.1.5.1 DNA processing

5.1.5.1.1 Cell counting

PBMC cryovials were defrosted by transferring them from liquid nitrogen to a 4°C fridge. A cell count was performed on the PBMC-freezing medium mixture to determine the volume that would be required for DNA extraction.

Twelve microlitres of the PBMC-freezing medium mixture were added to twelve microlitres of Trypan Blue and mixed. 10 µl were then placed in duplicate in a cell counting slide and placed in an automated cell counter (Biorad, California, USA). The cell sizes to be counted were set at 6–17 µM as immature PBMCs are usually 6–10 µM in diameter and mature PBMCs are usually 8–17 µM in diameter. The

two slots on each slide were read twice and the average number of cells present per millilitre was calculated from the four readings taken.

5.1.5.1.2 DNA extraction

Genomic DNA was extracted from the PBMCs of the selected cases and controls using the QIAamp DNA Blood Mini Kit (QIAGEN) following manufacturer's instructions. To up to 5 x 10^6 lymphocytes in a total volume of 200 µl, 20 µl of proteinase-K was added in a 1.5 ml microcentrifuge tube for cell digestion followed by followed by 0.4 mg of RNase A for residual RNA elimination and incubation at room temperature for one minute. Then, 200 µl of lysis buffer AL was added and the tubes mixed by pulse-vortexing for 15 s and incubated at 56°C for 10 min. The microcentrifuge tubes were briefly centrifuged and 200 µl of ethanol was added and the tubes mixed by pulse-vortexing for 15 s. The solution from each tube was then applied to a silica gel membrane spin column and centrifuged at maximum speed for one minute. The filtrate was discarded and the column placed back into the tube. The membrane-bound DNA was washed with 500 µl of two different buffers to remove leftover ethanol. The column was then placed in a new collection tube and centrifuged at full speed for one minute to eliminate possible wash buffer carry-over. Lastly, 50 µl of the elution buffer containing 10 mM Tris-Cl and 0.5 mM EDTA pH 9.0 was added and the column incubated at room temperature for five minutes and centrifuged at 5800 RCF for one minute. The eluted DNA was stored at 4°C prior to shipping and not frozen to avoid the shearing forces during freezing/thawing.

5.1.5.1.3 DNA quality validation

The quality of extracted DNA was determined by agarose gel electrophoresis and spectrophotometry. Five microlitres of each DNA sample was electrophoresed on a 1% agarose gel at 70 V for one hour

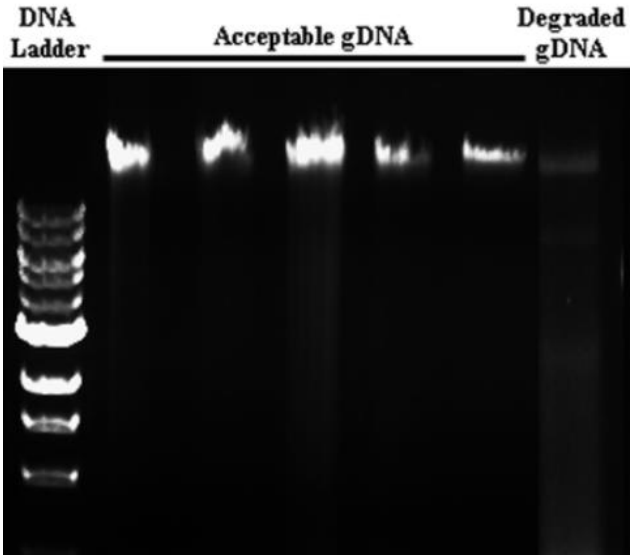


Figure 5.1-2 Example of agarose gel electrophoresis showing acceptable and degraded genomic DNA (gDNA) samples

and the gel observed under ultraviolet light at a wavelength of 254 nm using the Platinum HD Gel Documentation System (UVItec Limited, Cambridge, UK) and the image acquired using the UVIband-1D gel analysis software. Figure 5.1-2 shows an example of DNA of acceptable quality. The purified DNA was also quantitated using the NanoDrop® ND-100 (ThermoFisher Scientific). The concentration and purity of the nucleic acid were automatically calculated with the "Nucleic Acid" Application module of the NanoDrop Software Version 3.1.0. Optical density (OD) ratios A260/A280 ≥ 1.8 and A230/A280 ≥ 1.9 were considered acceptable for WES.

5.1.5.1.4 Ethanol precipitation

DNA extracts with OD ratios outside of the desired ranges were purified by ethanol precipitation. 0.1 volume of 3 M sodium acetate pH 5.2 and 2.5 volumes of ice-cold 100% ethanol were added to DNA extracts, mixed, and stored at -20°C overnight to precipitate the DNA. The precipitated DNA was recovered by centrifugation at full speed in a microcentrifuge for 20 minutes. The ethanol was then poured off and the DNA pellet washed twice with 600 µl room temperature 70% ethanol, centrifuged at full speed for 10 minutes, and the supernatant discarded. The DNA pellet was allowed to air-dry for 15 minutes and resuspended in 25 µl of an elution buffer containing 10 mM Tris-Cl and 0.5 mM EDTA pH 9.0.

5.1.5.2 Whole exome sequencing

The exome is the protein-coding region of the human genome and although it represents less than 2% of the whole genome, it contains more than 85% of disease-causing variants. Since this study aimed to detect novel variants potentially causing early-onset HBV-related HCC development, whole exome sequencing was the most efficient approach as it focused on the genes most likely to affect phenotype. WES covers a relatively small area of the human genome (< 2%) but this corresponds to a region that has been extensively characterised making it easier to interpret identified variants in a more meaningful manner. Moreover, whole exome sequencing allowed a shorter turnaround time and produced a smaller data set allowing more manageable, faster, and easier analysis (Biesecker, Shianna & Mullikin, 2011).

Samples were couriered to Otogenetics Corporation (Atlanta, GA, USA) for whole exome sequencing. Otogenetics is Clinical Laboratory Improvement Amendments (CLIA) compliant, accredited by three American federal agencies (the Food and Drug Administration [FDA], Center for Medicaid Services [CMS], and the Center for Disease Control [CDC]) responsible for monitoring quality laboratory testing. They perform rigorous quality control checks for every step of the sequencing pipeline to guarantee delivery of the best possible sequencing data. Moreover, Otogenetics has experienced specialists in the field who have worked on more than 25 000 Next Generation Sequencing projects and have developed protocols and workflows to get the best possible sequencing data from challenging samples (such as samples containing degraded DNA).

The workflow for whole exome sequencing can be described in five basic steps:

1. library preparation by fragmentation of sample DNA and ligation with specialised adapters on both ends of the fragments produced;
2. exome enrichment using hybridisation probes specific to the exonic regions;
3. cluster amplification of each labelled fragment bound to a flow cell surface through bridge amplification;
4. sequencing using terminator-based technology detecting emission wavelength and intensity to identify the bases as they are incorporated;

5.1.5.2.1 Library preparation

The amount of input DNA was between 100 ng and 3000 ng as recommended by Otogenetics. Genomic DNA was mechanically sheared with an Adaptive Focused Acoustics™ instrument (Covaris, Massachusetts, USA) to create random DNA fragments which were selected for an average size of 230 bp. Quality control checks to confirm fragment sizes were performed using a 2200 TapeStation (Agilent Technologies, California, USA) that detects fluorescently stained double-stranded DNA including genomic DNA.

The next steps of library preparation were performed using the automated SPRIworks HT System (Beckman Coulter, California, USA). DNA fragments ends were blunted and 5′ phosphorylated, A-tails were added to the 3′ ends to facilitate ligation to sequencing adapters, and Illumina compatible adapters and indices were added to the ends of the fragments. This was followed by a PCR reaction to enrich for product with adapters at both ends. After each step, the sample was purified using a bead-based purification system.

5.1.5.2.2 Exome enrichment

The SureSelect Human All Exon v5 plus UTR kit (Agilent Technologies) was used for exome enrichment as per manufacturer's instructions. This kit targets 71 MB of genomic sequence representing 335 765 exons in 21 058 genes and corresponding untranslated regions (UTRs) and uses an in-solution hybridisation capture method which utilises long 120-mer, biotinylated cRNA baits for enriching exome regions from genomic DNA fragments. These biotinylated cRNA baits were incubated with the library for 16 hours and the targeted regions selected by a pull-down assay using magnetic streptavidin beads. All libraries constructed with this kit included an index tag that was added following enrichment and that enabled multiplex sequencing. Post-hybridisation and capture, the sample was purified using a bead-based purification system and the targeted regions were further amplified by PCR using the appropriate indexing primer for each sample to produce a sequence-ready library. The quality and

quantity of the indexed library were determined using the 2200 TapeStation (Agilent Technologies) and High Sensitivity D1000 ScreenTape before sequencing.

5.1.5.2.3 Cluster amplification

This library was then loaded onto a flow cell, which is a glass slide with lanes, where the fragments were captured on a lawn of surface-bound oligos complementary to the library adapters. A polymerase created a complement of the hybridised fragment. The double-stranded molecule was then denatured and the original template washed away. The strands were then isothermally amplified into distinct clonal clusters through bridge amplification. In this process, the strand folded over and the adaptor hybridised to a second type of oligo present on the flow cell. Polymerases created a complementary strand forming a double-stranded bridge. This bridge was denatured forming two single-stranded copies of the same molecule that were tethered to the flow cell. The process was then repeated over and over and was repeated simultaneously for millions of clusters resulting in clonal amplification of all the fragments. After clonal amplification, the reverse strands were cleaved and washed off, leaving only the forward strands. The 3′ ends were then blocked to prevent unwanted priming.

5.1.5.2.4 Sequencing and base calling

Sequencing was performed on the HiSeq 2500 system (Illumina, Inc., San Diego, CA, USA) with a paired-end read length of 100 bp and 50 x coverage. The Illumina platform was chosen as it applies a sequencing-by-synthesis approach where only one nucleotide per sequencing cycle is incorporated using reversible dye terminators. All four bases are present at the same time and compete for incorporation. This natural competition minimises incorporation bias and greatly reduces raw error rates compared to other platforms (Liu, Li, Li, Hu, He, *et al.*, 2012).

During the sequencing process, the sequencing primer was extended to produce the first read. With each cycle, fluorescently-tagged nucleotides competed for addition to the growing chain and only one was incorporated based on the template sequence. After the addition of each nucleotide, the clusters were excited by a light source and a characteristic fluorescent signal was emitted. The emission wavelength along with the intensity determined the base call. For a given cluster, all identical strands were read simultaneously. Hundreds of millions of clusters were thus sequenced in a massively parallel process. After the completion of the first read, the read product was washed away. The index read primer was then introduced and hybridised to the template and the read generated similar to the first read. After completion of the index read, the read product was washed off and the 3′ ends of the template deprotected. The template folded over and bound to the second oligo on the flow cell. Index 2 was read in the same manner as index 1 and polymerases extended the second oligo forming a double-stranded bridge which was then linearized and the 3′ ends blocked. The original forward strand was cleaved off and washed away leaving only the reverse strand. Read 2 began with the introduction of the sequencing

primer. As with read 1, the sequencing steps were repeated until the desired read length was achieved. The read 2 product was then washed away. This entire process generated millions of reads representing all the fragments.

5.1.6 Post-testing phase

5.1.6.1 Sequencing data analysis

Sequencing data analysis involved the following five steps:

1. Assessment of raw data and read quality
2. Alignment of sequences to a reference genome
3. Variant identification
4. Variant annotation
5. Variant selection

The sequencing instrument generated BCL basecall files which were converted to standard FASTQ format using bcl2fastq Conversion Software. The quality of the raw reads was assessed and reads that did not meet the quality standards were trimmed, corrected, or removed using the FASTX-Toolkit. The sequence reads were assembled and mapped to the University of Santa Cruz (UCSC) human reference genome hg19 [GRCh37.p13 (GCF_000001405.25)] using the NOVOALIGN short-read aligner (www.novocraft.com), producing Sequence Alignment/Map files. The average coverage of each sample was then assessed using the software *Mosdepth* (Pedersen & Quinlan, 2018). Post-processing of the reads was then done to remove PCR duplicates using Picard (http://sourceforge.net/projects/picard/). The Genome Analysis Tool Kit (GATK) HaplotypeCaller was used for refining alignments and accurate variant calling, producing Variant Call Files (VCF).

After alignment and creation of the VCF, a second script filtering was performed to remove variants that were homozygous recessive in the cases but absent or heterozygous in their respective controls. These modified VCF files were uploaded onto wANNOVAR, the online version of the software ANNOVAR (wannovar.wglab.org) (Chang & Wang, 2012; Wang, Li & Hakonarson, 2010) for the functional annotation of the genetic variants that passed the final quality filter (quality score of > 50) with information such as variant location (e.g. exonic), class (e.g. non-synonymous), allele frequencies in public databases (e.g. the Genome Aggregation Database [gnomAD]) and for non-synonymous variants, functional effect prediction scores (e.g. Functional Analysis through Hidden Markov Models [FATHMM]). The generated results were downloaded in csv. format and visualised in Microsoft Excel (2010).

Variants were subdivided into their respective classes (truncating, splice site, and missense). Truncating and splice site variants were further filtered on rarity and variants with a gnomAD score of less than 1% (< 0.01) or formerly unseen variants (indicated by a ".") were selected. After the same filters were applied for non-synonymous variants, the predicted functional impact of the filtered variants was examined using the Combined Annotation Dependent Depletion (CADD), deleterious annotation of genetic variants using neural networks (DANN), and FATHMM algorithms. Variants with CADD phred quality scores of > 15 (equivalent to a 95% probability that the variant has some effect), or DANN scores of ≥ 0.99, or identified as being deleterious by FATHMM were selected. Further filtering was done by excluding any variants with a minor variant allele frequency of > 0.01 in the 1000 Genomes Project database for the general population and the African population.

Priority variants were selected from this list based on whether they were flagged by more than one algorithm for their known functional effect. This list of potential variants was further narrowed down by identifying their functions on Genecards® and DisGenet (www.disgenet.org) and selecting priority variants that were most likely to contribute to HCC development. Novel variants not described in HCC literature were prioritised in terms of whether they had previously been described to contribute to cancer development or been associated with other types of cancers.

5.1.6.2 Confirmation of genetic variants by Sanger sequencing

Next-generation-sequencing is prone to low levels of detectable errors making it necessary to confirm the results obtained by a different method such as Sanger sequencing. Therefore, following the identification of the three most promising potentially cancer-predisposing point mutations per case-control group, confirmation was performed by Sanger-based capillary electrophoresis.

5.1.6.2.1 Primer design for identification of variants

A genomic region of approximately 500 bases upstream and 500 bases downstream of each point mutation of interest was obtained from NCBI Gene Viewer and input in Primer 3, an online primer designing software. Primers flanking the point mutation were generated according to the following criteria: minimum primer size of 18 bp, maximum of 27 bp and optimal of 25 bp; minimum primer melting temperature of 50°C, maximum of 60°C, and optimal of 55°C. The specificity of each designed primer pair for the target region was verified using NCBI BLAST.

5.1.6.2.2 Verification of presence of variants in cases and sibling controls

5.1.6.2.2.1 Amplification using PCR

PCRs were performed on DNA previously extracted from each case and control to confirm the presence of the identified point mutations using the designed primers and following a standard protocol with annealing temperatures dependent on the melting temperatures of the designed primers.

5.1.6.2.2.2 Gel electrophoresis

A 2% agarose gel was prepared by melting 1.5 g of agarose powder (SeaKem® LE Agarose, Maine, USA) in 75 ml of 1X SB Buffer. Five microlitres of each PCR product was mixed with one microlitre of 6X Novel Juice (GeneDireX Inc., Taoyuan, Taiwan) and pipetted into wells on the agarose gel to separate any PCR product according to size. The samples were electrophoresed alongside a 1 Kb DNA ladder (GeneRuler™ 100 bp DNA Ladder, ThermoFisher Scientific) for 30 minutes at 100 V. The agarose gel was then visualised under ultraviolet light at a wavelength of 254 nm using the Platinum HD Gel Documentation System (UVItec Limited, Cambridge, UK) and the image acquired using the UVIband-1D gel analysis software. PCR products with visible bands of the correct size were selected for further testing downstream.

5.1.6.2.2.3 Purification of PCR products

PCR products were purified using the MinElute PCR Purification Kit (QIAGEN) by following manufacturer's instructions. A buffer was first added to the PCR products and the mixture applied to silica gel membrane spin columns to allow efficient binding of the amplified DNA to the column membrane. This was followed by a wash step using an ethanol-containing buffer to ensure the removal of primers and other impurities that could inhibit sequencing downstream. A centrifugation step in an empty tube then ensured the removal of residual ethanol-containing buffer. The DNA was finally eluted in 10 µl of elution buffer containing 10 mM Tris-Cl, pH 8.5.

5.1.6.2.2.4 Sequencing PCR

Sequencing PCR reactions were carried out in a 96-well plate using the corresponding PCR primers to sequence the region of interest. A master mix consisting of the components listed in Table 5.1-1 was prepared and distributed in volumes of 8 µl into the appropriate number of wells. Next, 1 µl of each primer and 1 µl of purified template DNA from each sample was added to the appropriate wells. The cycling conditions shown in Table 5.1-2 were used.

Table 5.1-1 Sequencing reaction master mix, adapted from manufacturer's instructions (ThermoScientific)

Reagent	Volume/µl
ABI Sequencing Buffer	3
Big Dye Terminator	1

Primer, 2 µM	1
Water	3
Total volume	**8**

µl: microlitre; µM: micromolar

Table 5.1-2 Cycling parameters for sequencing reaction, adapted from manufacturer's instructions (ThermoScientific)

Cycling parameter	Cycles	Temperature	Time
Denaturation	30	96°C	20 s
Annealing		50°C	20 s
Extension		60°C	4 min

°C: degrees Celsius; min: minute; s: second

5.1.6.2.2.5 Sequencing PCR purification

The sequencing PCR reaction products were purified using the BigDye® Xterminator Purification Kit consisting of SAM™ and XTerminator® solutions (ThermoScientific) as per manufacturer's instructions; 45 µl of SAM solution and 10 µl of Xterminator® solution were added to each well before the wells were sealed with adhesive film. The 96-well plate was vortexed for 30 minutes at 2000 RCF and centrifuged for 1 minute at 1000 RCF.

5.1.6.2.2.6 Capillary electrophoresis and sequencing data analysis

Capillary electrophoresis was performed on the samples using the ABI Prism 3130XL Genetic Analyzer (ThermoFisher Scientific). The information obtained was converted to raw data files using DNA sequencing analysis software (ThermoFisher Scientific). The length of the capillaries allowed sequence reads of approximately 1 Kb.

The raw trace files were further analysed using Geneious R11 (Biomatters Ltd., Auckland, New Zealand). The quality of each sequence was improved individually by looking at the chromatograms and trimming the ends where necessary. A reference sequence for the amplified fragment containing the wild-type allele was created using the corresponding primer sequences and was used to trim the sequences beyond the primer region. Mismatches or ambiguities were verified manually.

It was expected that the cases would carry the mutant alleles identified and that the controls would carry the wild-type allele or be heterozygous.

5.1.6.2.3 Confirmation of variants in population controls

Individuals with CHB, without HCC, and who were older than 30 years of age were screened for the selected variants to act as general population controls to further confirm rarity of the variants identified.

These individuals were selected from a parent project ongoing at the Division of Medical Virology, with prior ethics approval from the Stellenbosch University HREC (N11-09-284) and from the newly diagnosed HBV-positive individuals from Study 1 (S15/08/179). DNA was extracted from either the PBMCs or plasma collected from the study participants as described in Sections 5.1.4.9 and 3.1.10.2.3.1 respectively. PCRs followed by Sanger sequencing were performed on the DNA extracts as described in Section 5.1.6.2.2. It was expected that the frequency of the SNPs in the general population controls would be low, thus confirming that they were rare variants.

5.2 RESULTS

5.2.1 Pre-testing phase

5.2.1.1 Description of selected HBV-related HCC cases

For this exploratory study, stringent selection criteria were used on the available cohort, which resulted in two HCC patients who met the selection criteria. Cases 1 and 2 were both deceased Coloured males diagnosed with HBV-related HCC at the age of 21 and 30 years, respectively. They had no other known risk factor for HCC; they had no alcohol dependency, were negative for HIV and HCV, and had no known family history of liver cancer.

5.2.1.2 Screening and selection of controls

First-generation relatives of each case (Figures 5.2-1 and 5.2-2) were screened using serological, molecular, and clinical biochemical testing (Tables 5.2-1 to 5.2-4) for potential enrolment in the study as controls based on the study inclusion criteria.

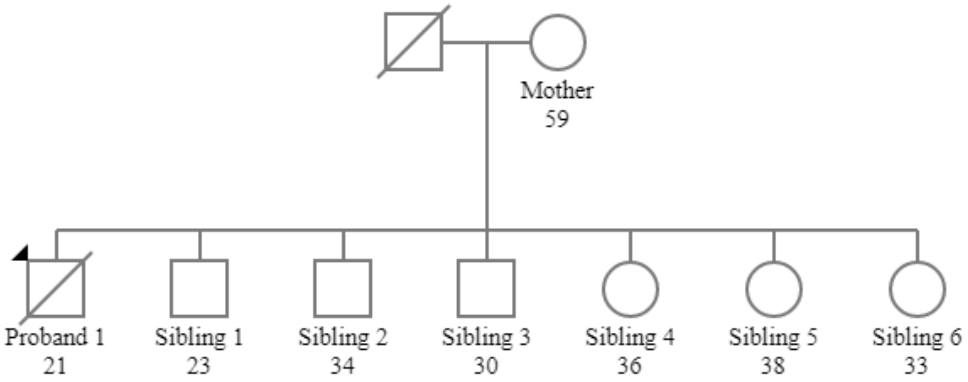


Figure 5.2-1 First-generation relatives of Case 1

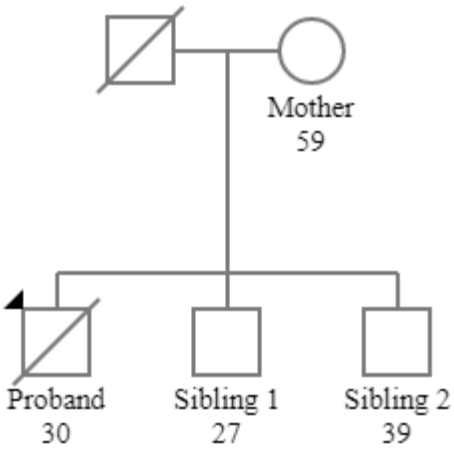


Figure 5.2-2 First-generation relatives of Case 2

Table 5.2-1 Serological and virological test results of Case 1 and male siblings

	HBsAg	anti-HBc IgM	anti-HBc IgG	HBeAg	anti-HBe	anti-HBs	HBV VL IU/ml	Anti-HCV	HIV-1
Case 1	–	+	Unknown	–	+	Unknown	Unknown	–	–
Sibling 1	+	–	–	+	–	–	5 910 000	–	–
Sibling 2	–	–	+	–	–	+	LDL	–	–
Sibling 3	+	–	+	–	+	–	< 20	–	+

Sibling 2 had cleared his hepatitis B virus (HBV) infection and therefore had an undetectable level of HB viral DNA (LDL: lower than detectable limit). Sibling 3 was co-infected with human immunodeficiency virus-1 (HIV-1) and was on antiretroviral therapy including tenofovir, hence his detectable but unquantifiable HB viral load (< 20 IU/ml). HBsAg: hepatitis B surface antigen; anti-HBs: antibody to hepatitis B surface antigen; HBeAg: hepatitis B envelope antigen; anti-HBe: antibody to hepatitis B envelope antigen; anti-HBc: antibody to hepatitis B core antigen; IgM: Immunoglobulin M; IgG: Immunoglobulin G; HCV: hepatitis C virus; VL: viral load; IU/ml: International Units per millilitre; <: less than; +: positive; –: negative.

Table 5.2-2 Liver function test results of Case 1 and male siblings

	Albumin g/L Normal: 35-52	Total bilirubin µmol/L Normal: 5-21	ALT U/L Normal: 10-40	AST U/L Normal: 15-40	AFP µg/L Normal: 0.0-8.0	FibroScan Kpa Normal: < 7.2 kPa
Case 1	33	66	407	117	17098	Unknown
Sibling 1	43	8	63	42	2.2	5.9
Sibling 2	43	7	14	20	3.3	4.9
Sibling 3	45	5	27	30	2.6	5.6

g/L: grams per litre; µmol/L: micromole per litre; U/L: units per litre; µg/L: micrograms per litre; kPa: kilopascal; <: less than

Table 5.2-3 Serological and virological test results of Case 2 and siblings

	HBsAg	anti-HBc IgM	anti-HBc IgG	HBeAg	anti-HBe	anti-HBs	HBV VL IU/ml	Anti-HCV	HIV-1
Case 2	+	–	+	+	–	Unknown	40 000	–	–
Sibling 1	+	–	+	+	–	–	32	–	–
Sibling 2	+	–	+	–	–	Unknown	< 20	–	–

HBsAg: hepatitis B surface antigen; anti-HBs: antibody to hepatitis B surface antigen; HBeAg: hepatitis B envelope antigen; anti-HBe: antibody to hepatitis B envelope antigen; anti-HBc: antibody to hepatitis B core antigen; IgM: Immunoglobulin M; IgG: Immunoglobulin G; HCV: hepatitis C virus; HIV-1: human immunodeficiency virus-1; HBV: hepatitis B virus; VL: viral load; IU/ml: International Units per millilitre; <: less than; +: positive; –: negative.

Table 5.2-4 Liver function test results of Case 2 and siblings

	Albumin g/L Normal: 35-52	Total bilirubin µmol/L Normal: 5-21	ALT U/L Normal: 10-40	AST U/L Normal: 15-40	AFP µg/L Normal: 0.0-8.0	FibroScan Kpa Normal: < 7.2 kPa
Case 2	46	66	139	315	Unknown	Unknown
Sibling 1	44	18	22	Unknown	8.2	5.1
Sibling 2	Unknown	6	19	20	6.3	6.6

g/L: grams per litre; µmol/L: micromole per litre; U/L: units per litre; µg/L: micrograms per litre; kPa: kilopascal; <: less than

Based on the study selection criteria, only male siblings with current or past HBV infections and no other risk factors of HCC development were screened as potential controls.

Case 1 had three male Coloured siblings who tested positive for markers of present or past HBV infection (Table 5.2-1). Sibling 3 was co-infected with HIV-1, which disqualified him as a potential control. Siblings 1 and 2 were male and were older than the case at the time of testing. Sibling 1 had no history of liver disease besides a newly-diagnosed chronic HBV infection. Sibling 2 also had no history of liver disease although he showed past exposure to HBV and had since cleared the infection (negative for HBsAg and positive for total anti-HBc and anti-HBs). Both had no other known risk factors of HCC; they had no alcohol dependency and were negative for both HIV and HCV. Their FibroScan results were within normal range and ultrasound scans were clear and showed no cirrhosis or space-occupying lesions. Therefore, siblings 1 and 2 were selected as the best possible controls for Case 1 and code-labelled Control 1 and Control 2 respectively.

Case 2 had two male Coloured siblings with known positive HBV status. At the time of testing for the present study, both siblings had been on treatment with tenofovir and were regularly followed-up by a gastroenterologist for three years. Sibling 1 had no past history of liver disease besides a chronic HBV infection. He had no history of alcohol misuse and was negative for both HIV-1 and HCV. His liver enzyme levels were normal and his Fibroscan results were within normal range. For these reasons, although he was younger than the case at time of testing, he was included in the study as a control and code-labelled Control 3. Sibling 2 was older than Case 2 and had a similar profile to Sibling 1. He was included in the present study as a second control and code-labelled Control 4.

5.2.1.3 Management of newly diagnosed HBV-positive individuals and close contacts

Siblings 1 and 3 of Case 1 were newly diagnosed with HBV through the present study. They were both referred to and reviewed by a gastroenterologist at the Liver Clinic of the Division of Gastroenterology and Hepatology at Tygerberg Hospital. Sibling 3 was co-infected with HIV and was already on antiretroviral therapy including tenofovir and in regular follow up. Sibling 1 reached treatment threshold because of his very high viral load (5 910 000 IU/ml) and elevated AST and ALT levels (Table 5.2-2) and was placed on treatment with tenofovir. The immediate contacts (children and sexual partners) of Siblings 1, 2, and 3 were tested for HBV markers and found to be negative for active HBV infection.

5.2.2 Testing phase

5.2.2.1 Whole exome sequencing

5.2.2.1.1 DNA quality validation

The quality of samples shipped for whole exome sequencing is given in Table 5.2-5.

Table 5.2-5 Nanodrop readings of samples sent for whole exome sequencing

Sample	Amount of DNA, ng/µl	260/280 ratio	230/280 ratio
Case 1	21.64	2.33	2.20
Control 1	86.61	1.95	1.89
Control 2	58.66	1.91	1.90
Case 2	3.5	2.53	1.11*
Control 3	56.68	1.90	1.86
Control 4	37.74	1.98	1.94

*did not meet quality control of 230/280 ratio > 1.9 but was still sent for sequencing following consultations with Mr Brantley Wyatt (senior project technician specialising in library preparation and Next Generation Sequencing, Otogenetics USA); ng/ul – nanogram per microliter

5.2.2.1.2 Quality of reads

The average coverage of each sample, calculated using the software *Mosdepth* (Pedersen & Quinlan, 2018), was > 50X for all samples (Table 5.2-6) making them suitable for further analysis.

Table 5.2-6 Average coverage of whole exome sequenced samples

Sample	Average coverage
Case 1	59.41
Control 1	63.07
Control 2	61.88
Case 2	56.73
Control 3	51.45
Control 4	61.97

5.2.3 Post-testing phase

5.2.3.1 Sequence analysis

Variants that were homozygous recessive in the cases, but absent or heterozygous in their respective controls were annotated using wANNOVAR and resulted in an initial list of 1826 variants for Case-Control Group 1 and 1159 variants for Case-Control Group 2.

5.2.3.2 Preliminary screening of variants using algorithms

As per protocol, different filters were further applied to the different algorithms within this list, which generated an initial list of potential cancer-predisposing variants for each case-control group (Tables 5.2-7 and 5.2-8).

Table 5.2-7 List of preliminary variants for Case-control Group 1

Genes	Point mutation detail	dbSNP number	Class	1000G_ALL	fathmm-MKL_coding_pred	CADD-phred	DANN_score
HLA-DRB5	*HLA-DRB5*:NM_002125:exon2:c.C112T:p.Q38X	rs1071747	stopgain	.	Neutral	11,33	0,906
SPANXN2	*SPANXN2*:NM_001009615:exon1:c.71dupA:p.N24fs	rs782333345	frameshift insertion	.	.	.	.
KLHL17	*KLHL17*:NM_198317:exon8:c.G1234A:p.V412M	rs369390543	nonsynonymous	.	Deleterious	24,1	0,991
PERM1	*PERM1*:NM_001291366:exon3:c.G2190C:p.Q730H	rs374445170	nonsynonymous	.	Neutral	24,4	0,994
TTLL10	*TTLL10*:NM_001130045:exon16:c.G1711A:p.D571N	.	nonsynonymous	.	Neutral	13,76	0,992
ITIH6	*ITIH6*:NM_198510:exon8:c.C2030T:p.S677F	rs112265894	nonsynonymous	0,0053	Neutral	23,3	0,991
PTX3	*PTX3*:NM_002852:exon2:c.C476T:p.T159M	rs112277608	nonsynonymous	0,0076	Neutral	21,1	0,948
CCDC22	*CCDC22*:NM_014008:exon8:c.C926A:p.A309D	rs183877705	nonsynonymous	0,0008	Deleterious	15,99	0,977
SMARCA1	*SMARCA1*:NM_001282874:exon3:c.A415C:p.I139L	.	nonsynonymous	.	Deleterious	14	0,943
USP26	*USP26*:NM_031907:exon1:c.C74A:p.A25E	rs867828251	nonsynonymous	.	Neutral	19,73	0,824
SAMD11	*SAMD11*:NM_152486:exon13:c.A1757C:p.N586T	rs146548754	nonsynonymous	0,0012	Deleterious	4,235	0,699

dbSNP: Single Nucleotide Polymorphism database; CADD: Combined Annotation Dependent Depletion, DANN: deleterious annotation of genetic variants using neural networks, and FATHMM: Functional Analysis through Hidden Markov Models algorithms. 1000G: 1000 Genomes Project. Variants with CADD phred quality scores of > 13 (equivalent to a 95% probability that the variant has some effect), or DANN scores of ≥ 0.99, or identified as being deleterious by FATHMM were selected. Further filtering was done by excluding any variants with a minor variant allele frequency of > 0.01 in the 1000 Genomes Project database for the general population. *HLA-DRB5*: Major Histocompatibility Complex, Class II, DR Beta 5; *SPANXN2*: Sperm Protein Associated with the Nucleus on the X chromosome N2; *KLHL17*: Kelch Like Family member 17; *PERM1*: Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 And Estrogen-Related Receptor-Induced Regulator In Muscle 1; *TTLL10*: Tubulin Tyrosine Ligase Like 10; *ITIH6*: Inter-Alpha-Trypsin Inhibitor Heavy Chain Family Member 6; *PTX3*: Pentraxin 3; *CCDC22*: Coiled-Coil Domain Containing 22; *SMARCA1*: SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A, Member 1; *USP26*: Ubiquitin Specific Peptidase 26; *SAMD11*: Sterile Alpha Motif Domain Containing 11

Table 5.2-8 List of preliminary variants for Case-control Group 2

Gene	Point Mutation Detail	dbSNP	Class	1000G_ALL	fathmm-MKL_coding_pred	CADD-phred	DANN_score
VEGFA	*VEGFA*:NM_001171623:exon6:c.540delC:p.G180fs	rs374210528	Frameshift deletion	Unknown	Unknown	Unknown	Unknown
OR11G2	*OR11G2*:NM_001005503:exon1:c.682dupA:p.C227fs	rs77164062	Frameshift deletion	Unknown	Unknown	Unknown	Unknown
TNN	*TNN*:NM_022093:exon15:c.C3311T:p.T1104M	rs113025105	nonsynonymous	0,0022	Deleterious	31	0,999
CCDC125	*CCDC125*:NM_001297696:exon2:c.G143C:p.S48C	rs111417600	nonsynonymous	0,0012	Deleterious	25,4	0,992
C6orf132	*C6orf132*:NM_001164446:exon4:c.G3149A:p.G1050D	rs113527767	nonsynonymous	0,0012	Neutral	25	0,996
RAB19	*RAB19*:NM_001008749:exon3:c.C232T:p.R78C	.	nonsynonymous	.	Deleterious	35	0,999
HGH1	*HGH1*:NM_016458:exon3:c.G778A:p.E260K	rs370307214	nonsynonymous	.	Deleterious	33	0,999
BRCA2	*BRCA2*:NM_000059:exon27:c.C9875T:p.P3292L	rs56121817	nonsynonymous	0,0002	Deleterious	33	0,999
MYLK3	*MYLK3*:NM_182493:exon12:c.A2303C:p.E768A	rs113145242	nonsynonymous	0,001	Deleterious	24,2	0,996
ASPHD2	*ASPHD2*:NM_020437:exon2:c.G214A:p.V72M	rs374177116	nonsynonymous	0,0006	Deleterious	17,48	0,993
ZNRF3	*ZNRF3*:NM_032173:exon8:c.T1898C:p.L633S	rs201483333	nonsynonymous	0,001	Deleterious	6,038	0,993
C22orf31	*C22orf31*:NM_015370:exon3:c.C739T:p.L247F	rs374421493	nonsynonymous	.	Deleterious	27,9	0,999
SEC14L6	*SEC14L6*:NM_001193336:exon7:c.A542C:p.N181T	rs112068496	nonsynonymous	.	Deleterious	25,2	0,995
BAIAP2L2	*BAIAP2L2*:NM_025045:exon7:c.G529C:p.E177Q	rs540408118	nonsynonymous	Novel	Deleterious	23,6	0,997

MARVELD2	*MARVELD2*:NM_001244734:exon2:c.C215G:p.A72G	rs112938244	nonsynonymous	Novel	Deleterious	12,58	0,816
TMEM14A	*TMEM14A*:NM_014051:exon3:c.T172C:p.F58L	rs112397916	nonsynonymous	.	Deleterious	15,1	0,895
KDM7A	*KDM7A*:NM_030647:exon17:c.C2306T:p.P769L	rs113398071	nonsynonymous	0,0024	Deleterious	18,89	0,977
CHST7	*CHST7*:NM_019886:exon1:c.C934T:p.H312Y	rs750851012	nonsynonymous	0,0019	Deleterious	16,69	0,982
ABCB7	*ABCB7*:NM_001271696:exon12:c.A1589G:p.Y530C	rs199687417	nonsynonymous	0,0011	Deleterious	19,04	0,989
HLA-DRB1	*HLA-DRB1*:NM_002124:exon2:c.G125T:p.R42M	rs1136759	nonsynonymous	Unknown	Neutral	0.001	0.4

dbSNP: Single Nucleotide Polymorphism database; CADD: Combined Annotation Dependent Depletion, DANN: deleterious annotation of genetic variants using neural networks, and FATHMM: Functional Analysis through Hidden Markov Models algorithms. 1000G: 1000 Genomes Project. Variants with CADD phred quality scores of > 13 (equivalent to a 95% probability that the variant has some effect), or DANN scores of ≥ 0.99, or identified as being deleterious by FATHMM were selected. Further filtering was done by excluding any variants with a minor variant allele frequency of > 0.01 in the 1000 Genomes Project database for the general population. *VEGFA*: Vascular Endothelial Growth Factor A; *OR11G2*: Olfactory Receptor Family 11 Subfamily G Member 2; *TNN*: Tenascin N; *CCDC125*: Coiled-Coil Domain Containing 125; *C6orf132*: Chromosome 6 Open Reading Frame 132; *RAB19*: Member RAS Oncogene Family; *HGH1*: HGH1 homolog; *BRCA2*: Breast And Ovarian Cancer Susceptibility Protein 2; *MYLK3*: Myosin Light Chain Kinase 3; *ASPHD2*: Aspartate Beta-Hydroxylase Domain Containing 2; *ZNRF3*: Zinc And Ring Finger 3; *C22orf31*: Chromosome 22 Open Reading Frame 31; *SEC14L6*: SEC14 Like Lipid Binding 6; *BAIAP2L2*: Brain-Specific Angiogenesis Inhibitor 1-Associated Protein 2-Like Protein 2; *MARVELD2*: MARVEL domain-containing protein 2; *TMEM14A*: Transmembrane Protein 14A; *KDM7A*: Lysine Demethylase 7A; *CHST7*: Carbohydrate Sulfotransferase 7; *ABCB7*: ATP Binding Cassette Subfamily B Member 7; *HLA-DRB1*: Major Histocompatibility Complex, Class II, DR Beta 1

5.2.3.3 Final selection of variants

The function and disease-association of each gene on the two lists was determined and from these data, three priority variants were selected per case-control group. For Case-Control Group 1, the *CCDC22*, *SMARCA1*, and *PTX3* variants were selected and for case-control Group 2, the *TNN*, *RAB19*, and *ZNRF3* variants were selected.

Primers were designed (Table 5.2-9) and used to confirm the presence of these point mutations by Sanger sequencing.

Table 5.2-9 List of primers used to confirm WES variants

Name of primer	Sequence	Amplicon size
CCDC22-F	5′-tacccacatagtcacagtct-3′	222 bp
CCDC22-R	5′-cactctgctcacctgttc-3′	
SMARCA1-F	5′-gaaatcagtagaaccagcct-3′	283 bp
SMARCA1-R	5′-gaacatgaaattgggacgtc-3′	
PTX3-F	5′-cttcatcatgctggagaact-3′	369 bp
PTX3-R	5′-cttgcctccttacctgcc-3′	
TNN-F	5′-gtctgtacaccatctacctg-3′	182 bp
TNN-R	5′-tagggctccaaacactctat-3′	
RAB19-F	5′-gatgtaggtgaaggagttcc-3′	234 bp
RAB19-R	5′-ttgcagctccatatttctct-3′	
ZNRF3-F	5′-cccaatagctctacctcaga-3′	248 bp
ZNRF3-R	5′-ccgtacaagccctgggag-3′	

5′: 5 prime; 3′: 3 prime; bp: base pair.

5.2.3.4 Sanger sequencing confirmation results

5.2.3.4.1 Case-control Group 1

Sanger sequencing confirmed the presence of the *CCDC22* non-synonymous C>A transversion (rs183877705, Refseq mRNA accession NM_014008), which causes an alanine to aspartic acid amino acid change at codon 309 (A309D), in Case 1 (Figure 5.2-3). The two sibling controls were homozygous for the wild-type C-allele (Figure 5.2-3).

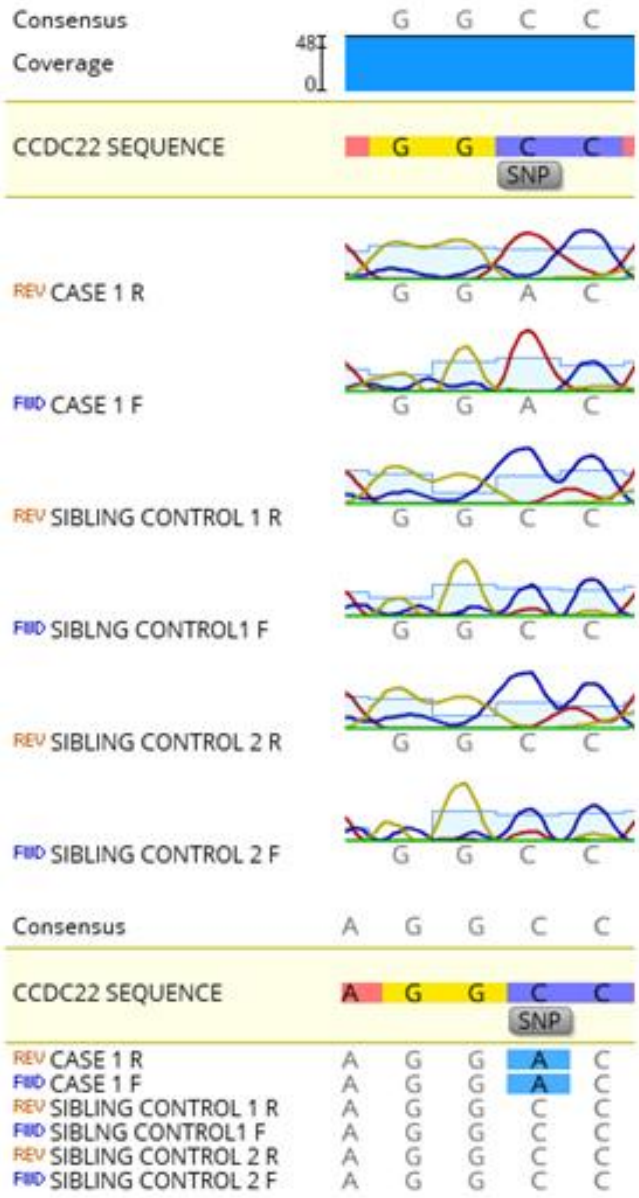


Figure 5.2-3 Representative chromatograms for Case-control Group 1. The chromatograms observed for *CCDC22* A309D confirming the C>A transversion in Case 1. Sanger sequencing analyses similarly confirmed the absence/presence of sequence changes for *SMARCA1* and *PTX3* (not shown).

Similarly, the novel *SMARCA1* non-synonymous C>A transversion at nucleotide position 415, which causes an isoleucine to lysine amino acid change at codon 139 (I139L) (Refseq mRNA accession NM_001282874), was present in Case 1. The two sibling controls were homozygous for the wild-type C-allele.

Lastly, the *PTX3* non-synonymous C>T transition at nucleotide 476 (rs183877705, Refseq mRNA accession NM_002852), which causes a threonine to methionine amino acid change at codon 159 (T159M) was present in Case 1. The two sibling controls were homozygous for the wild-type C-allele.

5.2.3.4.2 Case-control Group 2

Sanger sequencing confirmed that the *TNN* non-synonymous C>T transition at nucleotide position 3311 (rs113025105, Refseq mRNA accession NM_022093), which causes a threonine to methionine amino acid change at codon 1104 (T1104M) was present in Case 1. Sibling control 3 carried the variant T-allele (heterozygous) and sibling control 4 was homozygous for the wild-type C-allele (Figure 5.2-4).

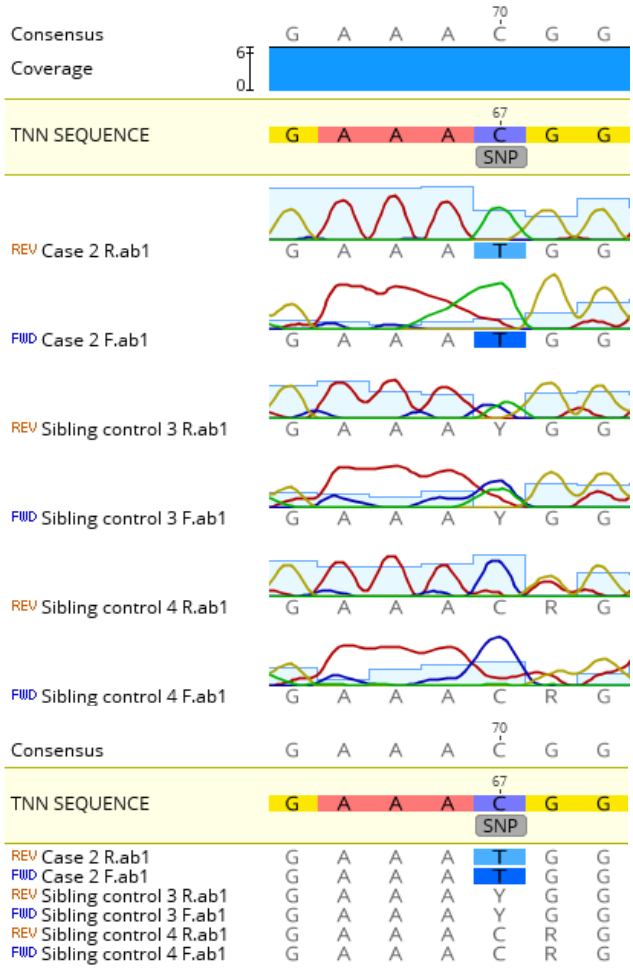


Figure 5.2-4 Representative chromatograms for Case-control Group 2. The chromatograms observed for *TNN* T1104M confirming the C>T transition in Case 2. Sanger sequencing analyses similarly confirmed the absence/presence of sequence changes for *RAB19* and *ZNRF3* (not shown).

The novel *RAB19* non-synonymous C>T transition at nucleotide 232 (Refseq mRNA accession NM_001008749), which causes an arginine to cysteine amino acid change at codon 78 (R78C) was confirmed in Case 1 by Sanger sequencing. The two sibling controls were heterozygous for the SNP.

Lastly, the *ZNRF3* non-synonymous T>C transversion at nucleotide 1898 (rs201483333, Refseq mRNA accession NM_032173), which causes a threonine to methionine amino acid change at codon 159 (T159M) was present in Case 1. The two sibling controls were homozygous for the wild-type T-allele.

5.2.3.5 Sanger sequencing confirmation results in population controls

5.2.3.5.1 Case-control Group 1

A total of 35 CHB controls were analysed for the *CCDC22* non-synonymous SNP rs183877705. The variant A-allele was observed at a frequency of 0.129 in the controls (Figure 5.2-5) (rs183877705 A-allele frequencies in global populations range between 0.00–0.02; 1000 Genomes Project Phase 3).

A total of 33 CHB controls were analysed for the novel *SMARCA1* non-synonymous mutation I139L. None of the controls harboured the variant A-allele.

A total of 15 CHB controls were analysed for the *PTX3* non-synonymous SNP rs112277608. The variant T-allele was observed at a frequency of 0.167 (rs112277608 T-allele frequencies in global populations range between 0.00–0.04; 1000 Genomes Project Phase 3).

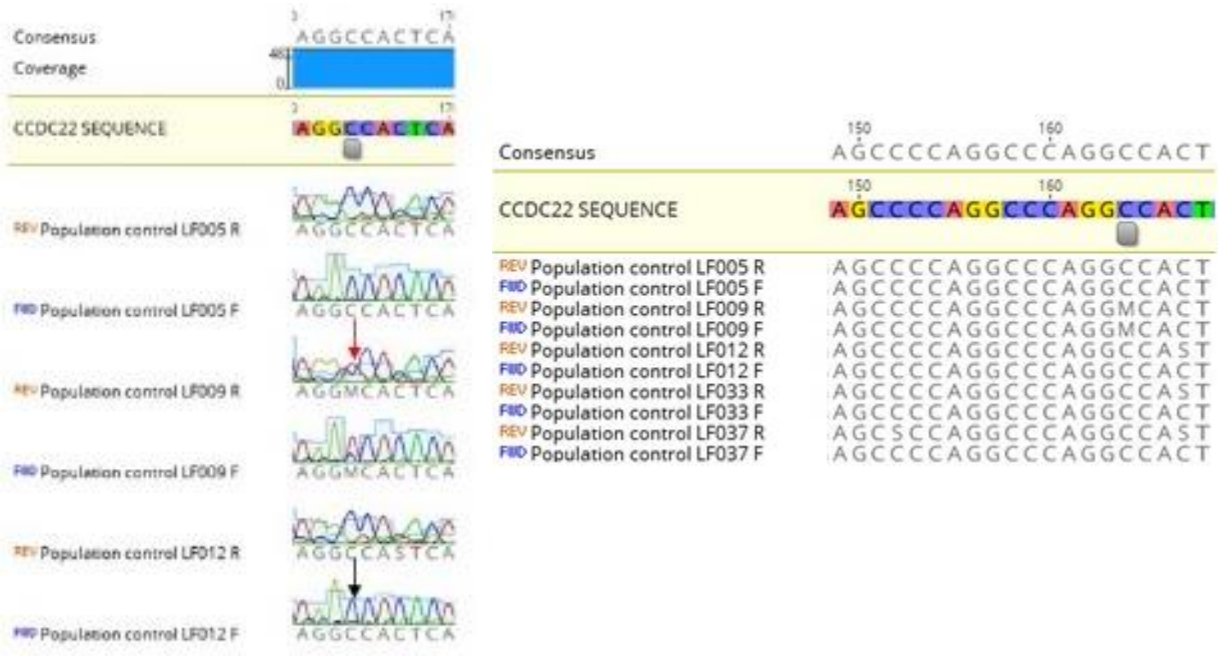


Figure 5.2-5 Representative chromatograms for Case-control Group 1. The chromatograms observed in population controls for *CCDC22* A309D. Sanger sequencing analyses similarly confirmed the absence/presence of sequence changes for *SMARCA1* and *PTX3* in population controls (not shown).

5.2.3.5.2 Case-control Group 2

A total of 32 CHB controls were analysed for the *TNN* non-synonymous SNP rs113025105. The variant T-allele was observed at a frequency of 0.047 in the controls (Figure 5.2-6; rs113025105 T-allele frequencies in global populations range between 0.00–0.02; 1000 Genomes Project Phase 3).

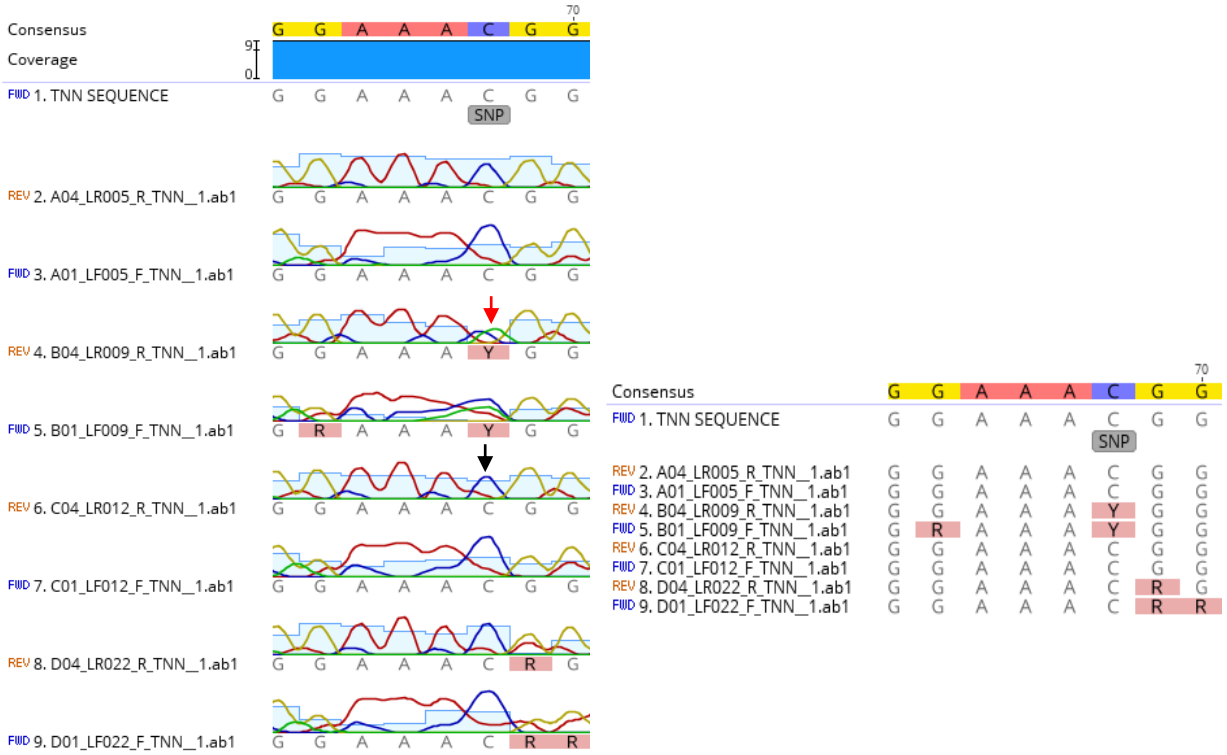


Figure 5.2-6 Representative chromatograms for Case-control Group 2. The chromatograms observed in population controls for *TNN* T1104M. Sanger sequencing analyses similarly confirmed the absence/presence of sequence changes for *RAB19* and *ZNRF3* in population controls (not shown).

A total of 35 CHB controls were assessed for the novel *RAB19* non-synonymous mutation R78C. Of these, none harboured the variant allele.

A total of 20 CHB controls were assessed for the *ZNRF3* non-synonymous variant rs201483333 (T159M). Of these, none harboured the variant allele.

5.3 DISCUSSION

The present study aimed to use whole exome sequencing and use comparisons between cases and sibling controls using recommended cut-offs for three different algorithms, CADD, DANN, and FATHMM, to identify driver mutations most likely to be implicated in hepatocarcinogenesis. The list of variants was further prioritised to three mutations per group based on gene function and prior reported involvement with HCC or cancer in general. These selected SNPs were also shown to be rare variants that were present in homozygous form only in the cases, and were either in heterozygous form or absent in the sibling controls and general population controls.

There were no overlapping genes in the two case-control groups indicating that any mutations observed could be private mutations restricted to the respective families. This is not surprising as it is known that the African genome is so diverse that it shows the highest proportion of private alleles and SNPs when compared to non-African genomes (Mayaphi, Martin, Mphahlele, Blackard & Bowyer, 2013). This finding also supports the theory that hepatocarcinogenesis is a complex process targeting multiple signalling pathways (Chen & Wang, 2015; Hayato & Shin, 2012).

In case-control Group 1, the *SMARCA1* variant identified was a novel non-synonymous point mutation annotated as being potentially deleterious by FATHMM and CADD. Since it is a novel variant, there was no associated frequency data from various human whole genome databases such as the 1000 Genomes Project. None of the unaffected siblings and population controls (0/33) had the novel variant adding weight to the possibility that this was a causal variant.

SMARCA1 is a gene producing an ATP-dependent chromatin-remodelling enzyme that is part of the SWI/SNF complexes that catalyse chromatin remodelling during cellular events that require rearrangements of chromatin structure, such as transcription, DNA repair, and replication (Narlikar, Sundaramoorthy & Owen-Hughes, 2013). Chromatin remodelling has been described to be a potential major pathway in carcinogenesis with 19.6% of human cancers showing mutations in the different SWI/SNF subunits (Kadoch, Hargreaves, Hodges, Elias, Ho, *et al.*, 2013) including hepatocellular carcinoma (Zhong, Liu, Tian, Wang, Tian, *et al.*, 2014). The role that chromatin remodelling has in maintaining genomic stability could explain the link between the SWI/SNF complexes, and therefore *SMARCA1*, and cancer (Narlikar *et al.*, 2013). Although mutations in *SMARCA1* have been previously described in an exome sequencing study conducted on French patients with HCC of varying aetiologies, these mutations were not recurrent (Guichard, Amaddeo, Imbeaud, Ladeiro, Pelletier, *et al.*, 2012). The genes predominantly mutated in the SWI/SNF complexes in that study and another whole-genome sequencing study on Japanese HCC patients (Fujimoto, Totoki, Abe, Boroevich, Hosoda, *et al.*, 2012) were *ARID1A* and *ARID2* but this was not observed in the present study indicating that although the

chromatin remodelling pathway is targeted in hepatocarcinogenesis, different genes on that pathway can be affected in different populations.

The *CCDC22* SNP rs183877705 was a non-synonymous point mutation with a minor allele frequency (MAF) of 0.0008 based on the 1000 Genomes Project and was annotated as having a potentially deleterious effect by FATHMM and CADD. The unaffected siblings were homozygous for the wild-type C allele. However, 12.9% of the general population control group investigated in this study carried the variant allele indicating that this variant is not a rare variant in the South African population. This finding is most likely caused by the underrepresentation of African genomes in online genomic databases such as the 1000 Genomes Project and supports the call to increase genomic research on African populations because variants that are rare in other populations may be common in this setting and vice-versa (Popejoy & Fullerton, 2016).

CCDC22 codes for a protein involved in the regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling by interacting with copper metabolism Murr1 domain-containing (COMMD) proteins. CCDC22 is required for NF-κB activation (Starokadomskyy, Gluck, Li, Chen, Wallis, *et al.*, 2013) and its silencing leads to the impaired activation of genes targeted by NF-κB. NF-κB is a pro-inflammatory transcription factor whose signalling pathway is tightly regulated. It has been shown to have oncogenic properties as it is involved in some of the classical cancer pathways such as angiogenesis (Xia, Shen & Verma, 2014) and its suppression in tumour cells has been shown to generally cause tumour regression. However, it would seem that NF-κB also has anti-tumour effects in certain parts of the body, such as the liver. In fact, in chemically-induced HCC models, inhibition of the NF-κB pathway was shown to increase tumourigenesis (Maeda, Kamata, Luo, Leffert & Karin, 2005). Extrapolating from these data, it is possible that the deleterious effect of rs183877705 and subsequent potential silencing of *CCDC22* could have led to the inhibition of the NF-κB pathway. This, in turn, could be a potential contributor to the hepatocarcinogenic process and development of HCC in HBV-infected individuals.

The *PTX3* SNP rs112277608 was a non-synonymous point mutation with a MAF of 0.0076 based on the 1000 Genomes Project and was annotated as having a potentially deleterious effect by *CADD*. The unaffected siblings were homozygous for the wild-type C-allele. However, 16.7% of the general population control group investigated in this study carried the variant T-allele indicating that this variant is also not a rare variant in the South African population. As only a small number of controls were tested for this particular variant, this finding must be interpreted with caution.

PTX3 is the prototype member of the long-pentraxin subfamily (Breviarios, Aniellos, Golay, Bottazzis, Bairochll, *et al.*, 1992) and is mainly involved in innate immunity and inflammation. While it has been implicated in the inhibition of angiogenesis (Rusnati, Camozzi, Moroni, Bottazzi, Peri, *et al.*, 2004),

which is one of the hallmarks of cancer (Hanahan & Weinberg, 2011), higher levels of PTX3 in-vivo have also been described in certain cancers such as HCV-associated HCC (Carmo, Aroucha, Vasconcelos, Pereira, Moura, *et al.*, 2016). It is therefore possible that *PTX3* has both pro- and anti-tumour effects that are cancer dependent (Garlanda, Bottazzi, Magrini, Inforzato & Mantovani, 2018; Giacomini, Ghedini, Presta & Ronca, 2018). It must be noted that SNP rs112277608 was recently part of an *in-silico* analysis that described it to have a "neutral" effect by several algorithms (Thakur & Shankar, 2016) just as two of the algorithms used in the present study, FATHMM and DANN, did. When Thakur & Shankar (2016) calculated the difference in folding free energy ΔG (folding) between the wild-type protein and the mutant, they found it to be positive (0.43), suggesting this SNP stabilises PTX3. In the light of this information, it is therefore possible that *PTX3* function is enhanced by SNP rs112277608 leading to pro-cancerous behaviour in individuals with HBV.

In case-control group 2, the *TNN* SNP rs113025105 was a non-synonymous point mutation with a MAF of 0.0022 based on the 1000 Genomes Project, which is marginally higher than the frequency observed in global populations, and was annotated as having a potentially deleterious effect by all three algorithms. The variant T-allele was observed at a frequency of 4.7% in the control population in this study.

TNN is part of the tenascin family and is therefore an extracellular matrix glycoprotein. *TNN* has been described as being overexpressed in a number of tumours such as breast, colon (Degen, Brellier, Schenk, Driscoll, Zaman, *et al.*, 2008; Scherberich, Tucker, Degen, Brown-Luedi, Andres, *et al.*, 2005), pancreas, kidney (Brellier, Martina, Degen, Heuzé-Vourc'h, Petit, *et al.*, 2012), and brain cancer (Martina, Degen, Rüegg, Merlo, Lino, *et al.*, 2010). No functional or knockout mice studies have looked at the potential effect of abrogation of *TNN* expression making it difficult to comment on potential pathways that could be affected. Moreover, although *TNN* is primarily involved in osteogenesis, it remains the least studied member of the tenascin family and as more functions for genes are being discovered regularly, it is possible that *TNN* is involved in a pathway that has yet to be described.

The *RAB19* variant identified was a novel non-synonymous point mutation annotated as being potentially deleterious by all three algorithms. Since it is a novel SNP, there was no associated frequency data from various human whole genome databases such as the 1000 Genomes Project. The unaffected siblings in the present study were heterozygous for this variant whereas it was completely absent in the general population controls (0/35). It is therefore possible that this variant is a private mutation segregating in this family and that the heterozygous siblings are at risk of transmitting it to future generations although they are not affected themselves.

RAB19 is a member of the small guanosine-5'-triphosphatase (GTPase) superfamily involved in membrane trafficking and post-translational modification of proteins. Just as for several of the 70

members in the GTPase family, the roles of *RAB19* remain ill-defined. However, there are previous reports of the involvement of Rab proteins in carcinogenesis; Rab5a, for example, is overexpressed in hepatocellular carcinoma (Fukui, Tamura, Wada, Kamada, Igura, *et al.*, 2007) but other Rab proteins such as Rab14 and Rab20 are downregulated in bladder cancer (Ho, Chapeaublanc, Kirkwood, Nicolle, Benhamou, *et al.*, 2012). Similarly, future studies on *RAB19* may yet show the involvement of this gene either as an oncogene or tumour suppressor gene and how it could potentially contribute to HBV-related HCC.

The *ZNRF3* variant identified (rs201483333) was a non-synonymous point mutation annotated as being deleterious by two FATHMM and DANN and has a MAF of 0.001 as reported by the 1000 Genomes Project. None of the unaffected siblings and population controls (0/20) had the variant adding weight to the possibility that this was a causal variant although the sample size is too small to state this conclusively.

This gene belongs to the E3 ubiquitin ligases family and has been shown to act as a tumour suppressor in gastric cancer (Zhou, Lan, Wang, Shi, Lan, *et al.*, 2013) and nasopharyngeal carcinoma cells (Wang, Wang, Ren, Jin & Guo, 2017) by regulating the Wnt/beta-catenin/T-cell factor signalling pathway. Defects in the latter pathway have been implicated in gastrointestinal carcinogenesis, including HCC, (Kolligs, Bommer & Göke, 2002) indicating that SNP rs201483333 could be a potential driver mutation of HCC. In fact, *ZNRF3* has been described in a previous exome sequencing study as being a recurrent target of mutations in HCC (Schulze *et al.*, 2015).

5.3.1 Study strengths and limitations

The strengths of the present study lie in that it is the first African study using WES to identify germline driver mutations of HCC. WES has the advantage of focusing on the coding regions of the human genome, enabling greater depth of coverage. WES covers a relatively small area of the human genome (<2%) but this corresponds to a region that has been extensively characterised making it easier to interpret identified variants in a more meaningful manner. Moreover, most high-penetrance variants are mediated by non-synonymous, frame-shifting, and splice variants present within the human exome. Lastly, WES is a more appropriate technique to use than other types of approaches such as GWAS because the African genome is more genetically diverse than the genome of non-African populations, or whole genome sequencing because it is more cost-effective.

However, these results must be considered within the context of the study limitations. Firstly, it was impossible to predict whether the controls used in the study would develop HCC in future. However, tests such as abdominal ultrasound scans and clinical biochemical tests were performed to ensure that they did not have HCC at the time of sampling. Secondly, the study looked at the biological plausibility of identified variants to contribute to carcinogenesis and therefore only one possible cancer-

predisposing mechanism was studied making it highly unlikely that the variants identified alone could cause HCC, although they may have predisposed the patient to develop cancer. There are several others major cancer-causing pathways that were not considered in the present study such as epigenetics, loss of allele heterozygosity, and large structural or copy number variations and chromosomal rearrangements. Lastly, although some of the variants were not found in general population controls, the sample size was too small to conclusively state that they were rare alleles in a South African setting.

5.4 CONCLUSION

The present study aimed to use whole exome sequencing to identify inheritable driver mutations most likely to be implicated in hepatocarcinogenesis. Two novel variants, in *SMARCA1* and *RAB19*, were identified and confirmed to be present in cases and absent or present in heterozygous form in the sibling and general population controls; while *SMARCA1* has previously been implicated in HCC and other cancers, *RAB19* had no known link to cancer.

This is the first study to use WES in HCC South African HCC cases and is therefore also the first to identify germline mutations that could have potentially predisposed South African men to develop early-onset HBV-related HCC. These results show that there are genetic inheritable variants that could have potentially predisposed the cases to develop HCC, although their functional impact is unknown.

Moreover, as no overlapping variants or genes were identified in this study between the two case-control groups, it was not possible to pinpoint common pathways that could have been disrupted and contributed to early hepatocarcinogenesis. In addition, due to the diversity of the genomes encountered in SSA, it may be difficult to identify high penetrance mutations in this population. It is likely that most heritable forms of HCC in SSA will present with their own private mutations.

The present study has contributed to a better understanding of HCC pathogenesis through the identification of variants with the potential of posing an inheritable risk of HCC. It is not, however, expected that these results can be translated into clinical practice. Future studies should focus on the identified variants in the present study and conduct larger epidemiological studies to conclusively demonstrate that they were rare variants, as well as *in-silico* and proteomic studies to identify the biological impact of those mutations. This study and its results could also form the basis for future larger WES studies in an African setting.

6. FINAL CONCLUSION

This study has investigated HBV and HCC from an African perspective and tackled these public health issues by incorporating three key components for early diagnosis of HBV-related HCC: HBV screening, HCC biomarkers, and HBV-related HCC genomics.

The case for the implementation of HBV screening in South Africa has been presented by providing further evidence of the magnitude of the HBV health problem in South Africa and new evidence that the POCT test performs well in the field, is accepted by the community and health care providers, and that patients diagnosed with the test can be successfully linked to treatment and long-term follow-up. Moreover, it has shown that there are biomarkers that may be used to screen for HCC, paving the way for future studies looking into developing HCC risk scores for early diagnosis of HCC. Finally, it has shown that there are germline mutations that, in the absence of other risk factors besides HBV infection, may contribute to early-onset HBV-related HCC although further studies need to be conducted in larger studies to determine the functional impact of the variants identified in the present study.

These data provide evidence that early diagnosis of HBV-related HCC in SSA is possible particularly if a multi-targeted approach is taken. The easiest approach to minimise the incidence of HCC in SSA would be to implement HBV screening, at the very least in pregnant women, to break the transmission cycle of HBV. The screening programmes could be implemented within antenatal programmes or HIV-1 clinics that are already in place in most of SubSaharan African countries. Prior to the implementation of any screening programme, HBV information campaigns should be extensively conducted to familiarise SubSaharan Africans with HBV and its long-term consequences.

The newly diagnosed HBV-infected individuals will require lifelong follow-up and management and therefore future HCC risk scores could be useful in stratifying HCC risk, thus narrowing down the number of people who need to be stringently followed-up and therefore alleviating the financial burden on the local healthcare system. Therefore, the use of biomarkers identified in the present study, such as genotype A and *RASSF1A* methylation levels, should be investigated further in larger studies in SSA to determine their potential for use within risk scores to identify those at highest risk of developing HCC among those who have HBV. Furthermore, these markers should be investigated in non-invasive samples that would enable them to be implemented in a clinical setting in a SubSaharan African setting.

7. REFERENCES

Abdel-Rahman, O., Helbling, D., Schöb, O., Eltobgy, M., Mohamed, H., Schmidt, J., Giryes, A., Mehrabi, A., et al. 2017. Cigarette smoking as a risk factor for the development of and mortality from hepatocellular carcinoma: An updated systematic review of 81 epidemiological studies. *Journal of Evidence-Based Medicine*. 10(4):245–254.

Adkins, R.M., Krushkal, J., Tylavsky, F.A. & Thomas, F. 2011. Racial differences in gene-specific DNA methylation levels are present at birth. *Birth Defects Research Part A: Clinical and Molecular Teratology*. 91(8):728–736.

Akinyemiju, T., Abera, S., Ahmed, M., Alam, N., Alemayohu, M.A., Allen, C., Al-Raddadi, R., Alvis-Guzman, N., et al. 2017. The Burden of Primary Liver Cancer and Underlying Etiologies From 1990 to 2015 at the Global, Regional, and National Level. *JAMA Oncology*. 98121(12):1683–1691.

Ally, A., Balasundaram, M., Carlsen, R., Chuah, E., Clarke, A., Dhalla, N., Holt, R.A., Jones, S.J.M., et al. 2017. Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma. *Cell*. 169(7):1327–1341.e23.

Altavilla, G., Caputo, A., Lanfredi, M., Piola, C., Barbanti-Brodano, G. & Corallini, A. 2000. Enhancement of Chemical Hepatocarcinogenesis by the HIV-1 tat Gene. *The American Journal of Pathology*. 157(4):1081–1089.

Alward, W.L., McMahon, B.J., Hall, D.B., Heyward, W.L., Francis, D.P. & Bender, T.R. 1985. The long-term serological course of asymptomatic hepatitis B virus carriers and the development of primary hepatocellular carcinoma. *The Journal of infectious diseases*. 151(4):604–9. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/2982971.

Andersson, M.I., Maponga, T.G., Ijaz, S., Barnes, J., Theron, G.B., Meredith, S.A., Preiser, W. & Tedder, R.S. 2013. The epidemiology of hepatitis B virus infection in HIV-infected and HIV-uninfected pregnant women in the Western Cape, South Africa. *Vaccine*. 31(47):5579–5584.

Andriamandimby, S.F., Olive, M.M., Shimakawa, Y., Rakotomanana, F., Razanajatovo, I.M., Andrianinarivomanana, T.M., Ravalohery, J.P., Andriamamonjy, S., et al. 2017. Prevalence of chronic hepatitis B virus infection and infrastructure for its diagnosis in Madagascar: Implication for the WHO's elimination strategy. *BMC Public Health*. 17(1):1–9.

Araújo, O.C., Rosa, A.S., Fernandes, A., Niel, C., Villela-Nogueira, C.A., Pannain, V. & Araujo, N.M. 2016. RASSF1A and DOK1 Promoter Methylation Levels in Hepatocellular Carcinoma, Cirrhotic

and Non-Cirrhotic Liver, and Correlation with Liver Cancer in Brazilian Patients. *PLoS ONE*. 11(4):e0153796.

Arauz-Ruiz, P., Norder, H., Robertson, B.H. & Magnius, L.O. 2002. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *Journal of General Virology*. 83(8):2059–2073.

Arends, P., Sonneveld, M.J., Zoutendijk, R., Carey, I., Brown, A., Fasano, M., Mutimer, D., Deterding, K., et al. 2015. Entecavir treatment does not eliminate the risk of hepatocellular carcinoma in chronic hepatitis B: Limited role for risk scores in Caucasians. *Gut*. 64(8):1289–1295.

Azechi, H., Nishida, N., Fukuda, Y., Nishimura, T., Minata, M., Katsuma, H., Kuno, M., Ito, T., et al. 2001. Disruption of the p16/cyclin D1/retinoblastoma protein pathway in the majority of human hepatocellular carcinomas. *Oncology*. 60(4):346–54.

Baecker, A., Liu, X., La Vecchia, C. & Zhang, Z.F. 2018. Worldwide incidence of hepatocellular carcinoma cases attributable to major risk factors. *European Journal of Cancer Prevention*. 27(3):205–212.

Baker, G., Babb, C., Schnugh, D., Nayler, S., Louw, M., Goedhals, J., Bringuier, P., Blay, J., et al. 2013. Molecular characterisation of gastrointestinal stromal tumours in a South African population. *Oncology letters*. 5(1):155–160.

Baleta, A. & Mitchell, F. 2014. Country in Focus: Diabetes and obesity in South Africa. *The Lancet Diabetes and Endocrinology*. 2(9):687–688.

Baptista, M., Kramvis, A. & Kew, M.C. 1999. High prevalence of 1762T 1764A mutations in the basic core promoter of hepatitis B virus isolated from black africans with hepatocellular carcinoma compared with asymptomatic carriers. *Hepatology*. 29(3):946–953.

Barbaro, G. & Barbarini, G. 2006. Highly active antiretroviral therapy-associated metabolic syndrome and cardiovascular risk. *Chemotherapy*. 52(4):161–5.

Barghini, V., Donnini, D., Uzzau, A. & Soardo, G. 2013. Signs and Symptoms. In Vol. 206. InTech *Hepatocellular Carcinoma - Future Outlook*. 1063–1065.

Barth, R.E., Huijgen, Q., Taljaard, J. & Hoepelman, A.I.M. 2010. Hepatitis B/C and HIV in sub-Saharan Africa: an association between highly prevalent infectious diseases. A systematic review and meta-analysis. *International Journal of Infectious Diseases*. 14(12):e1024–e1031.

Beasley, R.P., Trepo, C., Stevens, C.E. & Szmuness, W. 1977. The e antigen and vertical transmission

of hepatitis B surface antigen. *American Journal of Epidemiology*. 105(2):94–98.

Beasley, R.P., Hwang, L.Y., Lin, C.C. & Chien, C.S. 1981. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22 707 men in Taiwan. *The Lancet*. 2(8256):1129–33. [Online], Available: http://www.scopus.com/inward/record.url?eid=2-s2.0-0019451351&partnerID=tZOtx3y1.

Beasley, R.P., Hwang, L.Y., Lin, C.C., Leu, M.L., Stevens, C.E., Szmuness, W. & Chen, K.P. 1982. Incidence of hepatitis B virus infections in preschool children in Taiwan. *The Journal of infectious diseases*. 146(2):198–204. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/7108271.

Bick, D. & Dimmock, D. 2011. Whole exome and whole genome sequencing. *Current opinion in pediatrics*. 23(6):594–600.

Biesecker, L.G., Shianna, K. V. & Mullikin, J.C. 2011. Exome sequencing: The expert view. *Genome Biology*. 12(9):12–14.

Bilgüvar, K., Öztürk, A.K., Louvi, A., Kwan, K.Y., Choi, M., Tatlı, B., Yalnızoğlu, D., Tüysüz, B., et al. 2010. Whole-exome sequencing identifies recessive WDR62 mutations in severe brain malformations. *Nature*. 467(7312):207–210.

Di Bisceglie, A.M. 2009. Hepatitis B and hepatocellular carcinoma. *Hepatology*. 49(S5):S56–S60.

Blumberg, B.S., Gerstley, B.J., Hungerford, D.A., London, W.T. & Sutnick, A.I. 1967. A serum antigen (Australia antigen) in Down's syndrome, leukemia, and hepatitis. *Annals of internal medicine*. 66(5):924–31.

Bock, C.T., Schranz, P., Schröder, C.H. & Zentgraf, H. 1994. Hepatitis B virus genome is organized into nucleosomes in the nucleus of the infected cell. *Virus genes*. 8(3):215–29. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/7975268.

Bortoluzzi, I., Gambato, M., Albertoni, L., Mescoli, C., Pacenti, M., Cusinato, R., Germani, G., Senzolo, M., et al. 2013. Use of grafts from Anti-HBc-positive donors in liver transplantation: A 5-year, single-center experience. *Transplantation Proceedings*. 45(7):2707–2710.

Botha, J.F., Dusheiko, G.M., Ritchie, M.J.J., Mouton, H.W.K. & Kew, M.C. 1984. Hepatitis B virus carrier state in black children in Ovamboland: role of perinatal and horizontal infection. *The Lancet*. 323(8388):1210–1212.

Bouffard, P., Lamelin, J.-P., Zoulim, F., Pichoud, C. & Trepo, C. 1990. Different forms of hepatitis B virus DNA and expression of HBV antigens in peripheral blood mononuclear cells in chronic

hepatitis B. *Journal of Medical Virology*. 31(4):312–317.

Boyles, T.H. & Cohen, K. 2011. The prevalence of hepatitis B infection in a rural South African HIV clinic. *South African Medical Journal*. 101(7):470–471.

Brandt, A., Bermejo, J.L., Sundquist, J. & Hemminki, K. 2008a. Age of onset in familial cancer. *Annals of Oncology*. 19(12):2084–2088.

Brandt, A., Bermejo, J.L., Sundquist, J. & Hemminki, K. 2008b. Age of onset in familial cancer. *Annals of Oncology*. 19(12):2084–2088.

Brellier, F., Martina, E., Degen, M., Heuzé-Vourc'h, N., Petit, A., Kryza, T., Courty, Y., Terracciano, L., et al. 2012. Tenascin-W is a better cancer biomarker than tenascin-C for most human solid tumors. *BMC Clinical Pathology*. 12(1):14.

Bressac, B., Kew, M., Wands, J. & Ozturk, M. 1991. Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. *Nature*. 350(6317):429–431. [Online], Available: http://www.scopus.com/inward/record.url?eid=2-s2.0-0025828517&partnerID=tZOtx3y1.

Breviarios, F., Aniellos, E.M., Golay, J., Bottazzis, B., Bairochll, A., Sacconell, S., Marzellass, R., Predazziss, V., et al. 1992. Interleukin-1-inducible Genes in Endothelial Cells. *Journal of Biological Chemistry*. 267(31):22190–22197.

Bruix, J. & Sherman, M. 2005. Management of hepatocellular carcinoma. *Hepatology*. 42(5):1208–1236.

Burnett, R.J., Francois, G., Kew, M.C., Leroux-Roels, G., Meheus, A., Hoosen, A.A. & Mphahlele, M.J. 2005. Hepatitis B virus and human immunodeficiency virus co-infection in sub-Saharan Africa: a call for further investigation. *Liver International*. 25(2):201–213.

Cabrerizo, M., Bartolomé, J., Caramelo, C., Barril, G. & Carreño, V. 2000. Molecular analysis of hepatitis B virus DNA in serum and peripheral blood mononuclear cells from hepatitis B surface antigen–negative cases. *Hepatology*. 32(1):116–123.

Carman, W.F., Jacyna, M.R., Hadziyannis, S., Karayiannis, P., McGarvey, M.J., Makris, A. & Thomas, H.C. 1989. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *The Lancet*. 2(8663):588–91.

Carmo, R.F., Aroucha, D., Vasconcelos, L.R.S., Pereira, L.M.M.B., Moura, P. & Cavalcanti, M.S.M. 2016. Genetic variation in PTX3 and plasma levels associated with hepatocellular carcinoma in patients with HCV. *Journal of Viral Hepatitis*. 23(2):116–122.

Carr, A. & Cooper, D.A. 1997. Restoration of immunity to chronic hepatitis B infection in HIV-infected patient on protease inhibitor. *The Lancet*. 349(9057):995–996.

Carr, B.I., Kanke, F., Wise, M. & Satomura, S. 2007. Clinical evaluation of lens culinaris agglutinin-reactive alpha-fetoprotein and des-gamma-carboxy prothrombin in histologically proven hepatocellular carcinoma in the United States. *Digestive diseases and sciences*. 52(3):776–82.

Castera, L., Forns, X. & Alberti, A. 2008. Non-invasive evaluation of liver fibrosis using transient elastography. *Journal of Hepatology*. 48(5):835–847.

Chang, X. & Wang, K. 2012. wANNOVAR: annotating genetic variants for personal genomes via the web. *Journal of medical genetics*. 49(7):433–6.

Chang, M.-H., Chen, C.-J., Lai, M.-S., Hsu, H.-M., Wu, T.-C., Kong, M.-S., Liang, D.-C., Shau, W.-Y., et al. 1997. Universal Hepatitis B Vaccination in Taiwan and the Incidence of Hepatocellular Carcinoma in Children. *New England Journal of Medicine*. 336(26):1855–1859.

Chang, M. -H, Hwang, L. -Y, Hsu, H. -C, Lee, C. -Y & Beasley, R.P. 1988. Prospective study of asymptomatic hbsag carrier children infected in the perinatal period: Clinical and liver histologic studies. *Hepatology*. 8(2):374–377.

Chen, C. & Wang, G. 2015. Mechanisms of hepatocellular carcinoma and challenges and opportunities for molecular targeted therapy. *World journal of hepatology*. 7(15):1964–70.

Chen, C., Yang, H.-I., Su, J., Jen, C.-L., You, S.-L., Lu, S.-N., Huang, G.-T., Iloeje, U.H., et al. 2006. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA*. 295(1):65–73.

Chen, C.H., Huang, G.T., Lee, H.S., Yang, P.M., Chen, D.S. & Sheu, J.C. 1998. Clinical impact of screening first-degree relatives of patients with hepatocellular carcinoma. *Journal of clinical gastroenterology*. 27(3):236–9.

Chen, C.J., Wang, L.Y., Lu, S.N., Wu, M.H., You, S.L., Zhang, Y.J., Wang, L.W. & Santella, R.M. 1996. Elevated aflatoxin exposure and increased risk of hepatocellular carcinoma. *Hepatology*. 24(1):38–42.

Chen, C.L., Yang, H.I., Yang, W.S., Liu, C.J., Chen, P.J., You, S.L., Wang, L.Y., Sun, C.A., et al. 2008. Metabolic Factors and Risk of Hepatocellular Carcinoma by Chronic Hepatitis B/C Infection: A Follow-up Study in Taiwan. *Gastroenterology*. 135(1):111–121.

Chen, D.S., Sung, J.L., Shed, J.C., Lai, M.Y., How, S.W., Hsu, H.C., Lee, C.S. & Wei, T.C. 1984.

Serum α-Fetoprotein in the Early Stage of Human Hepatocellular Carcinoma. *Gastroenterology*. 86(6):1404–1409.

Chen, Y.-C., Chu, C.-M. & Liaw, Y.-F. 2010. Age-specific prognosis following spontaneous hepatitis B e antigen seroconversion in chronic hepatitis B. *Hepatology*. 51(2):435–44.

Chen, Y., Wang, L., Xu, H., Liu, X. & Zhao, Y. 2013. Exome capture sequencing reveals new insights into hepatitis B virus-induced hepatocellular carcinoma at the early stage of tumorigenesis. *Oncology Reports*. 30(4):1906–1912.

Cheng, J., Wei, D., Ji, Y., Chen, L., Yang, L., Li, G., Wu, L., Hou, T., et al. 2018. Integrative analysis of DNA methylation and gene expression reveals hepatocellular carcinoma-specific diagnostic biomarkers. *Genome Medicine*. 10(1):42.

Chirico, N., Vianelli, A. & Belshaw, R. 2010. Why genes overlap in viruses. *Proceedings of the Royal Society B: Biological Sciences*. 277(1701):3809–3817.

Chisenga, C.C., Musukuma, K., Chilengi, R., Zürcher, S., Munamununugu, V., Siyunda, A., Ojok, D., Bauer, S., et al. 2018. Field performance of the Determine HBsAg point-of-care test for diagnosis of hepatitis B virus co-infection among HIV patients in Zambia. *Journal of Clinical Virology*. 98:5–7.

Cholongitas, E., Papatheodoridis, G. V. & Burroughs, A.K. 2010. Liver grafts from anti-hepatitis B core positive donors: A systematic review. *Journal of Hepatology*. 52(2):272–279.

Chotun, N., Strobele, S., Maponga, T.G., Andersson, M.I. & Etienne De La Ray, N. (in press). Successful Treatment of a South African Pediatric Case of Acute Liver Failure Caused By Perinatal Transmission of Hepatitis B. *The Pediatric infectious disease journal*. (March, 27):epub ahead of print.

Chotun, N., Nel, E., Cotton, M.F., Preiser, W. & Andersson, M.I. 2015. Hepatitis B virus infection in HIV-exposed infants in the Western Cape, South Africa. *Vaccine*. 33(36):4618–22.

Chotun, N., Preiser, W., van Rensburg, C.J., Fernandez, P., Theron, G.B., Glebe, D. & Andersson, M.I. 2017. Point-of-care screening for hepatitis B virus infection in pregnant women at an antenatal clinic: A South African experience. *PLoS ONE*. 12(7):e0181267.

Chou, R., Cuevas, C., Fu, R., Devine, B., Wasson, N., Ginsburg, A., Zakher, B., Pappas, M., et al. 2015. Imaging techniques for the diagnosis of hepatocellular carcinoma: A systematic review and meta-analysis. *Annals of Internal Medicine*. 162(10):697–711.

Choudhuri, S. 2014. Genomic Technologies. In Elsevier *Bioinformatics for Beginners*. 55–72.

Chu, C.M. & Liaw, Y.F. 1990. The incidence of fulminant hepatic failure in acute viral hepatitis in Taiwan: increased risk in patients with pre-existing HBsAg carrier state. *Infection*. 18(4):200–3. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/2210850.

Chu, C.-M., Hung, S.-J., Lin, J., Tai, D.-I. & Liaw, Y.-F. 2004. Natural history of hepatitis B e antigen to antibody seroconversion in patients with normal serum aminotransferase levels. *The American journal of medicine*. 116(12):829–34.

Chu, C. -M, Liaw, Y. -F, Pao, C.C. & Huang, M. -J. 1989. The etiology of acute hepatitis superimposed upon previously unrecognized asymptomatic HBsAg carriers. *Hepatology*. 9(3):452–6.

Chuang, S.C., Lee, Y.C.A., Hashibe, M., Dai, M., Zheng, T. & Boffetta, P. 2010. Interaction between Cigarette Smoking and Hepatitis B and C Virus Infection on the Risk of Liver Cancer: A Meta-analysis. *Cancer Epidemiology Biomarkers & Prevention*. 19(5):1261–1268.

Clifford, G.M., Rickenbach, M., Polesel, J., Dal Maso, L., Steffen, I., Ledergerber, B., Rauch, A., Probst-Hensch, N.M., et al. 2008. Influence of HIV-related immunodeficiency on the risk of hepatocellular carcinoma. *AIDS*. 22(16):2135–2141.

Comino-Méndez, I., Gracia-Aznárez, F.J., Schiavi, F., Landa, I., Leandro-García, L.J., Letón, R., Honrado, E., Ramos-Medina, R., et al. 2011. Exome sequencing identifies MAX mutations as a cause of hereditary pheochromocytoma. *Nature genetics*. 43(7):663–7.

Coppola, N., Alessio, L., Gualdieri, L., Pisaturo, M., Sagnelli, C., Minichini, C., Di Caprio, G., Starace, M., et al. 2017. Hepatitis B virus infection in undocumented immigrants and refugees in Southern Italy: Demographic, virological, and clinical features. *Infectious Diseases of Poverty*. 6(1):1–9.

Corrao, G., Bagnardi, V., Zambon, A. & La Vecchia, C. 2004. A meta-analysis of alcohol consumption and the risk of 15 diseases. *Preventive medicine*. 38(5):613–9.

Coursaget, P., Depril, N., Chabaud, M., Nandi, R., Mayelo, V., LeCann, P. & Yvonnet, B. 1993. High prevalence of mutations at codon 249 of the p53 gene in hepatocellular carcinomas from senegal. *British Journal of Cancer*. 67(6):1395–1397.

Crowther, R.A., Kiselev, N.A., Böttcher, B., Berriman, J.A., Borisova, G.P., Ose, V. & Pumpens, P. 1994. Three-dimensional structure of hepatitis B virus core particles determined by electron cryomicroscopy. *Cell*. 77(6):943–50. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/8004680.

Dammann, R., Schagdarsurengin, U., Strunnikova, M., Rastetter, M., Seidel, C., Liu, L., Tommasi, S. & Pfeifer, G.P. 2003. Epigenetic inactivation of the Ras-association domain family 1 (RASSF1A) gene and its function in human carcinogenesis. *Histology and Histopathology*. 18(2):665–677.

Dane, D.S., Cameron, C.H. & Briggs, M. 1970. Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. *The Lancet*. 1(7649):695–8. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/4190997.

Degen, M., Brellier, F., Schenk, S., Driscoll, R., Zaman, K., Stupp, R., Tornillo, L., Terracciano, L., et al. 2008. Tenascin-W, a new marker of cancer stroma, is elevated in sera of colon and breast cancer patients. *International journal of cancer*. 122(11):2454–61.

Delius, H., Gough, N.M., Cameron, C.H. & Murray, K. 1983. Structure of the hepatitis B virus genome. *Journal of virology*. 47(2):337–343.

Diale, Q., Pattinson, R., Chokoe, R., Masenyetse, L. & Mayaphi, S. 2015. Antenatal screening for hepatitis B virus in HIV-infected and uninfected pregnant women in the Tshwane district of South Africa. *South African Medical Journal*. 106(1):97.

Dill, J.A., Camus, A.C., Leary, J.H., Di Giallonardo, F., Holmes, E.C. & Ng, T.F.F. 2016. Distinct Viral Lineages from Fish and Amphibians Reveal the Complex Evolutionary History of Hepadnaviruses. *Journal of Virology*. 90(17):7920–7933.

Donato, F., Gelatti, U., Chiesa, R., Albertini, A., Bucella, E., Boffetta, P., Tagger, A., Ribero, M.L., et al. 1999. A case-control study on family history of liver cancer as a risk factor for hepatocellular carcinoma in North Italy. Brescia HCC Study. *Cancer causes & control*. 10(5):417–21. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/10530612.

Dong, X., He, H., Zhang, W., Yu, D., Wang, X. & Chen, Y. 2015. Combination of serum RASSF1A methylation and AFP is a promising non-invasive biomarker for HCC patient with chronic HBV infection. *Diagnostic pathology*. 10:133.

Drexler, J.F., Geipel, A., Konig, A., Corman, V.M., van Riel, D., Leijten, L.M., Bremer, C.M., Rasche, A., et al. 2013. Bats carry pathogenic hepadnaviruses antigenically related to hepatitis B virus and capable of infecting human hepatocytes. *Proceedings of the National Academy of Sciences*. 110(40):16151–16156.

Durand, F., Regimbeau, J.M., Belghiti, J., Sauvanet, A., Vilgrain, V., Terris, B., Moutardier, V., Farges, O., et al. 2001. Assessment of the benefits and risks of percutaneous biopsy before surgical resection of hepatocellular carcinoma. *Journal of Hepatology*. 35(2):254–258.

Dusheiko, G.M., Conradie, J.D., Brink, B. a, Marimuthu, T. & Sher, R. 1989. Differences in the regional prevalence of chronic hepatitis B in southern Africa--implications for vaccination. *South African medical journal*. 75(10):473–8. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/2524893.

Dyson, J., Jaques, B., Chattopadyhay, D., Lochan, R., Graham, J., Das, D., Aslam, T., Patanwala, I., et al. 2014. Hepatocellular cancer: The impact of obesity, type 2 diabetes and a multidisciplinary team. *Journal of Hepatology*. 60(1):110–117.

Ehrlich, M. 2002. DNA methylation in cancer: too much, but also too little. *Oncogene*. 21(35):5400–13.

Ervik, M., Lam, F., Ferlay, J., Mery, L., Soerjomataram, I. & Bray, F. 2016. *Cancer Today*. [Online], Available: http://gco.iarc.fr/today [2018, May 09].

Esser, D., Holze, N., Haag, J., Schreiber, S., Krüger, S., Warneke, V., Rosenstiel, P. & Röcken, C. 2017. Interpreting whole genome and exome sequencing data of individual gastric cancer samples. *BMC Genomics*. 18(1):1–15.

Fasinu, P.S., Gurley, B.J. & Walker, L.A. 2015. Clinically Relevant Pharmacokinetic Herb-drug Interactions in Antiretroviral Therapy. *Current drug metabolism*. 17(1):52–64.

Fattovich, G. 2003. Natural history of hepatitis B. *Journal of Hepatology*. 39:50–58.

Fattovich, G., Bortolotti, F. & Donato, F. 2008. Natural history of chronic hepatitis B: Special emphasis on disease progression and prognostic factors. *Journal of Hepatology*. 48(2):335–352.

Feng, Q., Stern, J.E., Hawes, S.E., Lu, H., Jiang, M. & Kiviat, N.B. 2010. DNA methylation changes in normal liver tissues and hepatocellular carcinoma with different viral infection. *Experimental and Molecular Pathology*. 88(2):287–292.

Ferenci, P., Fried, M., Labrecque, D., Bruix, J., Sherman, M., Omata, M., Heathcote, J., Piratsivuth, T., et al. 2010. World Gastroenterology Organisation Guideline. Hepatocellular carcinoma (HCC): a global perspective. *Journal of Gastrointestinal and Liver Diseases*. 19(3):311–317. [Online], Available: http://www.scopus.com/inward/record.url?eid=2-s2.0-78650150055&partnerID=tZOtx3y1.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., Forman, D., et al. 2015. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *International journal of cancer*. 136(5):E359-86.

Firnhaber, C., Reyneke, A., Schulze, D., Malope, B., Maskew, M., MacPhail, P., Sanne, I. & Di Bisceglie, A. 2008. The prevalence of hepatitis B co-infection in a South African urban government HIV clinic. *South African Medical Journal*. 98(7):541–544. [Online], Available: http://www.scopus.com/inward/record.url?eid=2-s2.0-48749133300&partnerID=tZOtx3y1.

Fong, Y., Sun, R.L., Jarnagin, W. & Blumgart, L.H. 1999. An Analysis of 412 Cases of Hepatocellular Carcinoma at a Western Center. *Annals of Surgery*. 229(6):790.

Forner, A., Reig, M. & Bruix, J. 2009. Alpha-fetoprotein for hepatocellular carcinoma diagnosis: the demise of a brilliant star. *Gastroenterology*. 137(1):26–9.

Foulkes, W.D. 2008. Inherited susceptibility to common cancers. *The New England journal of medicine*. 359(20):2143–53.

Friedt, M., Gerner, P., Lausch, E., Trübel, H., Zabel, B. & Wirth, S. 1999. Mutations in the basic core promotor and the precore region of hepatitis B virus and their selection in children with fulminant and chronic hepatitis B. *Hepatology*. 29(4):1252–8.

Fritzsche, C., Becker, F., Hemmer, C.J., Riebold, D., Klammt, S., Hufert, F., Akam, W., Kinge, T.N., et al. 2013. Hepatitis B and C: neglected diseases among health care workers in Cameroon. *Transactions of The Royal Society of Tropical Medicine and Hygiene*. 107(3):158–164.

Frommer, M., McDonald, L.E., Millar, D.S., Collis, C.M., Watt, F., Grigg, G.W., Molloy, P.L. & Paul, C.L. 1992. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proceedings of the National Academy of Sciences*. 89(5):1827–1831.

Fujimoto, A., Totoki, Y., Abe, T., Boroevich, K.A., Hosoda, F., Nguyen, H.H., Aoki, M., Hosono, N., et al. 2012. Whole-genome sequencing of liver cancers identifies etiological influences on mutation patterns and recurrent mutations in chromatin regulators. *Nature Genetics*. 44(7):760–764.

Fukui, K., Tamura, S., Wada, A., Kamada, Y., Igura, T., Kiso, S. & Hayashi, N. 2007. Expression of Rab5a in hepatocellular carcinoma: Possible involvement in epidermal growth factor signaling. *Hepatology Research*. 37(11):957–965.

Galanter, J.M., Gignoux, C.R., Oh, S.S., Torgerson, D., Pino-Yanes, M., Thakur, N., Eng, C., Hu, D., et al. 2017. Differential methylation between ethnic sub-groups reflects the effect of genetic ancestry and environmental exposures. *eLife*. 6:1–24.

Gao, X.D., Qu, J.H., Chang, X.J., Lu, Y.Y., Bai, W.L., Wang, H., Xu, Z.X., An, L.J., et al. 2014.

Hypomethylation of long interspersed nuclear element-1 promoter is associated with poor outcomes for curative resected hepatocellular carcinoma. *Liver International*. 34(1):136–146.

Garlanda, C., Bottazzi, B., Magrini, E., Inforzato, A. & Mantovani, A. 2018. PTX3, a Humoral Pattern Recognition Molecule, in Innate Immunity, Tissue Repair, and Cancer. *Physiological Reviews*. 98(2):623–639.

Garson, J., Grant, P., Ayliffe, U., Ferns, R. & Tedder, R. 2005. Real-time PCR quantitation of hepatitis B virus DNA using automated sample preparation and murine cytomegalovirus internal control. *Journal of virological methods*. 126(1–2):207–213. [Online], Available: http://www.sciencedirect.com/science/article/pii/S0166093405000820 [2014, December 04].

Giacomini, A., Ghedini, G.C., Presta, M. & Ronca, R. 2018. Long pentraxin 3: A novel multifaceted player in cancer. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*. 1869(1):53–63.

Goldgar, D.E., Easton, D.F., Cannon-Albright, L.A. & Skolnick, M.H. 1994a. Systematic population-based assessment of cancer risk in first-degree relatives of cancer probands. *Journal of the National Cancer Institute*. 86(21):1600–8. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/7932824.

Goldgar, D.E., Easton, D.F., Cannon-Albright, L.A. & Skolnick, M.H. 1994b. Systematic population-based assessment of cancer risk in first-degree relatives of cancer probands. *Journal of the National Cancer Institute*. 86(21):1600–8.

Gouas, D. a., Villar, S., Ortiz-Cuaran, S., Legros, P., Ferro, G., Kirk, G.D., Lesi, O. a., Mendy, M., et al. 2012. TP53 R249S mutation, genetic variations in HBX and risk of hepatocellular carcinoma in The Gambia. *Carcinogenesis*. 33(6):1219–1224.

Guaraldi, G., Squillace, N., Stentarelli, C., Orlando, G., D'Amico, R., Ligabue, G., Fiocchi, F., Zona, S., et al. 2008. Nonalcoholic fatty liver disease in HIV-infected patients referred to a metabolic clinic: prevalence, characteristics, and predictors. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 47(2):250–7.

Guichard, C., Amaddeo, G., Imbeaud, S., Ladeiro, Y., Pelletier, L., Maad, I. Ben, Calderaro, J., Bioulac-Sage, P., et al. 2012. Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. *Nature Genetics*. 44(6):694–698.

Gupta, S., Govindarajan, S., Fong, T.-L. & Redeker, A.G. 1990. Spontaneous Reactivation in Chronic Hepatitis B: Patterns and Natural History. *Journal of Clinical Gastroenterology*. 12(5):562–568.

Gust, I.D., Burrell, C.J., Coulepis, A.G., Robinson, W.S. & Zuckerman, A.J. 1985. Taxonomic

classification of human hepatitis B; virus. *Intervirology*. 25(1):14–29.

Hadziyannis, S.J. & Papatheodoridis, G. V. 2006. Hepatitis Be Antigen – Negative Chronic Hepatitis B : Natural History and Treatment. *Seminars in liver disease*. 26(2):130–141.

Hahn, C.M., Iwanowicz, L.R., Cornman, R.S., Conway, C.M., Winton, J.R. & Blazer, V.S. 2015. Characterization of a Novel Hepadnavirus in the White Sucker (Catostomus commersonii) from the Great Lakes Region of the United States. *Journal of Virology*. 89(23):11801–11811.

Hanahan, D. & Weinberg, R.A. 2011. Hallmarks of cancer: The next generation. *Cell*. 144(5):646–674.

Hansoti, B., Stead, D., Parrish, A., Reynolds, S.J., Redd, A.D., Whalen, M.M., Mvandaba, N. & Quinn, T.C. 2018. HIV testing in a South African Emergency Department: A missed opportunity. *PLoS ONE*. 13(3):1–14.

Hassan, M.M., Spitz, M.R., Thomas, M.B., Curley, S.A., Patt, Y.Z., Vauthey, J., Glover, K.Y., Kaseb, A., et al. 2009a. The association of family history of liver cancer with hepatocellular carcinoma: A case-control study in the United States. *Journal of Hepatology*. 50(2):334–341.

Hassan, M.M., Spitz, M.R., Thomas, M.B., Curley, S.A., Patt, Y.Z., Vauthey, J., Glover, K.Y., Kaseb, A., et al. 2009b. The association of family history of liver cancer with hepatocellular carcinoma: A case-control study in the United States. *Journal of Hepatology*. 50(2):334–341.

Hayato, N. & Shin, M. 2012. Inflammation- and stress-related signaling pathways in hepatocarcinogenesis. *World Journal of Gastroenterology*. 18(31):4071–4081.

Hecht, R., Hiebert, L., Spearman, W.C., Sonderup, M.W., Guthrie, T., Hallett, T.B., Nayagam, S., Razavi, H., et al. 2018. The investment case for hepatitis B and C in South Africa: adaptation and innovation in policy analysis for disease program scale-up. *Health policy and planning*. 33(4):528–538.

Hellerbrand, C., Hartmann, A., Richter, G., Knöll, A., Wiest, R., Schölmerich, J. & Lock, G. 2001. Hepatocellular Carcinoma in Southern Germany: Epidemiological and Clinicopathological Characteristics and Risk Factors. *Digestive Diseases*. 19(4):345–351.

Herath, N.I., Kew, M.C., Walsh, M.D., Young, J., Powell, L.W., Leggett, B.A. & MacDonald, G.A. 2002a. Reciprocal relationship between methylation status and loss of heterozygosity at the p14(ARF) locus in Australian and South African hepatocellular carcinomas. *Journal of gastroenterology and hepatology*. 17(3):301–7.

Herath, N.I., Kew, M.C., Walsh, M.D., Young, J., Powell, L.W., Leggett, B.A. & MacDonald, G.A.

2002b. Reciprocal relationship between methylation status and loss of heterozygosity at the p14(ARF) locus in Australian and South African hepatocellular carcinomas. *Journal of gastroenterology and hepatology*. 17(3):301–7.

Herath, N.I., Purdie, D.M., Kew, M.C., Smith, J.L., Young, J., Leggett, B.A. & Macdonald, G.A. 2009. Varying etiologies lead to different molecular changes in Australian and South African hepatocellular carcinomas. *International Journal of Oncology*. 35(5):1081–1089.

Hernandez-Vargas, H., Lambert, M.-P., Le Calvez-Kelm, F., Gouysse, G., McKay-Chopin, S., Tavtigian, S. V, Scoazec, J.-Y. & Herceg, Z. 2010. Hepatocellular Carcinoma Displays Distinct DNA Methylation Signatures with Potential as Clinical Predictors. *PLoS ONE*. 5(3):e9749.

Hirzel, C., Pfister, S., Gorgievski-Hrisoho, M., Wandeler, G. & Zuercher, S. 2015. Performance of HBsAg point-of-care tests for detection of diagnostic escape-variants in clinical samples. *Journal of Clinical Virology*. 69:33–35.

Ho, J.R., Chapeaublanc, E., Kirkwood, L., Nicolle, R., Benhamou, S., Lebret, T., Allory, Y., Southgate, J., et al. 2012. Deregulation of Rab and Rab effector genes in bladder cancer. *PLoS ONE*. 7(6):1–16.

Hoffmann, C.C.J., Mashabela, F., Cohn, S., Hoffmann, J.J.D., Lala, S., Martinson, N.A.N. & Chaisson, R.E.R. 2014. Maternal hepatitis B and infant infection among pregnant women living with HIV in South Africa. *Journal of the International AIDS Society*. 17:1–5. [Online], Available: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4032505/ [2014, December 03].

Howell, J., Ladep, N.G., Nayagam, S., Lemoine, M., Garside, D.A., Crossey, M.M.E., Okeke, E., Njie, R., et al. 2016. PROLIFICA: a story of West African clinical and research collaborations to target hepatitis B-related hepatocellular carcinoma in West Africa. *QJM: An International Journal of Medicine*. 109(6):373–375.

Hsieh, Y.-H., Hogan, M.T., Barnes, M., Jett-Goheen, M., Huppert, J., Rompalo, A.M. & Gaydos, C.A. 2010. Perceptions of an Ideal Point-of-Care Test for Sexually Transmitted Infections – A Qualitative Study of Focus Group Discussions with Medical Providers. *PLoS ONE*. 5(11):e14144.

Hsieh, Y.H., Gaydos, C.A., Hogan, M.T., Uy, O.M., Jackman, J., Jett-Goheen, M., Albertie, A., Dangerfield, D.T., et al. 2011. What qualities are most important to making a point of care test desirable for clinicians and others offering sexually transmitted infection testing? *PLoS ONE*. 6(4):2–6.

Hsu, I.C., Metcalf, R.A., Sun, T., Welsh, J.A., Wang, N.J. & Harris, C.C. 1991. Mutational hotspot in

the p53 gene in human hepatocellular carcinomas. *Nature*. 350(6317):427–8.

Hsu, Y.-C., Yip, T.C.-F., Ho, H.J., Wong, V.W.-S., Huang, Y.-T., El-Serag, H.B., Lee, T.-Y., Wu, M.-S., et al. 2018. Development of a scoring system to predict hepatocellular carcinoma in Asians on antivirals for chronic hepatitis B. *Journal of hepatology*. 69(2):278–285.

Hsu, Y.S., Chien, R.N., Yeh, C.T., Sheen, I.S., Chiou, H.Y., Chu, C.M. & Liaw, Y.F. 2002. Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. *Hepatology*. 35(6):1522–1527.

Hu, L., Chen, G., Yu, H. & Qiu, X. 2010. Clinicopathological significance of RASSF1A reduced expression and hypermethylation in hepatocellular carcinoma. *Hepatology International*. 4(1):423–432.

Huang, G., Krocker, J.D., Kirk, J.L., Merwat, S.N., Ju, H., Soloway, R.D., Wieck, L.R., Li, A., et al. 2014. Evaluation of INK4A promoter methylation using pyrosequencing and circulating cell-free DNA from patients with hepatocellular carcinoma. *Clinical Chemistry and Laboratory Medicine*. 52(6):899–909.

Huang, Y.H., Hsiao, L.T., Hong, Y.C., Chiou, T.J., Yu, Y. Bin, Gau, J.P., Liu, C.Y., Yang, M.H., et al. 2013. Randomized controlled trial of entecavir prophylaxis for rituximab-associated hepatitis B virus reactivation in patients with lymphoma and resolved hepatitis B. *Journal of Clinical Oncology*. 31(22):2765–2772.

Hui, C.K., Leung, N., Yuen, S.T., Zhang, H.Y., Leung, K.W., Lu, L., Cheung, S.K.F., Wong, W.M., et al. 2007. Natural history and disease progression in Chinese chronic hepatitis B patients in immune-tolerant phase. *Hepatology*. 46(2):395–401.

Huy, T.T.T., Ngoc, T.T. & Abe, K. 2008. New Complex Recombinant Genotype of Hepatitis B Virus Identified in Vietnam. *Journal of Virology*. 82(11):5657–5663.

International Agency for Research on Cancer. 2010. *Detection of TP53 mutations by direct sequencing*. [Online], Available: http://p53.iarc.fr/Download/TP53_DirectSequencing_IARC.pdf [2018, July 23].

Ive, P., MacLeod, W., Mkumla, N., Orrell, C., Jentsch, U., Wallis, C.L., Stevens, W., Wood, R., et al. 2013. Low Prevalence of Liver Disease but Regional Differences in HBV Treatment Characteristics Mark HIV/HBV Co-Infection in a South African HIV Clinical Trial. *PLoS ONE*. 8(12):e74900.

Jammeh, S., Tavner, F., Watson, R., Thomas, H.C. & Karayiannis, P. 2008. Effect of basal core

promoter and pre-core mutations on hepatitis B virus replication. *Journal of General Virology*. 89(4):901–909.

Jiang, D., Sun, J., Cao, G., Liu, Y., Lin, D., Gao, Y., Ren, W., Sun, L., et al. 2013. Genetic variants in STAT4 and HLA-DQ genes confer risk of hepatitis B virus – related hepatocellular carcinoma. 45(1).

Jin, M., Piao, Z., Kim, N.G., Park, C., Shin, E.C., Park, J.H., Jung, H.J., Kim, C.G., et al. 2000. p16 is a major inactivation target in hepatocellular carcinoma. *Cancer*. 89(1):60–8. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/10897001.

Jones, S., Hruban, R.H., Kamiyama, M., Borges, M., Zhang, X., Parsons, D.W., Lin, J.C.-H., Palmisano, E., et al. 2009. Exomic Sequencing Identifies PALB2 as a Pancreatic Cancer Susceptibility Gene. *Science*. 324(5924):217–217.

Kadoch, C., Hargreaves, D.C., Hodges, C., Elias, L., Ho, L., Ranish, J. & Crabtree, G.R. 2013. Proteomic and bioinformatic analysis of mammalian SWI/SNF complexes identifies extensive roles in human malignancy. *Nature Genetics*. 45(6):592–601.

Kao, J.H. & Chen, D.S. 2006. HBV genotypes: Epidemiology and implications regarding natural history. *Current Hepatitis Reports*. 5(1):5–13.

Kariem, R., Chotun, N., Preiser, W. & Andersson, M. 2015. *Evaluation of the Determine™ HBsAg rapid test as a point-of-care screening tool for the diagnosis of hepatitis B virus infection*.

Keane, E., Funk, A.L. & Shimakawa, Y. 2016. Systematic review with meta-analysis: the risk of mother-to-child transmission of hepatitis B virus infection in sub-Saharan Africa. *Alimentary Pharmacology and Therapeutics*. 44(10):1005–1017.

Kew, M.C. 1996. Progress towards the comprehensive control of hepatitis B in Africa: a view from South Africa. *Gut*. 38(2):31–36.

Kew, M.C. 2003. Synergistic interaction between aflatoxin B1 and hepatitis B virus in hepatocarcinogenesis. *Liver International*. 23(6):405–409. [Online], Available: http://www.scopus.com/inward/record.url?eid=2-s2.0-0348013131&partnerID=tZOtx3y1.

Kew, M.C. 2008. Hepatitis B virus infection: the burden of disease in South Africa. *The Southern African Journal of Epidemiology and Infection*. 23(1):4–8.

Kew, M.C. 2012. Hepatocellular carcinoma in developing countries: Prevention, diagnosis and treatment. *World Journal of Hepatology*. 4(3):99.

Kew, M.C. 2013. Epidemiology of hepatocellular carcinoma in sub-Saharan Africa. *Annals of Hepatology*. 12(2):173–182.

Kew, M.C. & Macerollo, P. 1988. Effect of age on the etiologic role of the hepatitis B virus in hepatocellular carcinoma in blacks. *Gastroenterology*. 94(2):439–442.

Kew, M.C., Dos Santos, H.A. & Sherlock, S. 1971. Diagnosis of primary cancer of the liver. *British medical journal*. 4(5784):408–11. [Online], Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1799483&tool=pmcentrez&rendertype=abstract.

Kew, M.C., van Staden, L. & Bellingan, A. 1995. Serum alpha-fetoprotein concentrations in urban and rural Southern African blacks with hepatocellular carcinoma. *Tropical gastroenterology*. 16(4):11–5. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/8854949.

Kew, M.C., Kramvis, A., Yu, M.C., Arakawa, K. & Hodkinson, J. 2005. Increased hepatocarcinogenic potential of hepatitis B virus genotype A in Bantu-speaking sub-saharan Africans. *Journal of Medical Virology*. 75(4):513–521.

Kew, M.C., Welschinger, R. & Viana, R. 2008. Occult hepatitis B virus infection in Southern African blacks with hepatocellular carcinoma. *Journal of gastroenterology and hepatology*. 23(9):1426–1430.

Kew, M.C., Smuts, H. & Stewart, A. 2010. Does HIV Infection Enhance the Hepatocarcinogenic Potential of Chronic Hepatitis B Virus Infection? *JAIDS Journal of Acquired Immune Deficiency Syndromes*. 53(3):413–414.

Kimbi, G.C., Kramvis, A. & Kew, M.C. 2004. Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. *Journal of General Virology*. 85(5):1211–1220.

Kimbi, G.C., Kew, M.C., Yu, M.C., Arakawa, K. & Hodkinson, J. 2005. 249ser p53 mutation in the serum of black southern African patients with hepatocellular carcinoma. *Journal of gastroenterology and hepatology*. 20(8):1185–90.

Kirk, G.D., Lesi, O.A., Mendy, M., Akano, A.O., Sam, O., Goedert, J.J., Hainaut, P., Hall, A.J., et al. 2004. The Gambia Liver Cancer Study: Infection with hepatitis B and C and the risk of hepatocellular carcinoma in West Africa. *Hepatology*. 39(1):211–219.

Koh, W.-P., Robien, K., Wang, R., Govindarajan, S., Yuan, J.-M. & Yu, M.C. 2011. Smoking as an independent risk factor for hepatocellular carcinoma: the Singapore Chinese Health Study. *British journal of cancer*. 105(9):1430–5.

Kolligs, F.T., Bommer, G. & Göke, B. 2002. Wnt/beta-catenin/Tcf signaling: A critical pathway in gastrointestinal tumorigenesis. *Digestion*. 66(3):131–144.

Kramvis, A. 2008. Molecular characterisation of the genotypes and mutants of hepatitis B virus from South Africa. *Southern African Journal of Epidemiology and Infection*. 23(1):29–32. [Online], Available: http://reference.sabinet.co.za/sa_epublication_article/mp_sajei_v23_n1_a7.

Kramvis, A. & Kew, M.C. 2007a. Epidemiology of hepatitis B virus in Africa, its genotypes and clinical associations of genotypes. *Hepatology Research*. 37(s1):S9–S19.

Kramvis, A. & Kew, M.C. 2007b. Molecular characterization of subgenotype A1 (subgroup Aa) of hepatitis B virus. In Vol. 37 *Hepatology Research*. 27–32.

Kramvis, A., Kew, M. & François, G. 2005. Hepatitis B virus genotypes. *Vaccine*. 23(19):2409–2423.

Kredo, T., Ford, N., Adeniyi, F.B. & Garner, P. 2013. Decentralising HIV treatment in lower- and middle-income countries. *The Cochrane database of systematic reviews*. (6):CD009987.

Kredo, T., Adeniyi, F.B., Bateganya, M. & Pienaar, E.D. 2014. Task shifting from doctors to non-doctors for initiation and maintenance of antiretroviral therapy. *The Cochrane database of systematic reviews*. (7):CD007331.

Kuhns, M., McNamara, A., Mason, A., Campbell, C. & Perrillo, R. 1992. Serum and liver hepatitis B virus DNA in chronic hepatitis B after sustained loss of surface antigen. *Gastroenterology*. 103(5):1649–1656.

Kumar, M., Chauhan, R., Gupta, N., Hissar, S., Sakhuja, P. & Sarin, S.K. 2009. Spontaneous Increases in Alanine Aminotransferase Levels in Asymptomatic Chronic Hepatitis B Virus-Infected Patients. *Gastroenterology*. 136(4):1272–1280.

Kurbanov, F., Tanaka, Y., Kramvis, A., Simmonds, P. & Mizokami, M. 2008. When Should "I" Consider a New Hepatitis B Virus Genotype? *Journal of Virology*. 82(16):8241–8242.

Kurdyukov, S. & Bullock, M. 2016. DNA Methylation Analysis: Choosing the Right Method. *Biology*. 5(1):3.

Lambert, M.-P., Paliwal, A., Vaissière, T., Chemin, I., Zoulim, F., Tommasino, M., Hainaut, P., Sylla, B., et al. 2011. Aberrant DNA methylation distinguishes hepatocellular carcinoma associated with HBV and HCV infection and alcohol intake. *Journal of hepatology*. 54(4):705–15.

Lampertico, P., Agarwal, K., Berg, T., Buti, M., Janssen, H.L.A., Papatheodoridis, G., Zoulim, F. & Tacke, F. 2017. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus

infection. *Journal of Hepatology*. 67(2):370–398.

Lanford, R.E., Chavez, D., Brasky, K.M., Burns, R.B. & Rico-Hesse, R. 1998. Isolation of a hepadnavirus from the woolly monkey, a New World primate. *Proceedings of the National Academy of Sciences of the United States of America*. 95(10):5757–61. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/9576957.

Laubscher, B., Gehri, M., Roulet, M., Wirth, S. & Gerner, P. 2005. Survival of infantile fulminant hepatitis B and treatment with Lamivudine. *Journal of pediatric gastroenterology and nutrition*. 40(4):518–20. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/15795605.

Lee, H.W. & Ahn, S.H. 2016. Prediction models of hepatocellular carcinoma development in chronic hepatitis B patients. *World Journal of Gastroenterology*. 22(37):8314–8321.

Lee, M., Yang, H., Liu, J., Batrla-Utermann, R., Jen, C., Iloeje, U.H., Lu, S., You, S., et al. 2013. Prediction models of long-term Cirrhosis and hepatocellular carcinoma risk in chronic hepatitis B patients: Risk scores integrating host and virus profiles. *Hepatology*. 58(2):546–554.

Lee, S., Lee, H.J., Kim, J.H., Lee, H.S., Jang, J.J. & Kang, G.H. 2003. Aberrant CpG island hypermethylation along multistep hepatocarcinogenesis. *American Journal of Pathology*. 163(4):1371–1378.

Lemoine, M. & Thursz, M.R. 2017. Battlefield against hepatitis B infection and HCC in Africa. *Journal of Hepatology*. 66(3):645–654.

Lemoine, M., Eholié, S. & Lacombe, K. 2015. Reducing the neglected burden of viral hepatitis in Africa: Strategies for a global approach. *Journal of Hepatology*. 62(2):469–476.

Lemoine, M., Shimakawa, Y., Nayagam, S., Khalil, M., Suso, P., Lloyd, J., Goldin, R., Njai, H.F., et al. 2016. The gamma-glutamyl transpeptidase to platelet ratio (GPR) predicts significant liver fibrosis and cirrhosis in patients with chronic HBV infection in West Africa. *Gut*. 65(8):1369–1376.

Lemoine, M., Shimakawa, Y., Njie, R., Taal, M., Ndow, G., Chemin, I., Ghosh, S., Njai, H.F., et al. 2016. Acceptability and feasibility of a screen-and-treat programme for hepatitis B virus infection in The Gambia: the Prevention of Liver Fibrosis and Cancer in Africa (PROLIFICA) study. *The Lancet Global Health*. 4(8):e559–e567.

Lewallen, S. & Courtright, P. 1998. Epidemiology in practice: Case-control studies. *Community Eye Health Journal*. 11(28):57–58.

Liaw, Y. -F, Sheen, I. -S, Chen, T. -J, Chu, C. -M & Pao, C. -C. 1991. Incidence, determinants and significance of delayed clearance of serum HBsAg in chronic hepatitis B virus infection: a prospective study. *Hepatology*. 13(4):627–31.

Liaw, Y.F., Pao, C.C., Chu, C.M., Sheen, I.S. & Huang, M.J. 1983. Changes of serum hepatitis B virus DNA in two types of clinical events preceding spontaneous hepatitis B e antigen seroconversion in chronic type B hepatitis. *Hepatology*. 7(1):1–3.

Liaw, Y.F., Chu, C.M., Lin, D.Y., Sheen, I.S., Yang, C.Y. & Huang, M.J. 1984. Age-specific prevalence and significance of hepatitis B e antigen and antibody in chronic hepatitis B virus infection in Taiwan: a comparison among asymptomatic carriers, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. *Journal of medical virology*. 13(4):385–91. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/6330293.

Liaw, Y.F., Pao, C.C., Chu, C.M., Sheen, I.S. & Huang, M.J. 1987. Changes of serum hepatitis B virus DNA in two types of clinical events preceding spontaneous hepatitis B e antigen seroconversion in chronic type B hepatitis. *Hepatology (Baltimore, Md.)*. 7(1):1–3. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/2433201.

Life Technologies. 2013. *Demonstrated protocol: sample quantification for Ion AmpliSeq library preparation using the TapMan RNase P detection reagents Kit*. [Online], Available: https://tools.thermofisher.com/content/sfs/manuals/MAN0007732_SampleQuant_AmpliSeq_TaqManRNaseP_UB.pdf [2018, July 23].

Lin, C.-L. & Kao, J.-H. 2013. Hepatitis B Virus Genotypes: Clinical Relevance and Therapeutic Implications. *Current Hepatitis Reports*. 12(2):124–132.

Lin, C.-L. & Kao, J.-H. 2017. Natural history of acute and chronic hepatitis B: The role of HBV genotypes and mutants. *Best Practice & Research Clinical Gastroenterology*. 31(3):249–255.

Lin, C.-W., Lin, C.-C., Mo, L.-R., Chang, C.-Y., Perng, D.-S., Hsu, C.-C., Lo, G.-H., Chen, Y.-S., et al. 2013. Heavy alcohol consumption increases the incidence of hepatocellular carcinoma in hepatitis B virus-related cirrhosis. *Journal of hepatology*. 58(4):730–5.

Lin, C.H., Hsieh, S.Y., Sheen, I.S., Lee, W.C., Chen, T.C., Shyu, W.C. & Liaw, Y.F. 2001. Genome-wide hypomethylation in hepatocellular carcinogenesis. *Cancer Research*. 61(10):4238–4243.

Lin, S.-M., Yu, M.-L., Lee, C.-M., Chien, R.-N., Sheen, I.-S., Chu, C.-M. & Liaw, Y.-F. 2007. Interferon therapy in HBeAg positive chronic hepatitis reduces progression to cirrhosis and hepatocellular carcinoma. *Journal of hepatology*. 46(1):45–52.

Liu, J., Yang, H.I., Lee, M.H., Lu, S.N., Jen, C.L., Wang, L.Y., You, S.L., Iloeje, U.H., et al. 2010. Incidence and determinants of spontaneous hepatitis B surface antigen seroclearance: A community-based follow-up study. *Gastroenterology*. 139(2):474–482.

Liu, L., Li, Y., Li, S., Hu, N., He, Y., Pong, R., Lin, D., Lu, L., et al. 2012. Comparison of Next-Generation Sequencing Systems. *Journal of Biomedicine and Biotechnology*. 2012(251364):1–11.

Liu, S., Zhang, H., Gu, C., Yin, J., He, Y., Xie, J. & Cao, G. 2009. Associations between hepatitis B virus mutations and the risk of hepatocellular carcinoma: a meta-analysis. *Journal of the National Cancer Institute*. 101(15):1066–82.

Lok, A.S. & Lai, C.L. 1988. A longitudinal follow-up of asymptomatic hepatitis B surface antigen-positive Chinese children. *Hepatology*. 8(5):1130–3. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/3417235.

Lok, A.S., Sterling, R.K., Everhart, J.E., Wright, E.C., Hoefs, J.C., Di Bisceglie, A.M., Morgan, T.R., Kim, H., et al. 2010. Des-γ-Carboxy Prothrombin and α-Fetoprotein as Biomarkers for the Early Detection of Hepatocellular Carcinoma. *Gastroenterology*. 138(2):493–502.

Lok, A.S.F., Hadziyannis, S.J., Weller, I. V.D., Karvountzis, M.G., Monjardino, J., Karayiannis, P., Montano, L. & Thomas, H.C. 1984. Contribution of low level HBV replication to continuing inflammatory activity in patients with anti-HBe positive chronic hepatitis B virus infection. *Gut*. 25(11):1283–1287.

Lok, A.S.F., Lai, C.-L., Wu, P.-C., Leung, E.K.Y. & Lam, T.-S. 1987. Spontaneous hepatitis B e antigen to antibody seroconversion and reversion in Chinese patients with chronic hepatitis B virus infection. *Gastroenterology*. 92(6):1839–1843.

Lok, A.S.F., McMahon, B.J., Brown, R.S., Wong, J.B., Ahmed, A.T., Farah, W., Almasri, J., Alahdab, F., et al. 2016. Antiviral therapy for chronic hepatitis B viral infection in adults: A systematic review and meta-analysis. *Hepatology*. 63(1):284–306.

Lopez, J.B., Balasegaram, M., Thambyrajah, V. & Timor, J. 1996. The value of liver function tests in hepatocellular carcinoma. *The Malaysian journal of pathology*. 18(2):95–9. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/10879229.

Lukhwareni, A., Burnett, R.J., Selabe, S.G., Mzileni, M.O. & Mphahlele, M.J. 2009. Increased detection of HBV DNA in HBsAg-positive and HBsAg-negative South African HIV/AIDS patients enrolling for highly active antiretroviral therapy at a Tertiary Hospital. *Journal of medical*

virology. 81(3):406–412.

Lv, X., Ye, G., Zhang, X. & Huang, T. 2017. p16 Methylation was associated with the development, age, hepatic viruses infection of hepatocellular carcinoma, and p16 expression had a poor survival. *Medicine*. 96(38):e8106.

Ma, X., Peng, J. & Hu, Y. 2014. Chinese Herbal Medicine-induced Liver Injury. *Journal of Clinical and Translational Hepatology*. 2(3):170–175.

Maeda, S., Kamata, H., Luo, J.L., Leffert, H. & Karin, M. 2005. IKKβ couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell*. 121(7):977–990.

Malungu Ngaira, J.A., Kimotho, J., Mirigi, I., Osman, S., Ng'ang'a, Z., Lwembe, R. & Ochwoto, M. 2016. Prevalence, awareness and risk factors associated with Hepatitis B infection among pregnant women attending the antenatal clinic at Mbagathi District Hospital in Nairobi, Kenya. *Pan African Medical Journal*. 24:1–7.

Mandishona, E., MacPhail, A.P., Gordeuk, V.R., Kedda, M.A., Paterson, A.C., Rouault, T.A. & Kew, M.C. 1998. Dietary iron overload as a risk factor for hepatocellular carcinoma in Black Africans. *Hepatology*. 27(6):1563–1566.

Mantzoukis, K., Rodriguez-Peralvarez, M., Buzzetti, E., Thorburn, D., Davidson, B.R., Tsochatzis, E. & Gurusamy, K.S. 2017. Pharmacological interventions for acute hepatitis B infection: an attempted network meta-analysis. *The Cochrane database of systematic reviews*. 3(3):CD011645.

Maponga, T.G. 2016. Hepatitis B virus-associated hepatocellular carcinoma in South Africa : epidemiology and impact of HIV-1 co-infection and immune dysregulation. Stellenbosch University, South Africa.

Marcellin, P., Ziol, M., Bedossa, P., Douvin, C., Poupon, R., De Lédinghen, V. & Beaugrand, M. 2009. Non-invasive assessment of liver fibrosis by stiffness measurement in patients with chronic hepatitis B. *Liver International*. 29(2):242–247.

Marcellin, P., Gane, E., Buti, M., Afdhal, N., Sievert, W., Jacobson, I.M., Washington, M.K., Germanidis, G., et al. 2013. Regression of cirrhosis during treatment with tenofovir disoproxil fumarate for chronic hepatitis B: A 5-year open-label follow-up study. *The Lancet*. 381(9865):468–475.

de Martel, C., Maucort-Boulch, D., Plummer, M. & Franceschi, S. 2015. World-wide relative contribution of hepatitis B and C viruses in hepatocellular carcinoma. *Hepatology*. 62(4):1190–

1200.

Martina, E., Degen, M., Rüegg, C., Merlo, A., Lino, M.M., Chiquet-Ehrismann, R. & Brellier, F. 2010. Tenascin-W is a specific marker of glioma-associated blood vessels and stimulates angiogenesis in vitro. *The FASEB Journal*. 24(3):778–787.

Martins-Filho, S.N., Paiva, C., Azevedo, R.S. & Alves, V.A.F. 2017. Histological Grading of Hepatocellular Carcinoma-A Systematic Review of Literature. *Frontiers in medicine*. 4(November):193.

Mason, W.S., Seal, G. & Summers, J. 1980. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. *Journal of virology*. 36(3):829–836.

Matsuda, Y., Ichida, T., Matsuzawa, J., Sugimura, K. & Asakura, H. 1999. p16(INK4) is inactivated by extensive CpG methylation in human hepatocellular carcinoma. *Gastroenterology*. 116(2):394–400.

Maucort-Boulch, D., de Martel, C., Franceschi, S. & Plummer, M. 2018. Fraction and incidence of liver cancer attributable to hepatitis B and C viruses worldwide. *International journal of cancer*. 142(12):2471–2477.

Mayaphi, S.H., Rossouw, T.M., Masemola, D.P., Olorunju, S.A.S., Mphahlele, M.J. & Martin, D.J. 2012. HBV/HIV co-infection : The dynamics of HBV in South African patients with AIDS. *South African Medical Journal*. 102(3):157–162.

Mayaphi, S.H., Martin, D.J., Mphahlele, M.J., Blackard, J.T. & Bowyer, S.M. 2013. Variability of the preC/C region of hepatitis B virus genotype A from a South African cohort predominantly infected with HIV. *Journal of Medical Virology*. 85(11):1883–1892.

McKillop, I. & Schrum, L. 2009. Role of Alcohol in Liver Carcinogenesis. *Seminars in Liver Disease*. 29(02):222–232.

McMahon, B.J. 2009. The natural history of chronic hepatitis B virus infection. *Hepatology*. 49(S5):S45–S55.

McMahon, B.J., Alward, W.L., Hall, D.B., Heyward, W.L., Bender, T.R., Francis, D.P. & Maynard, J.E. 1985. Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state. *The Journal of infectious diseases*. 151(4):599–603.

Mdlalose, N., Parboosing, R. & Moodley, P. 2016. The prevalence of hepatitis B virus infection in HIV-

positive and HIV-negative infants: KwaZulu-Natal, South Africa. *African Journal of Laboratory Medicine*. 5(1):8–12.

Meintjes, G., Moorhouse, M.A., Carmona, S., Davies, N., Dlamini, S., Van Vuuren, C., Manzini, T., Mathe, M., et al. 2017. Adult antiretroviral therapy guidelines 2017. *Southern African Journal of HIV Medicine*. 18(1).

Merlo, A., Herman, J.G., Mao, L., Lee, D.J., Gabrielson, E., Burger, P.C., Baylin, S.B. & Sidransky, D. 1995. 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nature medicine*. 1(7):686–92. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/7585152.

Mikeska, T., Felsberg, J., Hewitt, C.A. & Dobrovic, A. 2011. Analysing DNA Methylation Using Bisulphite Pyrosequencing. In *Epigenetics Protocols*. 33–53.

Miller, A.L., Garcia, P.L., Pressey, J.G., Beierle, E.A., Kelly, D.R., Crossman, D.K., Council, L.N., Daniel, R., et al. 2017. Whole exome sequencing identified sixty-five coding mutations in four neuroblastoma tumors. *Scientific Reports*. 7(1):1–12.

Mills, E., Foster, B.C., van Heeswijk, R., Phillips, E., Wilson, K., Leonard, B., Kosuge, K. & Kanfer, I. 2005. Impact of African herbal medicines on antiretroviral metabolism. *AIDS*. 19(1):95–7.

Mohamed, A.E., Kew, M.C. & Groeneveld, H.T. 1992. Alcohol consumption as a risk factor for hepatocellular carcinoma in urban southern African blacks. *International journal of cancer*. 51(4):537–41. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/1318267.

Mphahlele, M.J., Lukhwareni, A., Burnett, R.J., Moropeng, L.M. & Ngobeni, J.M. 2006. High risk of occult hepatitis B virus infection in HIV-positive patients from South Africa. *Journal of Clinical Virology*. 35(1):14–20.

Narlikar, G.J., Sundaramoorthy, R. & Owen-Hughes, T. 2013. Mechanisms and Functions of ATP-Dependent Chromatin-Remodeling Enzymes. *Cell*. 154(3):490–503.

Nassal, M. & Schaller, H. 1993. Hepatitis B virus replication. *Trends in microbiology*. 1(6):221–8. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/8137119.

Naugler, W.E., Sakurai, T., Kim, S., Maeda, S., Kim, K., Elsharkawy, A.M. & Karin, M. 2007. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. *Science*. 317(5834):121–4.

Nayagam, S., Conteh, L., Sicuri, E., Shimakawa, Y., Suso, P., Tamba, S., Njie, R., Njai, H., et al. 2016.

Cost-effectiveness of community-based screening and treatment for chronic hepatitis B in The Gambia: an economic modelling analysis. *The Lancet Global Health*. 4(8):e568–e578.

Ncube, E., Flett, B.C., Waalwijk, C. & Viljoen, A. 2010. Occurrence of aflatoxins and aflatoxin-producing Aspergillus spp. associated with groundnut production in subsistence farming systems in South Africa. *South African Journal of Plant and Soil*. 27(2):195–198. [Online], Available: http://www.worldcat.org/oclc/16664630732544.

Ng, A.W.T., Poon, S.L., Huang, M.N., Lim, J.Q., Boot, A., Yu, W., Suzuki, Y., Thangaraju, S., et al. 2017. Aristolochic acids and their derivatives are widely implicated in liver cancers in Taiwan and throughout Asia. *Science Translational Medicine*. 9(412):eaan6446.

Niederau, C., Heintges, T., Lange, S., Goldmann, G., Niederau, C.M., Mohr, L. & Häussinger, D. 1996. Long-Term Follow-up of HBeAg-Positive Patients Treated with Interferon Alfa for Chronic Hepatitis B. *New England Journal of Medicine*. 334(22):1422–1427.

Nishida, N., Nagasaka, T., Nishimura, T., Ikai, I., Boland, C.R. & Goel, A. 2008. Aberrant methylation of multiple tumor suppressor genes in aging liver, chronic hepatitis, and hepatocellular carcinoma. *Hepatology*. 47(3):908–918.

Njai, H.F., Shimakawa, Y., Sanneh, B., Ferguson, L., Ndow, G., Mendy, M., Sow, A., Lo, G., et al. 2015. Validation of rapid point-of-care (POC) tests for detection of hepatitis B surface antigen in field and laboratory settings in the Gambia, Western Africa. *Journal of Clinical Microbiology*. 53(4):1156–1163.

Norder, H., Couroucé, A.M. & Magnius, L.O. 1994. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology*. 198(2):489–503.

Norder, H., Couroucé, A.-M., Coursaget, P., Echevarria, J.M., Lee, S.-D., Mushahwar, I.K., Robertson, B.H., Locarnini, S., et al. 2004. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology*. 47(6):289–309.

Núñez, M. 2006. Hepatotoxicity of antiretrovirals: Incidence, mechanisms and management. *Journal of Hepatology*. 44:S132–S139.

Nyirenda, M., Beadsworth, M.B.J., Stephany, P., Hart, C.A., Hart, I.J., Munthali, C., Beeching, N.J. & Zijlstra, E.E. 2008. Prevalence of infection with hepatitis B and C virus and coinfection with HIV in medical inpatients in Malawi. *Journal of Infection*. 57(1):72–77.

O'Hara, G.A., McNaughton, A.L., Maponga, T., Jooste, P., Ocama, P., Chilengi, R., Mokaya, J., Liyayi,

M.I., et al. 2017. Hepatitis B virus infection as a neglected tropical disease. *PLoS Neglected Tropical Diseases*. 11(10):e0005842.

Ochwoto, M., Chauhan, R., Gopalakrishnan, D., Chen, C.-Y., Ng, Z., Okoth, F., Kioko, H., Kimotho, J., et al. 2013. Genotyping and molecular characterization of hepatitis B virus in liver disease patients in Kenya. *Infection, genetics and evolution*. 20:103–10.

Olinger, C.M., Jutavijittum, P., Hübschen, J.M., Yousukh, A., Samountry, B., Thammavong, T., Toriyama, K. & Muller, C.P. 2008. Possible new hepatitis B virus genotype, southeast Asia. *Emerging infectious diseases*. 14(11):1777–80.

Oliphant, J.W. 1944. Jaundice Following Administration of Human Serum. *Bulletin of the New York Academy of Medicine*. 20(8):429–445. [Online], Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1869983/#reference-sec.

Ott, J.J., Stevens, G.A., Groeger, J. & Wiersma, S.T. 2012. Global epidemiology of hepatitis B virus infection: New estimates of age-specific HBsAg seroprevalence and endemicity. *Vaccine*. 30(12):2212–2219.

Ott, J.J., Horn, J., Krause, G. & Mikolajczyk, R.T. 2017. Time trends of chronic HBV infection over prior decades – A global analysis. *Journal of Hepatology*. 66(1):48–54.

Ozturk, M. 1991. p53 mutation in hepatocellular carcinoma after aflatoxin exposure. *The Lancet*. 338(8779):1356–9. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/1682737.

Pai, N.P., Vadnais, C., Denkinger, C., Engel, N. & Pai, M. 2012. Point-of-Care Testing for Infectious Diseases: Diversity, Complexity, and Barriers in Low- And Middle-Income Countries. *PLoS Medicine*. 9(9):e1001306.

Papatheodoridis, G. V., Manolakopoulos, S., Liaw, Y.F. & Lok, A. 2012. Follow-up and indications for liver biopsy in HBeAg-negative chronic hepatitis B virus infection with persistently normal ALT: A systematic review. *Journal of Hepatology*. 57(1):196–202.

Papatheodoridis, G. V., Manolakopoulos, S., Touloumi, G., Nikolopoulou, G., Raptopoulou-Gigi, M., Gogos, C., Vafiadis-Zouboulis, I., Karamanolis, D., et al. 2015. Hepatocellular carcinoma risk in HBeAg-negative chronic hepatitis B patients with or without cirrhosis treated with entecavir: HepNet.Greece cohort. *Journal of viral hepatitis*. 22(2):120–7.

Paul, S., Saxena, A., Terrin, N., Viveiros, K., Balk, E.M. & Wong, J.B. 2016. Hepatitis B Virus Reactivation and Prophylaxis During Solid Tumor Chemotherapy: A Systematic Review and Meta-analysis. *Annals of internal medicine*. 164(1):30–40.

Pedersen, B.S. & Quinlan, A.R. 2018. Mosdepth: quick coverage calculation for genomes and exomes. *Bioinformatics*. 34(5):867–868.

Peeling, R.W., Holmes, K.K., Mabey, D. & Ronald, A. 2006. Rapid tests for sexually transmitted infections (STIs): the way forward. *Sexually transmitted infections*. 82(S5):v1-6.

Pfeifer, G.P. & Dammann, R. 2005. Methylation of the Tumor Suppressor Gene RASSF1A in Human Tumors. *Biochemistry*. 70(5):576–583.

Pisa, P.T., Vorster, H.H., Kruger, A., Margetts, B. & Loots, D.T. 2015. Association of alcohol consumption with specific biomarkers: a cross-sectional study in South Africa. *Journal of health, population, and nutrition*. 33(1):146–56. [Online], Available: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4438658/pdf/jhpn0033-0146.pdf.

Popejoy, A.B. & Fullerton, S.M. 2016. Genomics is failing on diversity. *Nature*. 538(7624):161–164.

Prozesky, O.W., Szmuness, W., Stevens, C.E., Kew, M.C., Harley, E.J., Hoyland, J.A., Scholtz, J.E., Mitchell, A.D., et al. 1983. Baseline epidemiological studies for a hepatitis B vaccine trial in Kangwane. *South African Medical Journal*. 64(23):891–893. [Online], Available: http://www.scopus.com/inward/record.url?eid=2-s2.0-0021048903&partnerID=tZOtx3y1.

Public Health England. 2007. *Recommended protocol for HBV genotyping and for antiviral resistance analysis*. [Online], Available: https://www.gov.uk/government/publications/hepatitis-b-hbv-genotyping-protocol [2018, July 20].

QU, Z., JIANG, Y., LI, H., YU, D.-C. & DING, Y.-T. 2015. Detecting abnormal methylation of tumor suppressor genes GSTP1, P16, RIZ1, and RASSF1A in hepatocellular carcinoma and its clinical significance. *Oncology Letters*. 10(4):2553–2558.

Raimondo, G., Allain, J.-P., Brunetto, M.R., Buendia, M.-A., Chen, D.-S., Colombo, M., Craxì, A., Donato, F., et al. 2008. Statements from the Taormina expert meeting on occult hepatitis B virus infection. *Journal of hepatology*. 49(4):652–657.

Reddy, P., Zuma, K., Shisana, O., Kim, J. & Sewpaul, R. 2015. Prevalence of tobacco use among adults in South Africa: Results from the first South African National Health and Nutrition Examination Survey. *South African Medical Journal*. 105(8):648.

Regimbeau, J.M., Colombat, M., Mognol, P., Durand, F., Abdalla, E., Degott, C., Degos, F., Farges, O., et al. 2004. Obesity and diabetes as a risk factor for hepatocellular carcinoma. *Liver Transplantation*. 10(S2):S69–S73.

Rhee, S., Margeridon-Thermet, S., Nguyen, M.H., Liu, T.F., Kagan, R.M., Beggel, B., Verheyen, J., Kaiser, R., et al. 2010. Hepatitis B virus reverse transcriptase sequence variant database for sequence analysis and mutation discovery. *Antiviral Research*. 88(3):269–275.

Robin, L., Mboumba Bouassa, R.-S., Nodjikouambaye, Z.A., Charmant, L., Matta, M., Simon, S., Filali, M., Mboup, S., et al. 2018. Analytical performances of simultaneous detection of HIV-1, HIV-2 and hepatitis C- specific antibodies and hepatitis B surface antigen (HBsAg) by multiplex immunochromatographic rapid test with serum samples: A cross-sectional study. *Journal of Virological Methods*. 253:1–4.

Robotin, M.C., Kansil, M.Q., Porwal, M., Penman, A.G. & George, J. 2014. Community-based prevention of hepatitis-B-related liver cancer: Australian insights. *Bulletin of the World Health Organization*. 92(5):374–379.

Rocco, J.W. & Sidransky, D. 2001. p16(MTS-1/CDKN2/INK4a) in cancer progression. *Experimental Cell Research*. 264(1):42–55.

Ruiz-Tachiquín, M.-E., Valdez-Salazar, H.-A., Juárez-Barreto, V., Dehesa-Violante, M., Torres, J., Muñoz-Hernández, O. & Alvarez-Muñoz, M.-T. 2007. Molecular analysis of hepatitis B virus "a" determinant in asymptomatic and symptomatic Mexican carriers. *Virology journal*. 4(1):6.

Rusnati, M., Camozzi, M., Moroni, E., Bottazzi, B., Peri, G., Indraccolo, S., Amadori, A., Mantovani, A., et al. 2004. Selective recognition of fibroblast growth factor-2 by the long pentraxin PTX3 inhibits angiogenesis. *Blood*. 104(1):92–99.

Russo, F.P., Imondi, A., Lynch, E.N. & Farinati, F. 2018. When and how should we perform a biopsy for HCC in patients with liver cirrhosis in 2018? A review. *Digestive and liver disease*. 50(7):640–646.

Salmon-Ceron, D., Rosenthal, E., Lewden, C., Bouteloup, V., May, T., Burty, C., Bonnet, F., Costagliola, D., et al. 2009. Emerging role of hepatocellular carcinoma among liver-related causes of deaths in HIV-infected patients: The French national Mortalité 2005 study. *Journal of hepatology*. 50(4):736–45.

Sarin, S.K., Kumar, M., Lau, G.K., Abbas, Z., Chan, H.L.Y., Chen, C.J., Chen, D.S., Chen, H.L., et al. 2016. Asian-Pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. *Hepatology International*. 10(1):1–98.

Sawai, H., Nishida, N., Mbarek, H., Matsuda, K., Mawatari, Y., Yamaoka, M., Hige, S., Kang, J., et al. 2012. No association for Chinese HBV-related hepatocellular carcinoma susceptibility SNP in

other East Asian populations. 13–16.

Scherberich, A., Tucker, R.P., Degen, M., Brown-Luedi, M., Andres, A.-C. & Chiquet-Ehrismann, R. 2005. Tenascin-W is found in malignant mammary tumors, promotes alpha8 integrin-dependent motility and requires p38MAPK activity for BMP-2 and TNF-alpha induced expression in vitro. *Oncogene*. 24(9):1525–32.

Schulze, K., Imbeaud, S., Letouzé, E., Alexandrov, L.B., Calderaro, J., Rebouissou, S., Couchy, G., Meiller, C., et al. 2015. Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets. *Nature Genetics*. 47(5):505–511.

Schweitzer, A., Horn, J., Mikolajczyk, R.T., Krause, G. & Ott, J.J. 2015. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. *The Lancet*. 386(10003):1546–1555.

Seck, A., Ndiaye, F., Maylin, S., Ndiaye, B., Simon, F., Funk, A.L., Fontanet, A., Takahashi, K., et al. (in press). Poor Sensitivity of Commercial Rapid Diagnostic Tests for Hepatitis B e Antigen in Senegal, West Africa. *The American journal of tropical medicine and hygiene*. (June, 4):epub ahead of print.

Seeger, C. & Mason, W.S. 2015. Molecular biology of hepatitis B virus infection. *Virology*. 479–480(2):672–686.

Seeger, C., Ganem, D. & Varmus, H.E. 1986. Biochemical and genetic evidence for the hepatitis B virus replication strategy. *Science*. 232(4749):477–84. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/3961490.

Seeger, C., Mason, W.S., Seeger, C. & Mason, W.S. 2000. Hepatitis B Virus Biology Hepatitis B Virus Biology. 64(1):51–68.

Seitz, H.K. & Stickel, F. 2006. Risk factors and mechanisms of hepatocarcinogenesis with special emphasis on alcohol and oxidative stress. *Biological chemistry*. 387(4):349–60.

Servant-Delmas, A., Duong, L.T., Hamon, C., Houdah, A.K. & Laperche, S. 2015. Comparative performance of three rapid HBsAg assays for detection of HBs diagnostic escape mutants in clinical samples. *Journal of Clinical Microbiology*. 53(12):3954–3955.

Shen, J., Wang, S., Zhang, Y.J., Kappil, M., Wu, H.C., Kibriya, M.G., Wang, Q., Jasmine, F., et al. 2012. Genome-wide DNA methylation profiles in hepatocellular carcinoma. *Hepatology*. 55(6):1799–1808.

Sherman, M. 2001. Alphafetoprotein: an obituary. *Journal of hepatology*. 34(4):603–5.

Sherman, M. 2008. Recurrence of Hepatocellular Carcinoma. *New England Journal of Medicine*. 359(19):2045–2047.

Sherman, M., Peltekian, K.M. & Lee, C. 1995. Screening for hepatocellular carcinoma in chronic carriers of hepatitis B virus: incidence and prevalence of hepatocellular carcinoma in a North American urban population. *Hepatology*. 22(2):432–8.

Shimakawa, Y., Lemoine, M., Bottomley, C., Njai, H.F., Ndow, G., Jatta, A., Tamba, S., Bojang, L., et al. 2015. Birth order and risk of hepatocellular carcinoma in chronic carriers of hepatitis B virus: A case-control study in The Gambia. *Liver International*. 35(10):2318–2326.

Shimakawa, Y., Lemoine, M., Njai, H.F., Bottomley, C., Ndow, G., Goldin, R.D., Jatta, A., Jeng-Barry, A., et al. 2016. Natural history of chronic HBV infection in West Africa: a longitudinal population-based study from The Gambia. *Gut*. 65(12):2007–2016.

Shimakawa, Y., Njie, R., Ndow, G., Vray, M., Mbaye, P.S., Bonnard, P., Sombié, R., Nana, J., et al. 2018. Development of a simple score based on HBeAg and ALT for selecting patients for HBV treatment in Africa. *Journal of Hepatology*.

Shitani, M., Sasaki, S., Akutsu, N., Takagi, H., Suzuki, H., Nojima, M., Yamamoto, H., Tokino, T., et al. 2012. Genome-wide analysis of DNA methylation identifies novel cancer-related genes in hepatocellular carcinoma. *Tumor Biology*. 33(5):1307–1317.

Shivkumar, S., Peeling, R., Jafari, Y., Joseph, L. & Pai, N.P. 2012. Rapid point-of-care first-line screening tests for hepatitis B infection: a meta-analysis of diagnostic accuracy (1980-2010). *The American journal of gastroenterology*. 107(9):1306–1313.

Shouval, D. & Shibolet, O. 2013. Immunosuppression and HBV Reactivation. *Seminars in Liver Disease*. 33(02):167–177.

Simonetti, J., Bulkow, L., McMahon, B.J., Homan, C., Snowball, M., Negus, S., Williams, J. & Livingston, S.E. 2010. Clearance of hepatitis B surface antigen and risk of hepatocellular carcinoma in a cohort chronically infected with hepatitis B virus. *Hepatology*. 51(5):1531–1537.

Singh, H. & Mahmud, S.M. 2009. Different study designs in the epidemiology of cancer: case-control vs. cohort studies. *Methods in molecular biology (Clifton, N.J.)*. 471(12):217–25.

Sloane, D., Chen, H. & Howell, C. 2006. Racial disparity in primary hepatocellular carcinoma: tumor stage at presentation, surgical treatment and survival. *Journal of the National Medical Association*.

98(12):1934–9. [Online], Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2569668&tool=pmcentrez&rendertype=abstract%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/17225837%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2569668.

Song, E. 2005. Acute and chronic viral hepatitis. *Continuing Medical Education*. 23(8):398–405.

South African National Cancer Registry. 2014. *Cancer in South Africa 2014*. Johannesburg. [Online], Available: www.ncr.ac.za. Accessed 08 July 2018.

Spearman, C.W., Afihene, M., Ally, R., Apica, B., Awuku, Y., Cunha, L., Dusheiko, G., Gogela, N., et al. 2017. Hepatitis B in sub-Saharan Africa: strategies to achieve the 2030 elimination targets. *The Lancet Gastroenterology and Hepatology*. 2(12):2121.

Spearman, C.W.N., Sonderup, M.W., Botha, J.F., Van der Merwe, S.W., Song, E., Kassianides, C., Newton, K.A. & Hairwadzi, H.N. 2013. South African guideline for the management of chronic hepatitis B: 2013. *South African Medical Journal*. 103(5):335–349.

Sprengel, R., Kaleta, E.F. & Will, H. 1988. Isolation and characterization of a hepatitis B virus endemic in herons. *Journal of virology*. 62(10):3832–9. [Online], Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=253529&tool=pmcentrez&rendertype=abstract.

Stadler, Z.K., Schrader, K.A., Vijai, J., Robson, M.E. & Offit, K. 2014. Cancer Genomics and Inherited Risk. *Journal of Clinical Oncology*. 32(7):687–698.

Starokadomskyy, P., Gluck, N., Li, H., Chen, B., Wallis, M., Maine, G.N., Mao, X., Zaidi, I.W., et al. 2013. CCDC22 deficiency in humans blunts activation of proinflammatory NF-κB signaling. *Journal of Clinical Investigation*. 123(5):2244–2256.

Statistics South Africa. 2016. *Mid-year population estimates: 2016*. [Online], Available: https://www.statssa.gov.za/publications/P0302/P03022016.pdf [2018, July 15].

Stockwell, T., Chikritzhs, T., Holder, H., Single, E., Elena, M., Jernigan, D. & Dawson, D. 2000. *International guide for monitoring alcohol consumption and related harm.* [Online], Available: http://apps.who.int/iris/bitstream/10665/66529/1/WHO_MSD_MSB_00.4.pdf [2018, July 22].

Stuyver, L., De Gendt, S., Van Geyt, C., Zoulim, F., Fried, M., Schinazi, R.F. & Rossau, R. 2000. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *The Journal of general virology*. 81(Pt 1):67–74.

Sulkowski, M.S. 2003. Hepatotoxicity associated with antiretroviral therapy containing HIV-1 protease inhibitors. *Seminars in liver disease*. 23(2):183–94.

Summers, J., Smolec, J.M. & Snyder, R. 1978. A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. *Proceedings of the National Academy of Sciences of the United States of America*. 75(9):4533–7. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/212758.

Sweat, F., Puchtler, H. & Rosenthal, S.I. 1964. Sirius Red F3BA as a stain for connective tissue. *Archives of pathology*. 78:69–72. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/14150734.

Szymańska, K., Lesi, O. a, Kirk, G.D., Sam, O., Taniere, P., Scoazec, J.-Y., Mendy, M., Friesen, M.D., et al. 2004. Ser-249TP53 mutation in tumour and plasma DNA of hepatocellular carcinoma patients from a high incidence area in the Gambia, West Africa. *International journal of cancer*. 110(3):374–9.

Tabrizian, P., Jibara, G., Shrager, B., Schwartz, M. & Roayaie, S. 2015. Recurrence of hepatocellular cancer after resection: patterns, treatments, and prognosis. *Annals of surgery*. 261(5):947–55.

Tanaka, K., Hirohata, T., Takeshita, S., Hirohata, I., Koga, S., Sugimachi, K., Kanematsu, T., Ohryohji, F., et al. 1992. Hepatitis B virus, cigarette smoking and alcohol consumption in the development of hepatocellular carcinoma: a case-control study in Fukuoka, Japan. *International journal of cancer*. 51(4):509–14. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/1318264.

Tassopoulos, N.C., Papaevangelou, G.J., Sjogren, M.H., Roumeliotou-karayannis, A., Gerin, J.L. & Purcell, R.H. 1987. Natural history of acute hepatitis B surface antigen-positive hepatitis in Greek adults. *Gastroenterology*. 92(6):1844–1850.

Tatematsu, K., Tanaka, Y., Kurbanov, F., Sugauchi, F., Mano, S., Maeshiro, T., Nakayoshi, T., Wakuta, M., et al. 2009. A Genetic Variant of Hepatitis B Virus Divergent from Known Human and Ape Genotypes Isolated from a Japanese Patient and Provisionally Assigned to New Genotype J. *Journal of Virology*. 83(20):10538–10547.

Terrault, N.A., Bzowej, N.H., Chang, K.-M., Hwang, J.P., Jonas, M.M. & Murad, M.H. 2016. AASLD guidelines for treatment of chronic hepatitis B. *Hepatology*. 63(1):261–283.

Terrault, N.A., Lok, A.S.F., McMahon, B.J., Chang, K.-M., Hwang, J.P., Jonas, M.M., Brown, R.S., Bzowej, N.H., et al. 2018. Update on prevention, diagnosis, and treatment of chronic hepatitis B: AASLD 2018 hepatitis B guidance. *Hepatology*. 67(4):1560–1599.

Thakur, R. & Shankar, J. 2016. In silico Analysis Revealed High-risk Single Nucleotide Polymorphisms in Human Pentraxin-3 Gene and their Impact on Innate Immune Response against Microbial Pathogens. *Frontiers in Microbiology*. 7:192.

The Cancer of the Liver Italian Program (CLIP) Investigators. 1998. A new prognostic system for hepatocellular carcinoma: a retrospective study of 435 patients: the Cancer of the Liver Italian Program (CLIP) investigators. *Hepatology*. 28(3):751–5.

Thio, C.L., Seaberg, E.C., Skolasky, R., Phair, J., Visscher, B., Muñoz, A. & Thomas, D.L. 2002. HIV-1, hepatitis B virus, and risk of liver-related mortality in the Multicenter Cohort Study (MACS). *The Lancet*. 360(9349):1921–1926.

Thumbiran, N. V., Moodley, D., Parboosing, R. & Moodley, P. 2014. Hepatitis B and HIV co-infection in pregnant women: Indication for routine antenatal hepatitis B virus screening in a high HIV prevalence setting. *South African Medical Journal*. 104(4):307.

Tischoff, I. & Tannapfel, A. 2008. DNA methylation in hepatocellular carcinoma. *World Journal of Gastroenterology*. 14(11):1741.

Tong, M.J., Huynh, T.T. & Siripongsakun, S. 2013. Familial clustering of hepatocellular carcinoma in HBsAg-positive patients in the United States. *Hepatology International*. 7(4):1019–1029.

Tong, S., Li, J., Vitvitski, L. & Trépo, C. 1990. Active hepatitis B virus replication in the presence of anti-HBe is associated with viral variants containing an inactive pre-C region. *Virology*. 176(2):596–603.

Tseng, T.-C., Liu, C.-J., Yang, H.-C., Su, T.-H., Wang, C.-C., Chen, C.-L., Kuo, S.F.-T., Liu, C.-H., et al. 2012. High levels of hepatitis B surface antigen increase risk of hepatocellular carcinoma in patients with low HBV load. *Gastroenterology*. 142(5):1140-1149.e3; quiz e13–4.

Tseng, T.-C., Liu, C.-J., Yang, H.-C., Su, T.-H., Wang, C.-C., Chen, C.-L., Hsu, C.-A., Fang-Tzu Kuo, S., et al. 2013. Serum hepatitis B surface antigen levels help predict disease progression in patients with low hepatitis B virus loads. *Hepatology*. 57(2):441–450.

Turati, F., Edefonti, V., Talamini, R., Ferraroni, M., Malvezzi, M., Bravi, F., Franceschi, S., Montella, M., et al. 2012. Family history of liver cancer and hepatocellular carcinoma. *Hepatology*. 55(5):1416–1425.

Um, T.H., Kim, H., Oh, B.K., Kim, M.S., Kim, K.S., Jung, G. & Park, Y.N. 2011. Aberrant CpG island hypermethylation in dysplastic nodules and early HCC of hepatitis B virus-related human multistep hepatocarcinogenesis. *Journal of Hepatology*. 54(5):939–947.

Vaissière, T., Cuenin, C., Paliwal, A., Vineis, P., Hainaut, P. & Herceg, Z. 2009. Quantitative analysis of DNA methylation after whole bisulfitome amplification of a minute amount of DNA from body fluids. *Epigenetics*. 4(4):221–230.

Vanhomwegen, J., Kwasiborski, A., Sauvage, V., Boizeau, L., Hoinard, D., Candotti, D., Laperche, S. & Shimakawa, Y. 2018. Pan-genotypic loop-mediated isothermal amplification assay for HBV: a simple, rapid and affordable point-of-care test to semi-quantify HBV DNA. *Journal of Hepatology*. 68:S483–S484.

Vardas, E., Mathai, M., Blaauw, D., McAnerney, J., Coppin, A. & Sim, J. 1999. Preimmunization epidemiology of hepatitis B virus infection in South African children. *Journal of medical virology*. 58(2):111–5.

Volk, M.L. & Lok, A.S.F. 2009. Is family history of liver cancer a risk factor for hepatocellular carcinoma? *Journal of Hepatology*. 50(2):247–248.

Wang, J., Wang, J.L., Yang, X., Xia, K., Hu, Z.M., Weng, L., Jin, X., Jiang, H., et al. 2010. TGM6 identified as a novel causative gene of spinocerebellar ataxias using exome sequencing. *Brain*. 133(12):3510–3518.

Wang, K., Li, M. & Hakonarson, H. 2010. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic acids research*. 38(16):e164.

Wang, Z., Wang, Y., Ren, H., Jin, Y. & Guo, Y. 2017. ZNRF3 Inhibits the Invasion and Tumorigenesis in Nasopharyngeal Carcinoma Cells by Inactivating the Wnt/β-Catenin Pathway. *Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics*. 25(4):571–577.

Weber, R., Sabin, C.A., Friis-Møller, N., Reiss, P., El-Sadr, W.M., Kirk, O., Dabis, F., Law, M.G., et al. 2006. Liver-related deaths in persons infected with the human immunodeficiency virus: the D:A:D study. *Archives of internal medicine*. 166(15):1632–41.

Wilson, A.S., Power, B.E. & Molloy, P.L. 2007. DNA hypomethylation and human diseases. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*. 1775(1):138–162.

Wilson, J.M.G., Jungner, G. & World Health Organization. 1968. *Principles and Practice of Screening for Disease*. [Online], Available: http://www.who.int/iris/handle/10665/37650 [2018, July 15].

Wolk, A., Gridley, G., Svensson, M., Nyrén, O., McLaughlin, J.K., Fraumeni, J.F. & Adam, H.O. 2001. A prospective study of obesity and cancer risk (Sweden). *Cancer causes & control*. 12(1):13–21.

Wong, D.J. & Locarnini, S.A. 2018. Molecular Virology and Life Cycle. In Singapore: Springer

Singapore *Hepatitis B Virus and Liver Disease*. 1–23.

Wong, D.K.H., Huang, F.Y., Lai, C.L., Poon, R.T.P., Seto, W.K., Fung, J., Hung, I.F.N. & Yuen, M.F. 2011. Occult hepatitis B infection and HBV replicative activity in patients with cryptogenic cause of hepatocellular carcinoma. *Hepatology*. 54(3):829–836.

Wong, M.C.S., Jiang, J.Y., Goggins, W.B., Liang, M., Fang, Y., Fung, F.D.H., Leung, C., Wang, H.H.X., et al. 2017. International incidence and mortality trends of liver cancer: a global profile. *Scientific reports*. 7(March):45846.

Wong, V.W.S., Chan, S.L., Mo, F., Chan, T.C., Loong, H.H.F., Wong, G.L.H., Lui, Y.Y.N., Chan, A.T.C., et al. 2010. Clinical scoring system to predict hepatocellular carcinoma in chronic hepatitis B carriers. *Journal of Clinical Oncology*. 28(10):1660–1665.

World Health Organization. 2001. *Hepatitis B Surface Antigen Assays : Operational Characteristics*. [Online], Available: http://www.who.int/diagnostics_laboratory/evaluations/en/hep_B_rep1.pdf [2018, July 22].

World Health Organization. 2010. *WHO prequalification of in vitro diagnostics programmes public report*. [Online], Available: http://www.who.int/diagnostics_laboratory/evaluations/hepb/161125_who_performance_criteria_hbsag_ivd.pdf [2018, July 22].

World Health Organization. 2015. *Guidelines for the prevention, care and treatment of persons with chronic hepatitis B infection*. [Online], Available: http://apps.who.int/iris/bitstream/10665/154590/1/9789241549059_eng.pdf?ua=1&ua=1 [2017, May 15].

World Health Organization. 2017a. *World Health Organization Hepatitis B Fact sheet*. [Online], Available: http://www.who.int/mediacentre/factsheets/fs204/en/ [2018, July 14].

World Health Organization. 2017b. *Global hepatitis report, 2017*. [Online], Available: http://www.who.int/hepatitis/publications/global-hepatitis-report2017/en/ [2018, July 22].

World Health Organization. 2017c. *WHO guidelines on hepatitis B and C testing*. [Online], Available: http://www.who.int/hepatitis/publications/guidelines-hepatitis-c-b-testing/en/ [2017, May 15].

World Health Organization. 2018. *List of WHO Prequalification Evaluating laboratories*. [Online], Available: http://www.who.int/diagnostics_laboratory/evaluations/180529_list_of_pq_laboratories.pdf [2018, July 22].

World Health Organization & Médecins Sans Frontières South Africa. 2003. *Antiretroviral therapy in primary health care : experience of the khayelitsha programme in South Africa*. [Online], Available: http://www.msf.org.za/docs/WHO_2003_case_study.pdf.

Wu, H.-C., Yang, H.-I., Wang, Q., Chen, C.-J. & Santella, R.M. 2017. Plasma DNA methylation marker and hepatocellular carcinoma risk prediction model for the general population. *Carcinogenesis*. 38(10):1021–1028.

Xia, Y., Shen, S. & Verma, I.M. 2014. NF- B, an Active Player in Human Cancers. *Cancer Immunology Research*. 2(9):823–830.

Yan, H., Zhong, G., Xu, G., He, W., Jing, Z., Gao, Z., Huang, Y., Qi, Y., et al. 2012. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *eLife*. 2012(1):1–28.

Yang, J.D. & Roberts, L.R. 2010. Hepatocellular carcinoma: A global view. *Nature reviews. Gastroenterology & hepatology*. 7(8):448–58.

Yang, J.D. & Roberts, L.R. 2017. Early age at diagnosis of hepatocellular carcinoma in sub-Saharan Africa – Authors' reply. *The Lancet Gastroenterology and Hepatology*. 2(6):394.

Yang, A.S., Estécio, M.R.H., Doshi, K., Kondo, Y., Tajara, E.H. & Issa, J.-P.J. 2004. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic acids research*. 32(3):e38.

Yang, B., Guo, M., Herman, J.G. & Clark, D.P. 2003. Aberrant promoter methylation profiles of tumor suppressor genes in hepatocellular carcinoma. *American Journal of Pathology*. 163(3):1101–1107. [Online], Available: http://www.scopus.com/inward/record.url?eid=2-s2.0-0041924707&partnerID=tZOtx3y1.

Yang, H.-I., Lu, S.-N., Liaw, Y.-F., You, S.-L., Sun, C.-A., Wang, L.-Y., Hsiao, C.K., Chen, P.-J., et al. 2002. Hepatitis B e Antigen and the Risk of Hepatocellular Carcinoma. *New England Journal of Medicine*. 347(3):168–174.

Yang, H.-I., Sherman, M., Su, J., Chen, P.-J., Liaw, Y.-F., Iloeje, U.H. & Chen, C.-J. 2010. Nomograms for risk of hepatocellular carcinoma in patients with chronic hepatitis B virus infection. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 28(14):2437–44.

Yang, H.-I., Yuen, M.-F., Chan, H.L.-Y., Han, K.-H., Chen, P.-J., Kim, D.-Y., Ahn, S.-H., Chen, C.-J., et al. 2011. Risk estimation for hepatocellular carcinoma in chronic hepatitis B (REACH-B):

development and validation of a predictive score. *The Lancet Oncology*. 12(6):568–574.

Yang, J.D., Gyedu, A., Afihene, M.Y., Duduyemi, B.M., Micah, E., Kingham, T.P., Nyirenda, M., Nkansah, A.A., et al. 2015. Hepatocellular carcinoma occurs at an earlier age in Africans, particularly in association with chronic Hepatitis B. *American Journal of Gastroenterology*. 110(11):1629–1631.

Yang, J.D., Altekruse, S.F., Nguyen, M.H., Gores, G.J. & Roberts, L.R. 2017. Impact of country of birth on age at the time of diagnosis of hepatocellular carcinoma in the United States. *Cancer*. 123(1):81–89.

Yin, J., Xie, J., Liu, S., Zhang, H., Han, L., Lu, W., Shen, Q., Xu, G., et al. 2011. Association between the various mutations in viral core promoter region to different stages of hepatitis B, ranging of asymptomatic carrier state to hepatocellular carcinoma. *The American journal of gastroenterology*. 106(1):81–92.

Yu, M.M., Gu, X.J., Xia, Y., Wang, G.J., Kan, N.Y., Jiang, H.X., Wu, K.H., Ji, Y., et al. 2012. Relationship between HBV cccDNA expression in the human ovary and vertical transmission of HBV. *Epidemiology and infection*. 140(8):1454–60.

Yu, M.W., Chang, H.C., Liaw, Y.F., Lin, S.M., Lee, S.D., Liu, C.J., Chen, P.J., Hsiao, T.J., et al. 2000. Familial risk of hepatocellular carcinoma among chronic hepatitis B carriers and their relatives. *Journal of the National Cancer Institute*. 92(14):1159–64. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/10904089.

Yuen, M.-F., Wong, D.K.-H., Fung, J., Ip, P., But, D., Hung, I., Lau, K., Yuen, J.C.-H., et al. 2008. HBsAg Seroclearance in chronic hepatitis B in Asian patients: replicative level and risk of hepatocellular carcinoma. *Gastroenterology*. 135(4):1192–9.

Yuen, M.-F., Tanaka, Y., Fong, D.Y.-T., Fung, J., Wong, D.K.-H., Yuen, J.C.-H., But, D.Y.-K., Chan, A.O.-O., et al. 2009. Independent risk factors and predictive score for the development of hepatocellular carcinoma in chronic hepatitis B. *Journal of hepatology*. 50(1):80–8.

Zhan, H., Jiang, J., Sun, Q., Ke, A., Hu, J., Hu, Z., Zhu, K., Luo, C., et al. 2017. Whole-Exome Sequencing-Based Mutational Profiling of Hepatitis B Virus-Related Early-Stage Hepatocellular Carcinoma. *Gastroenterology research and practice*. 2017(2029315):1–5.

Zhang, Y.-J. 2010. Interactions of chemical carcinogens and genetic variation in hepatocellular carcinoma. *World journal of hepatology*. 2(3):94–102.

Zhang, B.H., Yang, B.H. & Tang, Z.Y. 2004. Randomized controlled trial of screening for

hepatocellular carcinoma. *Journal of Cancer Research and Clinical Oncology*. 130(7):417–422.

Zhang, J.-C., Gao, B., Yu, Z.-T., Liu, X.-B., Lu, J., Xie, F., Luo, H.-J. & Li, H.-P. 2014. Promoter hypermethylation of p14 ARF, RB, and INK4 gene family in hepatocellular carcinoma with hepatitis B virus infection. *Tumor Biology*. 35(3):2795–2802.

Zhang, Y.J., Ahsan, H., Chen, Y., Lunn, R.M., Wang, L.Y., Chen, S.Y., Lee, P.H., Chen, C.J., et al. 2002. High frequency of promoter hypermethylation of RASSF1A and p16 and its relationship to aflatoxin B1-DNA adduct levels in human hepatocellular carcinoma. *Molecular Carcinogenesis*. 35(2):85–92.

Zhong, R., Liu, L., Tian, Y., Wang, Y., Tian, J., Zhu, B., Chen, W., Qian, J., et al. 2014. Genetic variant in SWI/SNF complexes influences hepatocellular carcinoma risk: a new clue for the contribution of chromatin remodeling in carcinogenesis. *Scientific reports*. 4:4147.

Zhong, S., Yeo, W., Tang, M.W., Wong, N., Lai, P.B.S. & Johnson, P.J. 2003. Intensive hypermethylation of the CpG island of Ras association domain family 1A in hepatitis B virus-associated hepatocellular carcinomas. *Clinical Cancer Research*. 9(9):3376–3382.

Zhou, J., Yu, L., Gao, X., Hu, J., Wang, J., Dai, Z., Wang, J.F., Zhang, Z., et al. 2011. Plasma microRNA panel to diagnose hepatitis B virus-related hepatocellular carcinoma. *Journal of Clinical Oncology*. 29(36):4781–4788.

Zhou, Y., Lan, J., Wang, W., Shi, Q., Lan, Y., Cheng, Z. & Guan, H. 2013. ZNRF3 acts as a tumour suppressor by the Wnt signalling pathway in human gastric adenocarcinoma. *Journal of Molecular Histology*. 44(5):555–563.

Zhu, Y.-Z., Zhu, R., Fan, J., Pan, Q., Li, H., Chen, Q. & Zhu, H.-G. 2010. Hepatitis B virus X protein induces hypermethylation of p16(INK4A) promoter via DNA methyltransferases in the early stage of HBV-associated hepatocarcinogenesis. *Journal of viral hepatitis*. 17(2):98–107.

Zimmermann, A. 2017. Immunohistochemistry of Hepatocellular Carcinoma. In Cham: Springer International Publishing *Tumors and Tumor-Like Lesions of the Hepatobiliary Tract*. 65–90.

APPENDIX A: ETHICS APPROVAL LETTER FOR HEPATITIS B VIRUS POINT-OF-CARE TESTING STUDY

UNIVERSITEIT•STELLENBOSCH•UNIVERSITY
jou kennisvennoot • your knowledge partner

Approval Notice
Response to Deferral

02-Feb-2016
Chotun, Bibi BN

Ethics Reference #: S15/08/179

Title: **Rapid-test-based screening for hepatitis B virus infection in a cohort of workers attending occupational health clinics in the Western Cape, South Africa.**

Dear Miss Bibi Chotun,

The **Response to Deferral** - ***(New Application)*** received on **28-Oct-2015**, was reviewed by members of **Health Research Ethics Committee 1** via Expedited review procedures on **02-Feb-2016** and was approved.
Please note the following information about your approved research protocol:

Protocol Approval Period: **02-Feb-2016 -01-Feb-2017**

Please remember to use your **protocol number** (**S15/08/179**) on any documents or correspondence with the HREC concerning your research protocol.

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

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

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

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

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Helene Visser at City Health (Helene.Visser@capetown.gov.za Tel:

APPENDIX B: VISUAL AID (FLIP-CHART) USED FOR PATIENT RECRUITMENT AT OCSA CLINICS

Testing for hepatitis B using a rapid test

By Nafiisah Chotun, Eveline Schurink and Natalie Venema

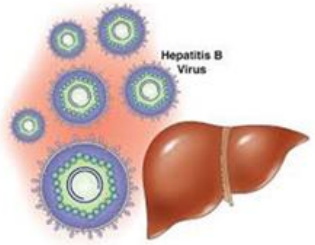
1 This is a university project that a student is doing

TYGERBERG HOSPITAL

STELLENBOSCH UNIVERSITY

2 She needs to find out how many people in Cape Town have hepatitis B

3 What is hepatitis B?

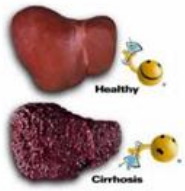


It is a very small virus that lives in the LIVER.

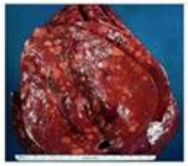
4 Is hepatitis B dangerous?

YES!
It can DAMAGE your liver and cause CANCER

Why is HBV infection dangerous?



Cirrhosis



Liver Cancer

5

How do you get hepatitis B?

From **MOTHER TO CHILD**

BETWEEN **CHILDREN**

THROUGH **SEXUAL** CONTACT

THROUGH **BLOOD** CONTACT
(transfusions, injections)

6

Where is the liver?

7

Is the liver important?

YES!

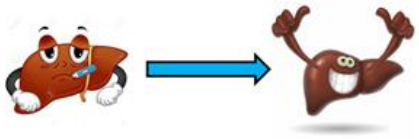
You CANNOT live without your liver!

PERSON WITH JAUNDICE (SYMPTOM OF LIVER DISEASE)

8

Can we treat hepatitis B?

YES! If we find out if you are sick **EARLY**, treatment can **STOP** the infection and even **REVERSE** the damage.



9 Why are you telling me this story?

This student needs your help! She wants to test **YOU** for hepatitis B.

She needs to test 1000 people, and she is asking **YOU** to help her by being part of her project.

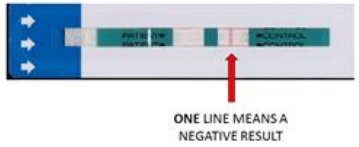
10 What will I have to do?

What if my result is negative?

11

It means you do **NOT** have hepatitis B!

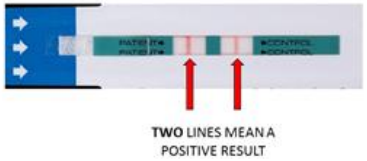
Your role stops here and you won't have anything else to do.



12 What if my result is positive?

It means you **have** hepatitis B.
You should see a doctor.

We will make an appointment for you to visit a doctor at **Tygerberg Hospital**.



13 How long will this visit take?

This doctor's visit will **only** take **2 hours** of your time and is **free**.

If you visit the doctor, you will get transport money in cash for coming to the hospital.

Mahalaaaaaaaa!

14 What will happen at the hospital?

The **doctor** will examine you and take your blood for tests.

These tests will help him decide if you need treatment or not.

The **student** will meet you and ask you some questions.

15 Why should I participate?

- You will know about your **health**!
- Since your family can also be sick, you can help **treat your family too**!

- You can get treatment if you are infected, and this will **improve your health**!

- You will help a student **pass** her exams!

16 Will my employer know about the result?

No!

The results are **confidential**.

Only you, the nurse and the student will know your result.

17 Can I change my mind later even if I said 'yes'?

OF COURSE!

You can change your mind at any time.

This will not affect your care in any way.

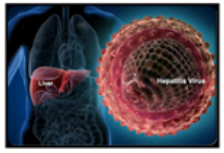
18 Is this a test for HIV?

NO!

Hepatitis B and HIV are two different things.

HIV lives in your **blood**.

Hepatitis B lives in your **liver**.



19 What if I don't want to participate?

YOU decide if you want to participate!

If you **CHOOSE NOT** to join the project, **NO PROBLEM**!

You will be treated the same way.

Remember: your participation is completely voluntary!

APPENDIX C: CONSENT FORM FOR PATIENT RECRUITMENT AT OCSA CLINICS

PARTICIPANT CONSENT FORM

TITLE OF THE RESEARCH PROJECT: Rapid-test-based screening for hepatitis B virus infection in a cohort of workers attending occupational health clinics in the Western Cape, South Africa

PRINCIPAL INVESTIGATOR: Nafiisah Chotun

ADDRESS: Division of Medical Virology

Stellenbosch University, Tygerberg Campus

CONTACT NUMBER: 021 938 9360

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the nurse any questions about any part that you do not understand.

This research project has been approved by the Health Research Ethics Committee at Stellenbosch University and it will be conducted according to international and locally accepted ethical guidelines for research, namely the Declaration of Helsinki, the SA Department of Health's 2004 Guidelines: *Ethics in Health Research: Principles, Structures and Processes* and the South African Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa.

What is this research project all about?

We want to test 1000 people to find out how many of them have hepatitis B. **H**epatitis **B** is found in the liver. In Africa, hepatitis B is mostly acquired in childhood and results in long-term infection. Long-term infection increases the risk of getting liver cancer. You may be infected with hepatitis B and not feel sick or show any signs of being sick.

Why have you been invited to participate?

Because you currently live in South Africa and are attending an occupational health clinic managed by OCSA and because you form part of the population at risk for infection with hepatitis B.

What will you have to do?

If you accept to take part in this project, the nurse will prick your finger and use a few drops of your blood for a rapid test for hepatitis B. This test is quick and the nurse will inform you of your result after 20 minutes.

If your result is negative: you will be informed of your result and your participation in the project will be complete. No further actions will be required from your side but your data will be recorded as part of the project data for analysis.

If your result is positive:

You will have to go to the hospital for more testing. The nurse will make an appointment for you. At the hospital, your blood will be taken and some tests (viral load, hepatitis B markers, liver enzyme levels and AFP level) will be done using that blood sample. You will also be offered two non-invasive scans (ultrasound scan and fibrosis scan). These tests will help the doctor decide if you need treatment or not. At the hospital, the same blood sample will also be used for other tests (genetics testing, bilirubin, albumin and platelet count) and those results will be used by the researchers of this project to answer some scientific questions. You will also be asked some questions so the researchers can understand

the disease (hepatitis B) better. These results will not be communicated to you as they will not affect your treatment in any way.

Will you benefit from taking part in this research?

The test is free and you will know whether you have hepatitis B or not, therefore giving insight on your own health status. Hepatitis B can be transmitted from mother to child, by sexual transmission and also through contact with infected blood. You will therefore also receive a letter so that your family and partners can go to Tygerberg Hospital to be tested for hepatitis B and get treated if they are positive or vaccinated if they are negative. This will be beneficial for them as they will know whether they need treatment or whether they need to be vaccinated to protect them.

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

As a result of the prick in your finger for the rapid test, there may be some minor pain or bruising.

If you need to go to the hospital, a blood sample will be taken for tests (described above) and as a result of the prick from the needle, there may be some minor pain or bruising.

If you do not agree to take part, what will happen?

Your participation is completely voluntary. If you do not want to participate, it will not affect your care in any way. You are also free to withdraw from the project at any point, even if you did agree to take part.

Who will have access to your medical records?

Only the researchers who are directly involved with the project or with your clinical care will have access to your medical records.

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

You will not be paid **to** take part in this project. However, all the tests associated with this project will be free. If you are positive for hepatitis B and have to go to the hospital, you will receive a refund in cash for your transport costs when you go to the hospital.

Is there anything else that you should know or do?

You can contact Miss Nafiisah Chotun at telephone number 021 938 9360 if you have any further questions or **encounter** any problems.

You can contact the Health Research Ethics Committee at telephone number 021 938 9207 if you have any concerns or complaints that have not been adequately addressed by your project nurse.

You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I ……..…… agree to take part in a research project entitled **Rapid-test-based screening for hepatitis B virus infection in a cohort of workers attending occupational health clinics in the Western Cape, South Africa**

I declare that:

- I have read this information and consent form and it is written in a language with which I am fluent and comfortable / Someone has read this information to me in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.

I understand that taking part in this project is **voluntary** and I have not been pressurised to take part. I may choose to leave the project at any time and will not be penalised or prejudiced in any way.

Signed at (place) .....…..…….. on (date) ……...…….. 20...

Signature of participant **Signature of witness**

Declaration by investigator

I (name) ……..…… declare that:

I explained the information in this document to ……..

I encouraged him/her to ask questions and took adequate time to answer them.
I am satisfied that he/she adequately understands all aspects of the research, as discussed above
I did/did not use an interpreter. (If an interpreter is used then the interpreter must sign the declaration below)

Signed at (place) .....…..…….. on (date) ……...…….. 20...

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

Declaration by interpreter

I (name) ……..…… declare that:

- I assisted the investigator (name) ……. to explain the information in this document to (name of participant) ……..…….. using the language medium of Afrikaans/Xhosa.
- I encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her questions satisfactorily answered.

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

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

APPENDIX D: STANDARDISED QUESTIONNAIRE FOR STUDY NURSES

CLINIC: **NURSE:** **AGE:** **GENDER: M ☐ F ☐**

Please mark your answers with a cross (☒). You may choose only one answer, unless specified otherwise.	
1.1 HBV rapid testing (the rapid test)	
1. On a scale of 1-5, how difficult (1) or easy (5) was it to perform the test?	1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐
2. Did you find any of the steps of the rapid test difficult?	Yes ☐ No ☐ I don't know ☐
3. **If you answered yes to question 2**, which steps did you find difficult when performing the test? Tick those that are relevant to you-multiple answers are possible. **If you answered no to question 2**, please proceed to question 4.	☐ Setting up the test and materials ☐ Opening the test strip ☐ Performing the finger prick ☐ Collecting the blood sample ☐ Adding the buffer ☐ Knowing when to read the result ☐ Reading the result ☐ None of the above ☐ Other (briefly describe below)
…….	
4. On a scale of 1-5, how confident were you that you read the test results correctly: not confident at all (1) - very confident (5)?	1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐
5. Do you think you received sufficient training to perform the HBV rapid test?	Yes ☐ No ☐ I don't know ☐
6. Did you trust the result of the HBV rapid test?	Yes ☐ No ☐ I don't know ☐
1.2 HBV rapid testing (general)	
1. Other than the HBV rapid test, have you previously performed a rapid test (e.g. HIV-1 rapid test)?	Yes ☐ No ☐ I don't know ☐
2. Did you have enough materials (lancets, cotton swabs, etc.) to carry out HBV rapid testing in your clinic?	Yes ☐ No ☐ I don't know ☐
3. Did HBV rapid testing interfere with providing other healthcare services?	Yes ☐ No ☐ I don't know ☐
4. On a scale of 1-5, how unimportant (1) or important (5) do you think it is to have a patient know their result after 20 minutes?	1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐
1.3 Adequacy of patient information testing	
1. Were you able to provide adequate pre-test information to the patient on HBV?	Yes ☐ No ☐ I don't know ☐
Elaborate if possible on your reasons why:	
2. Were you able to provide adequate post-test information to the patient on HBV?	Yes ☐ No ☐ I don't know ☐
Elaborate if possible on your reasons why:	

3. Were you able to provide adequate counselling to patients identified as being HBV positive?	Yes ☐ No ☐ I don't know ☐ Not applicable ☐
Elaborate if possible on your reasons why:	
4. On a scale of 1-5, how low (1) or how high (5) was the level of knowledge of the study participants on HBV?	1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐
5. On a scale of 1-5, how little (1) or how much (5) do you think patients understood the information they received about HBV?	1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐
Barriers to HBV rapid testing	
1. Were patients offended when offered HBV testing?	Yes ☐ No ☐ I don't know ☐
3. Were you comfortable discussing HBV rapid testing with patients?	Yes ☐ No ☐ I don't know ☐
4. Do you think HBV rapid testing may have had a negative effect on patients' opinion about your clinic?	Yes ☐ No ☐ I don't know ☐
5. On a scale of 1-5, how uninterested (1) or interested (5) were patients when offered the HBV rapid test?	1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐
6. Have a look at the potential barriers to HBV rapid testing listed. Were there any that were relevant to you? Multiple answers are possible. a. **If yes**, please tick all those that you think were relevant to you. b. **If none** were relevant to you, please tick "none of the above". c. **If you experienced** a barrier to HBV rapid testing **not listed**, please tick "Other" and specify your answer in the space provided.	☐ Lack of patient awareness on HBV ☐ Time taken for counselling ☐ Lack of patient interest in the test ☐ Language barriers ☐ Complexity of test (multiple steps) ☐ Time taken to read test ☐ Difficulty reading results ☐ Test interrupted routine workflow ☐ Test was unreliable ☐ Test was invasive ☐ None of the above ☐ Other (please specify below) ……
Confidentiality of HBV rapid testing	
1. Were patients concerned about the confidentiality of their result?	Yes ☐ No ☐ I don't know ☐
2. Were you able to convey the patient's result in a confidential manner?	Yes ☐ No ☐ I don't know ☐
Recommendations	
1. Do you think routine HBV testing is an important part of healthcare?	Yes ☐ No ☐ I don't know ☐
2. Would you recommend the use of this test at the clinic?	Yes ☐ No ☐ I don't know ☐
Elaborate on your reasons why:	

APPENDIX E: ETHICS APPROVAL LETTERS FOR BIOMARKER STUDY

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot • your knowledge partner

Approved with Stipulations
New Application

21-Oct-2013
CHOTUN, Bibi Nafiisah

Ethics Reference #: S13/04/084

Title: PILOT STUDY OF THE METHYLATION OF LIVER TISSUE FROM PATIENTS WITH HEPATOCELLULAR CARCINOMA (HCC)

DearMiss Bibi CHOTUN,

The **New Application** received on **25-Sep-2013**, was reviewed by members of **Health Research Ethics Committee 2** via Minimal Risk Review procedures on **02-Oct-2013**.

Please note the following information about your approved research protocol:

Protocol Approval Period: **21-Oct-2013 -21-Oct-2014**

The Stipulations of your ethics approval are as follows:
1. Waiver of informed consent granted.

Please remember to use your **protocol number** (S13/04/084) on any documents or correspondence with the HREC concerning your research protocol.

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

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

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

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

Provincial and City of Cape Town Approval

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

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

If you have any questions or need further assistance, please contact the HREC office at 0219389207.

Included Documents:
DEC LETTER WAINWRIGHT
COV LETTER
PROTOCOL
Application Form
DEC LETTERS

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6338 • **Facsimile** [021] 406 6411
Email: shuretta.thomas@uct.ac.za
Website: www.health.uct.ac.za/research/humanethics/forms

14 March 2014

HREC REF: 057/2014

Prof H Wainwright
Department of Pathology
D7
NGSH

Dear Prof Wainwright

PROJECT TITLE: *PILOT STUDY OF THE METHYLATION OF LIVER TISSUE FROM PATIENTS WITH HEPATOCELLULAR CARCINOMA (HCC)*

Thank you for your response letter to the Faculty of Health Sciences Human Research Ethics Committee.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30th March 2015

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/research/humanethics/forms)

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

Please quote the HREC reference no in all your correspondence.

Yours sincerely

pp T Burgess
PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN ETHICS
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938
This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.
The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

APPENDIX F: TABLE OF UNIVARIATE AND MULTIVARIATE LOGISTIC REGRESSION ANALYSES FOR *RASSF1A* PROMOTER HYPERMETHYLATION

Table Appendix-1 Univariate and multivariate logistic regression analyses for *RASSF1A* promoter hypermethylation status in HBsAg positive cases and controls

	Cases *n* = 23	Controls *n* = 10				
Hypermethylation status			**Crude OR (95% CI)**	**Significance**	**OR (95% CI)***	**Significance**
***RASSF1A* CpG site 1**						
Negative	17	10	1.0	-	1.0	-
Positive	6	0	-		-	
***RASSF1A* CpG site 2**						
Negative	18	10	1.0	-	1.0	-
Positive	5	0	-		-	
***RASSF1A* CpG site 3**						
Negative	14	10	1.0	-	1.0	-
Positive	9	0	-		-	
***RASSF1A* CpG site 4**						
Negative	12	10	1.0	-	1.0	-
Positive	11	0	-		-	
***RASSF1A* CpG site 5**			1.0		1.0	
Negative	14	10	-	-	-	-
Positive	9	0	1.0		1.0	
***RASSF1A* CpG site 6**			-		-	
Negative	15	10	1.0	-	1.0	-
Positive	8	0	-		-	
***RASSF1A* overall methylation**			1.0		1.0	
Negative	12	10	-	-	-	-
Positive	11	0	1.0		1.0	

RASSF1A: Ras association domain family 1 isoform A; CpG: CpG: deoxycytidylyl-deoxyguanosine dinucleotides. *adjusted for HBV genotype

APPENDIX G: ETHICS APPROVAL LETTER FOR WHOLE EXOME SEQUENCING STUDY

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot • your knowledge partner

Ethics Letter

18-Mar-2015

Ethics Reference #: N11/09/284A
Title: Whole exome sequencing of young South Africans with HBV-related hepatocellular carcinoma.

Dear Miss Bibi Chotun,

Your letter dated 14 January 2015 refers.

The Health Research Ethics Committee approved the amended documentation.

One stipulation:
A minor typo should be corrected in the revised informed consent form:
This research study has been approved by the ethics Health Research Ethics Committee at Stellenbosch University – should read:
This research study has been approved by the Health Research Ethics Committee at Stellenbosch University.

If you have any queries or need further help, please contact the REC Office 219389207.

Sincerely,

REC Coordinator
Mertrude Davids
Health Research Ethics Committee 2

APPENDIX H: HCC RISK FACTORS CASE RECORD FORM

HCC RISK CASE RECORD FORM

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

Patient folder number (*please place sticker here*):

DEMOGRAPHIC DATA

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

Gender: Male ☐ Female ☐

Weight *(in kilograms)*:_____ height *(in metres):*_____

Place of birth:_____

Area Born: Urban ☐ Rural ☐

Monthly income: _____

RISK FACTORS		
1. Family history of liver disease	**YES**	**NO**
2. If yes to family history of liver disease, please provide details		
3. Consumption of groundnuts		
4. Diagnosis of diabetes	**YES**	**NO**
5. Diagnosis of hyperlipidaemia	**YES**	**NO**
6. History of liver biopsy *If yes, please provide details (why was it performed?)*	**YES**	**NO**
7. Known diagnosis of any known cancer	**YES**	**NO**
8. HIV status (please tick appropriate response) ***If response is negative or unknown, skip to 17***	**POS**	**NEG**
9. If positive, state date of diagnosis		
10. Current CD4		

11. Nadir CD4		
12. HIV viral load		
13. HAART (please tick appropriate response) ***If response is no, please skip to 17***	**YES**	**NO**
14. Period of time on HAART		

15. If on HAART, please tick applicable below

Tenofovir

Lamivudine

Efavirenz

Nevirapine

AZT

Stavudine

DdI

Lopinivir/ritonavir

Azatanavir

Other (please state)

16. If on previous HAART, please tick applicable below

Tenofovir

Lamivudine

Efavirenz

Nevirapine

AZT

Stavudine

DdI

Lopinivir/ritonavir

Azatanavir

Other (please state)

17. **HBV status** HBsAg POS ☐ NEG ☐ UNKNOWN ☐

Anti-HBc	POS ☐	NEG ☐	UNKNOWN ☐
HBeAg	POS ☐	NEG ☐	UNKNOWN ☐
Anti-HBe	POS ☐	NEG ☐	UNKNOWN ☐

Date HBV diagnosed: _____

If HIV Negative:

Treatment for HBV: YES ☐ NO ☐

Treatment regimen: _____

Length of treatment:_____

Names of household and sexual contacts _____

_____

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

Interviewer name:

Signature: Date:

Blood collected by:

Signature: