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Comparison of the proteome of Huh7 cells transfected with different (sub)genotypes of Hepatitis B Virus prevailing in sub-Saharan Africa

Kiyasha Padarath (806617)

Thesis submitted to the Faculty of Health Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirement for the degree of Doctor of Philosophy (Ph.D.)

Declaration

I, Kiyasha Padarath, declare that this thesis is my own, unaided work and that all sources that I have used have been indicated by complete references. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



_____

Date: 8th of November 2024

Abstract

Hepatitis B Virus (HBV) is classified into nine genotypes, A to I, and at least 35 subgenotypes. In sub-Saharan Africa (SSA), (sub)genotypes A1, D3, and E prevail. Different mutations in the basic core promoter (BCP) and/or precore (PC) region that affect the expression of HBeAg can affect clinical outcomes and progression to hepatocellular carcinoma (HCC). Subgenotype A1, has high oncogenic potential and mutations affecting HBeAg expression at transcriptional, translation and post-translation levels, resulting in early HBeAg seroconversion. G1862T mutation, frequently found in subgenotype A1 HBV isolated from HCC patients, interferes with signal peptide cleavage and leads to a decreased expression of HBeAg. The effect of the SSA HBV (sub)genotypes and the G1862T mutation on protein expression and host signalling pathways has not been studied. Therefore, in this thesis Huh7 cells were transfected with replication-competent clones of (sub)genotypes A1, A2, D3 E and G1862T mutation to study the proteome using mass spectrometry. Proteomic analysis revealed significantly differentially expressed proteins between different SSA (sub)genotypes and A1 with G1862T compared to the wild-type. These differentially expressed proteins were classified into signalling pathways. The different (sub)genotypes and the G1862T mutant of HBV were shown to affect various signalling pathways involved in three hallmarks of cancer including: immune evasion and persistence, sustained proliferative signalling and genome instability and mutation. In particular, subgenotype A1 was shown to favour the dysregulation of MAPK (immune evasion and persistence) and PI3K/Akt/mTOR (sustained proliferative signalling) pathways and when accompanied by the G1862T mutation showed dysregulation of DNA synthesis pathway leading to uncontrolled proliferation (genome instability and mutation). This was the first study that demonstrated, using comprehensive proteomic analysis differences among SSA genotypes and the effect of G1862T in protein expression *in vitro*. It provides novel insights into the molecular underpinnings of the oncogenic potential of HBV.

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Research Outputs

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3. Padarath, K.; Deroubaix, A.; Naicker, P.; Stoychev, S.; Kramvis, A. Comparison of the Proteome of Huh7 Cells Transfected with Hepatitis B Virus Subgenotype A1, with or without G1862T. Curr. Issues Mol. Biol. 2024, 46, 7032-7047. https://doi.org/10.3390/cimb46070419

Conferences: At various stages of the study, the research was presented at several meetings. Presentations marked with an asterisk (*) received awards.

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2. Poster presentation at the International HBV Meeting, Kobe, Japan: Sept. 19th -23rd 2023
3. *Poster presentation at the Biannual School of Clinical Medicine Research Day, University of Witwatersrand, South Africa: Sept 28th 2023 (Awarded 2nd place for best poster)
4. *Poster presentation at Wits GP & Physician's update, University of Witwatersrand, South Africa: Oct 21st -22nd 2023 (Awarded the best poster prize).
5. Poster presentation (online) at the World Hepatitis Summit (WHS), Lisbon, Portugal: April 9th -11th 2024.
6. Poster presentation at African Viral Hepatitis Convention, Cape Town, South Africa: May 15th -18th 2024.

Padarath K, Deroubaix A and Kramvis A. Proteomic analyses of Huh7 cells transfected with replication-competent clones of subgenotype A1, with and without the G1862T mutation.

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2. HBV Meeting, Kobe, Japan: Sept. 19th -23rd 2023
3. Oral presentation at the Biannual School of Clinical Medicine Research Day, University of Witwatersrand, South Africa: Sept 28th 2023
4. Poster presentation (online) at the World Hepatitis Summit (WHS), Lisbon, Portugal: April 9th -11th 2024.

Preface

This work in this thesis was undertaken at the Hepatitis Virus Diversity Research Unit, in the Department of Internal Medicine at the University of Witwatersrand, between January 2021 and July 2024. No patient samples or patient data was used in this study therefore an ethical waiver was obtained from the University of the Witwatersrand Research Ethics Committee (Medical), Johannesburg, South Africa (HREC: W-CBP-210416-01).

The aim of this study was the comparison of the proteome of Huh7 cells transfected with different (sub)genotypes of HBV prevailing in sub-Saharan Africa to study the effect on host signalling pathways.

Objective 1: To transfect Huh7 cells with replication competent clones of (sub)genotypes A1, A2, D3 and E, to study the proteome using mass spectrometry

Objective 2: To transfect Huh7 cells with replication-competent clones of wild-type and mutant G1862T mutant subgenotype A1 to study the proteome using mass spectrometry

Objective 3: Using bioinformatics, to determine pathways differentially affected by the different (sub)genotypes and between the wild-type and the G1862T mutant of subgenotype A1.

The objectives of the study were achieved, and the thesis is presented in the following structure:

- **Chapter One:** Introduction: General Overview
- **Chapter Two:** Paper I: Literature review: The Complex Role of HBeAg and Its Precursors in the Pathway to Hepatocellular Carcinoma, *Status: Published in Viruses.*
- **Chapter Three:** Paper II: Article: Comparative proteomic analysis of Huh7 cells transfected with sub-Saharan African Hepatitis B Virus (sub)genotypes reveals potential oncogenic factors, *Status: Published in Viruses.*
- **Chapter Four:** Paper III: Article: Proteomic analyses of Huh7 cells transfected with replication-competent clones of subgenotype A1, with and without the G1862T mutation, *Status: Published in Current Issues in Molecular Biology (CIMB)*.
- **Chapter Five:** Discussion and Conclusion.
- **References:** References for literature cited in the published papers appear within the reference section of the papers themselves. References for all other citations appear in

the reference section at the end of the thesis. Some references may appear in both the papers and the thesis.

- **Appendices:** Ethics certificate, permission to use publications in thesis, experimental workflow, and plagiarism declaration and report

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Abbreviations list

A1 Subgenotype A1

A2 Subgenotype A2

ALT Alanine aminotransferase

ANOVA Analysis of variance

anti-Hbe Anti-hepatitis B e

anti-HBs Anti-hepatitis B s

anti-HBc Anti-hepatitis B c

anti-HBV Anti-hepatitis B virus

anti-HBc Hepatitis core antibody

APS Ammonium Perusulphate

AST Serum aspartate aminotransferase

ATP Adenosine triphosphate

BCA Bicinchronic Acid Protein

BCP Basic core promoter

BLAST Basic Local Alignment Search Tool

bp Base Pairs

BSA Bovine Serum Albumin

cccDNA Covalently Closed Circular DNA

cDNA Complementary deoxyribonucleic acid

CHB Chronic hepatitis B

CMJAH Charlotte Maxeke Johannesburg Academic Hospital

CMV Cytomegalovirus

CP Core promoter

CCKR Cholecystokinin Receptor

CIMB Current Issues in Molecular Biology

CTD C Terminal Domain

D3 Subgenotype D3

DAPI 4',6-Diamidino-2-Phenylindole, Dihydrochloride

dATP Deoxyadenosine triphosphate

DEPC Diethyl pyrocarbonate

DMEM Dulbecco's modified Eagle's Medium

DNA Deoxyribonucleic acid

dNTP Deoxy nucleoside triphosphate

DR Direct repeat

dsDNA Double stranded deoxyribonucleic acid

DSL-DNA Double stranded linear deoxyribonucleic acid

DEPs Differentially Expressed Proteins

DIA Data Independent Acquisition

E. coli Escherichia coli

EB Elution buffer

EDTA Ethylene Diamine Tetra-acetic acid

eGFP Enhanced Green Fluorescent Protein

ELISA Enzyme-linked immunosorbent assay

ER Endoplasmic Reticulum

ERGIC Endoplasmic Reticulum Golgi intermediate compartment

ESCRT Endosomal Sorting Complexes Required for Transport

ETV Entecavir

EtOH Ethanol

Exo Exogenous

EGF Epidermal Growth Factor

EMT Epithelial to Mesenchymal Transition

ERK extracellular Signal-Regulated Kinase

FBS Foetal Bovine Serum

FDR False Discovery Rate

FGF Fibroblast Growth Factor

GAPDH Glyceraldehyde 3-phosphate dehydrogenase

GSEA Gene Set Enrichment Analysis

HBcAg Hepatitis B core antigen

HBeAg Hepatitis B e antigen

HBsAg Hepatitis B surface antigen

HBcrAg Hepatitis B Core Related Antigen

HBV Hepatitis B Virus

HBx Hepatitis B X protein

HCC Hepatocellular carcinoma

HCL Hydrochloric Acid

HCV Hepatitis C virus

HEC Human Ethical Committee

HepG2 Hepatoma G2

HIV Human Immunodeficiency Virus

HLA Human leukocyte antigen

HNF Hepatocyte nuclear factors

HRP Horseradish Peroxidase

HSPG Heparan sulphate proteoglycans

Huh7 Human Hepatocellular Carcinoma Cells

IFN Interferon

IgG Immunoglobulin G

I**gM** Immunoglobulin M

iHeps Hepatocyte-like cells

INDEL Insertions and deletions

iPSCs Induced pluripotent stem cells

JNK c-Jun N-terminal Kinase

kb Kilobase pairs

kDa Kilodaltons

KOD Thermococcus kodakaraensis

LAM Lamivudine

LB Luria-Bertani

LHBs Large Hepatitis B Surface Antigen

MHBs Middle Hepatitis B Surface Antigen

MOPS 3-(N-morpholino)propanesulfonic acid

mRNA Messenger RNA

M Middle

MTCT Mother To Child Transmission

MS Mass Spectrometry

MAPK Mitogen-Activated Protein Kinase

NA Nucleot(s)ide analogues

NES Normalised Enrichment Score

NCBI National Centre of Bioinformatics

NIBSC National Institute for Biological Standards and Controls

NRF National Research Foundation

NTCP Sodium taurocholate co-transporting polypeptide

NLS Nuclear Localization Signal

ORF Open reading frame

OBI Occult Hepatitis B infection

P Polymerase gene

PAS polydenylation site

PBS Phosphate Buffered Saline

PCR Polymerase chain reaction

pgRNA Pre-genomic RNA

PI3K/Akt/mTOR phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR)

PHH Primary human hepatocytes

preC/C preCore/Core gene

preE preE antigen

preS/S preS/Surface

preS1 pre Surface 1

PCA Principal Component Analysis

PLC Primary Liver Cancer

RAxML Randomized Axelerated Maximum Likelihood

rcDNA relaxed circular Deoxyribonucleic acid

RH RNase H domain

RNA Ribonucleic acid

RNase Ribonuclease

ROX 6-carboxy-X-rhodamine

RT Reverse transcription

RT-PCR Real Time Polymerase Chain Reaction

RT-qPCR Reverse transcription quantitative polymerase chain reaction

RAS Rat sarcoma

RHOC Ras homolog gene family, member C

S Hepatitis B surface gene

SD Spacer domain

SDS Sodium Dodecyl Sulphate

SDS-PAGE Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SHBs Small Hepatitis B Surface Antigen

siRNA Small interfering RNA

SWATH-MS Sequential Window Acquisition of all Theoretical Mass Spectra

SSA Sub- Saharan Africa

SSC saline-sodium citrate

ssDNA single stranded Deoxyribonucleic acid

STRING Search Tool for the Retrieval of Interacting Genes/Proteins

SVPs Sub Viral Particles

TAE Tris Acetate EDTA

TATA thymine-, and adenine

TBE Tris Borate EDTA

TBP TATA binding protein

TBS Tris-buffered saline

TD Terminal domain

TDF Tenofovir disoproxil fumarate

TE Tris EDTA

TEMED Tetramethylethylenediamine

Tris Tris(hydroxymethyl)aminomethane

TP Terminal protein

TNF-α Tumour Necrosis factor alpha

UPR Unfolded Protein Response

URR Upstream regulatory region

USA United States of America

V Volts

WHO World Health Organization

X X gene

“Do the best you can until you know better. Then when you know better, do better.”

-Maya Angelou

Chapter 1: Introduction

Hepatitis B, is caused by the hepatitis B Virus (HBV), a hepatotropic DNA virus belonging to the family *Hepadnaviridae* (Marion, Cullen et al. 1987). The family *Hepadnaviridae* consists of enveloped, partially double-stranded DNA viruses, with small circular genomes, which replicate via an RNA intermediate (Summers and Mason 1982). All members of the family are hepatotropic and can cause liver disease, including hepatitis, fulminant hepatitis, fibrosis, cirrhosis and hepatocellular carcinoma (HCC; liver cancer). Despite the availability of an effective vaccine, hepatitis B continues to be a major health concern, with over 254 million people chronically infected with HBV, with 1.2 million new infections each year. In 2022, hepatitis B resulted in an estimated 1.1 million deaths globally as a result of severe liver disease (WHO 2024).

1.1 Structure and Life cycle of the HBV

The complete HBV virion, known as the Dane particle, consists of different components (Dane, 1970; Trepo, 2015). The viral envelope consists of a lipid bilayer membrane in which the large surface protein (LHBs), middle (MHBs) and small antigen (SHBs) of hepatitis B surface antigen (HBsAg) proteins are embedded (Block and Guo 2007, Baumert, Meredith et al. 2014). Collectively L, S, M envelope proteins are known as the (HBsAg) (İnan & Tabak, 2015; Kann et al., 2007; Yan et al, 2012). Enclosed in the envelope is the viral capsid, which is 42 nm in diameter and composed of hepatitis B core antigen (HBcAg). The capsid contains a loosely packed partially double-stranded circular DNA, with an incomplete positive strand and the HBV-encoded DNA polymerase (Tuttleman, Pourcel et al. 1986, Seeger and Mason 2015, Venkatakrishnan and Zlotnick 2016) (Figure 1A).

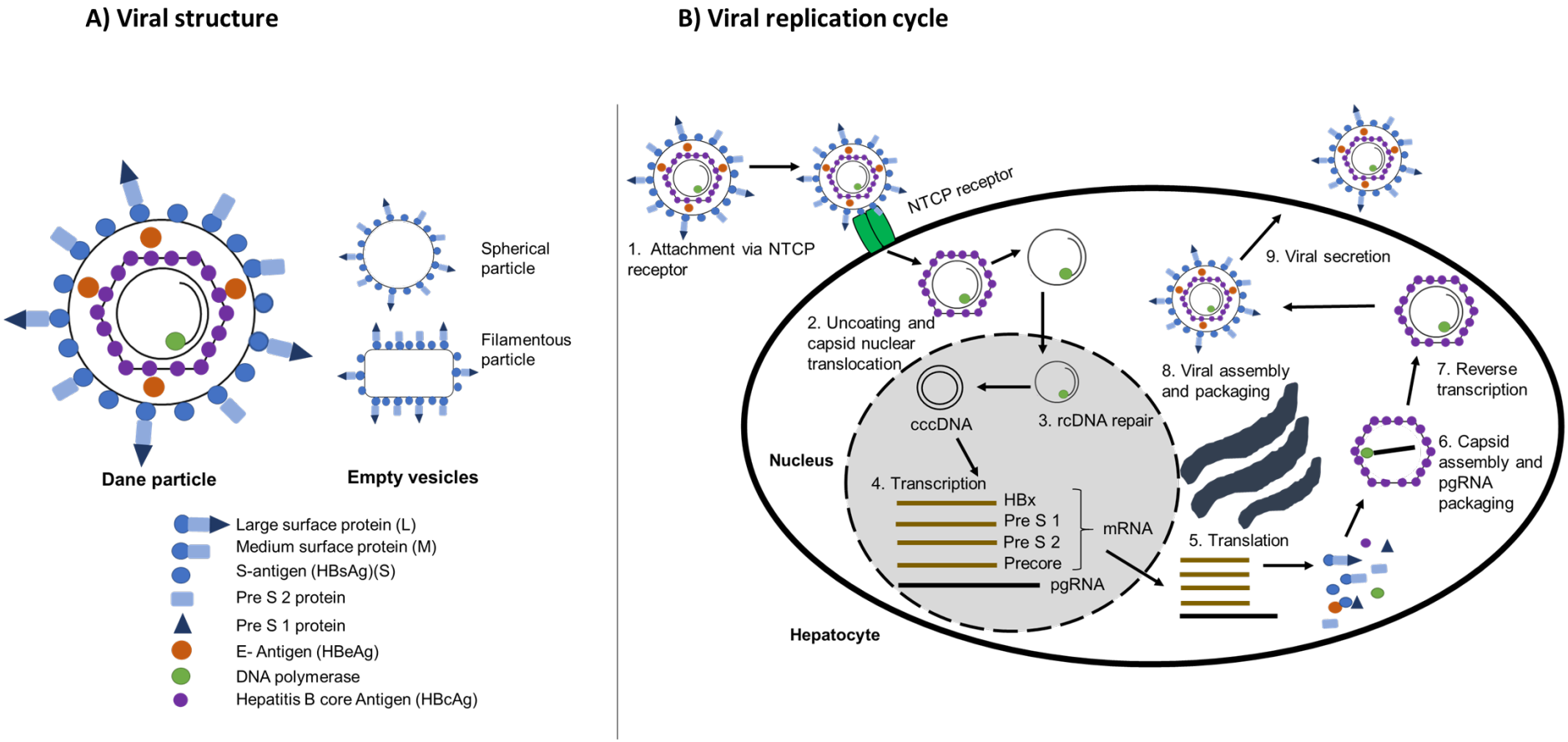


Figure 1: The structure (A) and replication cycle (B) of the HBV:

The image shows the structure of HBV (A) and replication cycle (B) of the HBV, adapted with permission from (İnan & Tabak, 2011)

To initiate an infection, the Dane particles, first bind loosely to heparan sulphate molecule on the surface of a hepatocytes (Nassal and Schaller 1996, Yan, Zhong et al. 2012, Venkatakrishnan and Zlotnick 2016), then the HBsAg initiates a high affinity binding with the sodium-taurocholate co-transporting polypeptide (NTCP) on the surface of hepatocytes, which allows the virion to be transported into the cytoplasm of the hepatocytes via endocytosis (Nassal and Schaller 1996, Yan, Zhong et al. 2012, İnan and Tabak 2015) (Figure 1B). After uncoating and release of the capsids in the cytoplasm, the DNA-filled capsids are imported into the nucleus, where disassembly occurs and the viral genome and the core proteins are released. The viral DNA is repaired through the host DNA repair mechanisms to become covalently closed circular DNA (cccDNA), which functions like host DNA (Wang and Seeger 1993, Seeger and Mason 2000, İnan and Tabak 2015) (Figure 1B). The cccDNA is transcribed using cellular mechanisms (Figure 1B). The 3.2 kilobases (kb) HBV genome has four overlapping reading frames and encodes for the five major RNA transcripts (Tiollais, Pourcel et al. 1985, Robaczewska, Narayan et al. 2005, Kramvis and Kew 2007, Spearman and Sonderup 2014). The RNA transcripts are two 3.5 kb, 2.4 kb, 2.1 kb and 0.7 kb in length (Levrero, Pollicino et al. 2009, Packianathan, Katen et al. 2010, Trépo, Chan et al. 2014). The two 3.5 kb transcripts function as the precore mRNA and the pre-genomic RNA (pgRNA), respectively (Packianathan, Katen et al. 2010). The mRNA molecules are transported out of the nucleus into the cytoplasm (Bartenschlager and Schaller 1992).

Regulatory and structural proteins are formed by the translation of the mRNA transcripts (Hirsch, Loeb et al. 1991, Urban, Schulze et al. 2010, Zhang, Hou et al. 2013). The 3.5 kb pgRNA is translated into the polymerase and HBcAg, whilst 3.5 kb precore mRNA is translated into the preCore protein. The 2.4 kb PreS1 mRNA is translated into the LHBs whilst the 2.1 kb PreS2/S mRNA is translated into MHBs and HBsAg. Lastly, the 0.7 kb HBx mRNA is translated into the X protein (Ganem and Varmus 1987, Beck and Nassal 2007, Urban, Schulze et al. 2010). (Figure 2).

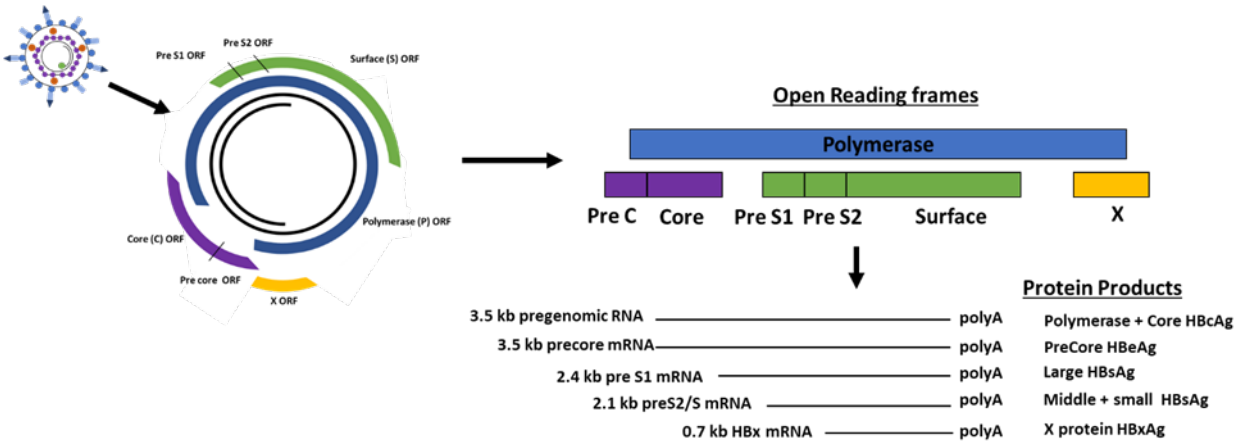


Figure 2: The genome of HBV.

The image shows the partially double-stranded DNA (dsDNA) and the four open reading frames shown in blue (polymerase ORF), green (PreS1/PreS2/S ORF for surface proteins, purple (precore/Core ORF), and yellow (X ORF). The translation of the mRNA and the protein products produced are also shown in the image. Image adapted with permission (İnan & Tabak, 2011)

The pgRNA template is used for the generation of the new viral DNA, however before this can occur, the pgRNA and the viral polymerase need to be encapsidated by HBcAg (Summers and Mason 1982, İnan and Tabak 2015). Following encapsidation, the viral polymerase binds to pgRNA and acts as a primer for the synthesis of the negative strand of the DNA chain through reverse transcription (Summers and Mason 1982, Summers, Smith et al. 1990, Wang and Seeger 1993, Beck and Nassal 2007, İnan and Tabak 2015, Seeger and Mason 2015). The negative strand and an RNase H are then used to synthesise an incomplete positive strand (Pugh, Zweidler et al. 1989, Loeb, Hirsch et al. 1991). During the synthesis of its DNA, HBV uses reverse transcriptase, lacking proofreading ability. Therefore, it is naturally prone to errors, which may end up with the evolution of rapidly mutant genes (Pugh, Zweidler et al. 1989, Trepo 2014, İnan and Tabak 2015) (Figure 1B).

Envelope proteins, which are translated by ribosomes on the endoplasmic reticulum (ER), are stored on the ER membrane and bud into the lumen after translation (Bruss, Lu et al. 1994, Bruss 2004). The HBeAg precursor p25 is translated and targeted to the ER membrane for post-translational modifications (Trépo, Chan et al. 2014, İnan and Tabak 2015). The envelope proteins are released via exocytosis as non-infectious sub-viral spherical and filamentous particles or infectious Dane particles contain the HBV DNA and nucleocapsid, which are

surrounded by envelope proteins (Blumberg 1965, Dane 1970, Thomas, Yoneda et al. 2015). The ability of HBV to release different structural states is characteristic of the virology of HBV and allows for evasion of the immune system (Figure 1B).

1.2 Viral proteins

1.2.1 HBsAg

The surface gene (S gene), which codes for the HBsAg, contains three in-frame start codons designated Pres1, Pres2 and S. This arrangement results in the production of proteins of 3 sizes (LHBs, MHBs and HBsAg), respectively. These proteins make up the viral envelope. HBsAg proteins also form non-infectious spherical and filamentous subviral particles (SVPs), approximately 22 nm in diameter (Figure 1A). These non-infectious SVPs are secreted in excess, approximately 1,000- to 100,000-fold higher than the infectious particles (Ganem and Prince 2004, Chai, Chang et al. 2008). Furthermore, several genetic variations in the three antigenic determinants of HBsAg determine the serological subtype (Blumberg et al., 1965; Magnius and Norder, 1995; Purdy et al., 2007). Besides being a structural protein, HBsAg can cause the deregulation of both innate and adaptive immune systems (Bruss 2007, Boltjes, van Montfoort et al. 2015, Le Bert, Gill et al. 2020, Carty, Guy et al. 2021).

1.2.2 HBV Polymerase

The 90 kDa viral DNA polymerase (pol) is the largest HBV protein. The polymerase is between 834 and 845 amino acids in length and is coded for by the polymerase gene which overlaps the surface gene (Seeger and Mason 2000). HBV DNA polymerase initiates viral replication by reverse transcription but lacks a proofreading function (Nowak, Bonhoeffer et al. 1996). First, the polymerase synthesises the single-stranded linear DNA by using the pgRNA as a template and subsequently synthesises partially double-stranded rcDNA (Yuen, Chen et al. 2018). The RNase H domain of the polymerase is responsible for RNA packaging and priming viral RNA binding, RNA packaging, protein priming, DNA synthesis, and RNA degradation (Hirsch, Lavine et al. 1990, Nowotny, Gaidamakov et al. 2005, Jones, Clark et al. 2014, Clark and Hu 2015).

1.2.3 HBx

HBx is the smallest HBV protein (154 amino acids), encoded by the X ORF, and is a non-structural protein (Seeger and Mason 2000, Bruss 2007, Slagle and Bouchard 2016). Several studies have shown HBx has various functions including transactivator of the viral and cellular promoters, epigenetic modifications, ubiquitination, autophagy and non-coding RNA regulation, stabilisation and activation of cccDNA (Cha, Ryu et al. 2009, Slagle and Bouchard 2016, Zhang, Han et al. 2017). Growing evidence shows that HBx plays an important role in viral persistence and liver pathogenesis, especially in hepatocarcinogenesis (Sartorius, Swadling et al. 2020, Sartorius, An et al. 2021, Zhao, Xie et al. 2021, Sivasudhan, Blake et al. 2022).

1.2.4 Molecular biology of HBeAg and HBcAg

HBeAg, is a 17 kDa secreted protein expressed by every member of the family *Hepadnaviridae,* though its expression is not required to maintain infection. HBeAg is thought to have an immune-modulatory role that allows for humoral and cell-mediated immune evasion (You, Yang et al. 2004, Milich 2019). In addition, studies have shown that HBeAg can protect hepatocytes from apoptosis, by directing the immune response away from infected hepatocytes and contributing towards the development of HBV chronicity. Therefore, the antigen is described as both an immunogen and a tolerogen. HBeAg has been used clinically as an index of viral replication, infectivity, severity of disease and response to treatment (Milich and Liang 2003, You, Yang et al. 2004, Milich 2019).

HBcAg is a 21 kDa protein, which shares the carboxyl end with HBeAg, and self-assembles to form the capsid (Schödel, Peterson et al. 1993, Crowther, Kiselev et al. 1994). Despite being a structural protein, it also binds to nuclear viral DNA, and is involved in the regulation of reverse transcription (Crowther, Kiselev et al. 1994). Furthermore, HBcAg affects the epigenetics of the virus and correlates with RNA specificity (Zlotnick, Venkatakrishnan et al. 2015).

The 25 kDa precursor of HBeAg (p25), is translated from the preCore/Core ORF on the precore mRNA, whilst HBcAg is translated from Core ORF on the pgRNA (Yuh, Chang et al. 1992, Yu and Mertz 1997, Kramvis, Kew et al. 2005) (Figure 3). The basic core promotor (BCP) of HBV controls the transcription of the precore/core from both transcripts. The translation of the

HBeAg precursor p25 from precore mRNA starts at the first ATG site, while the translation of the core protein on pgRNA starts at the second ATG (Bruss and Gerlich 1988, Garcia, Ou et al. 1988). p25 is translated and targeted to the ER membrane for post-translational modifications (Ou, Yeh et al. 1989, Trépo, Chan et al. 2014, İnan and Tabak 2015). In the ER, the 19 amino acid N terminal signal of p25 is proteolytically removed to yield the HBeAg precursor, p22 protein (Salhi, Messageot et al. 2001, Messageot, Salhi et al. 2003). Due to the presence of a nuclear localization signal (NLS) on the arginine-rich C-terminal domain (CTD) of p22, this protein can also be transported into the nucleus (Ou, Yeh et al. 1989, Yeh, Liaw et al. 1990). *In vitro* studies using sub-genomic p22, confirmed the protein's nuclear and cytoplasmic localization, however, the role of p22 in the nucleus is still to be determined (Ou, Yeh et al. 1989, Deroubaix and Kramvis 2021). The p22 protein undergoes further modification in the ER through the cleavage of the CTD by a furin endoprotease to give rise to the mature HBeAg, which is also known as p17 (Ito, Kim et al. 2009, Packianathan, Katen et al. 2010, Alexander, Jurgens et al. 2013, Venkatakrishnan and Zlotnick 2016). The mature HBeAg is secreted in a soluble form in the blood serum (Magnius and Espmark 1972, Di Bisceglie 2009, Padarath, Deroubaix et al. 2023) (Figure 3).

HBeAg can form part of a complex of three species of proteins known as HB core-related antigen (HBcrAg), which consists of related proteins sharing an identical 149 amino end sequence with different post-translational modifications on the carboxyl end: HBcAg (21 kDa), HBeAg (17 kDa), and a truncated 22 kDa precore protein (p22Cr). Like HBeAg, p22cr is also a processed product of the precore protein, but with protein processing only occurring at the C-terminal and not the amino end (Hong, Luckenbaugh et al. 2020) (Figure 3). Recent studies have shown that the detection of HBcrAg levels is a useful marker for disease monitoring, predicting treatment response, and disease outcome of chronic hepatitis B (CHB) and can also predict the risk of HCC (Honda, Shirasaki et al. 2016, Mak, Wong et al. 2018, Kramvis, Chang et al. 2022, Padarath, Deroubaix et al. 2023)

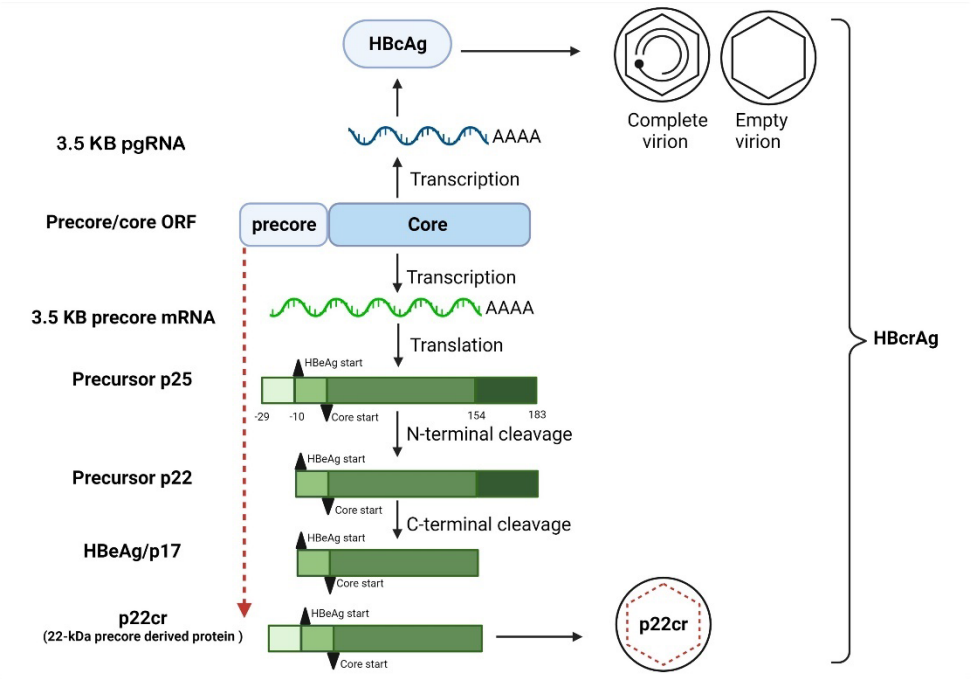


Figure 3: Biogenesis of HBeAg and its precursors.

This figure demonstrates HBeAg and HBcAg translation from the preCore/Core ORF, and has been used with permission from (Padarath, Deroubaix et al. 2023) (http://creativecommons.org/licenses/by/4.0/, accessed on 23 March 2024).

Precore and core mutations

Genetic mutations in the basic core promoter or precore region affect the expression of the HBeAg protein and its precursors leading to various clinical outcomes (Malik, Kumar et al. 2018). These mutations are (sub)genotype-specific and are discussed in more detail in Chapter 2 of this thesis.

1.3 Pathogenesis

When testing for HBV infection, several antigens and antibodies specific to HBV proteins are used, referred to as HBV biomarkers. These biomarkers are used to define different phases of HBV infection such as acute, chronic, or occult hepatitis B infection (OBI) (Kramvis, Chang et al. 2022) (Figure 4).

1.3.1 Acute infection

An acute HBV infection can be resolved within 6 months of exposure. During an acute infection, HBsAg and hepatitis B core antibody (anti-HBc) immunoglobulin M (IgM) are present (Conners, Panagiotakopoulos et al. 2023). HBsAg is positive in both acute and chronic HBV infection; however, the presence of IgM anti-HBc is a diagnostic marker of acute or recently acquired infection (Kramvis, Chang et al. 2022). When the infection has been resolved, the HBsAg is cleared while the total anti-HBc and anti-HBs remain positive (Conners, Panagiotakopoulos et al. 2023), the individual is therefore considered immune following natural HBV infection (Weber 2005).

1.3.2 Chronic infection

CHB infection is the persistence of HBsAg in the serum for longer than 6 months, without IgM anti-HBc. Several factors such as age, immune status of the individual and persistence of HBeAg for more than 3 months, may increase the risk of a patient developing CHB (WHO 2024). During CHB infection, HBeAg is used as a biomarker for viral infection and patient outcomes (Yuen, Sablon et al. 2002, Chauhan, Kazim et al. 2006). HBeAg seroconversion has an important impact on disease progression, with delayed seroconversion associated with poorer outcomes (Chu and Liaw 2007). For example, HBeAg loss associated with persistently elevated HBV DNA levels can lead to cirrhosis and hepatocellular carcinoma (HCC) (Alexopoulou and Karayiannis 2014). Therefore, HBeAg-negative CHB represents a clinical challenge (Ghosh, Mondal et al. 2012, Belyhun, Liebert et al. 2018).

1.3.3 Different phases of HBV infection

Briefly, the natural history of HBV can be divided into at least four phases, which are differentiated by the level of viral replication, the host immune response and HBeAg expression (Gish et al., 2015; Kramvis 2016) (Figure 4):

1. **The high replicative, low inflammatory phase:** is characterised by HBsAg/HBeAg-positive, with high HBV DNA levels (>2 × 10^5 IU/ml), with minimal liver inflammation, normal liver enzyme levels and asymptomatic.

2. **The immune clearance or reactive phase:** is characterised by fluctuating alanine amino transferase (ALT) and HBV DNA levels and ends with spontaneous HBeAg loss, by the emergence of precore and core mutations, HBeAg seroconversion is accompanied by elevated ALT and decreased HBV DNA levels.
3. **The HBeAg-negative chronic hepatitis phase**: is characterised by necroinflammation with high or fluctuating ALT levels and unsuccessful immune clearance, moderate to high viral loads and progressive liver disease.
4. **The low replicative phase (post-HBeAg seroconversion):** As a progression from the HBeAg-negative chronic hepatitis phase, this phase is also characterised by the absence of HBeAg, anti-HBe positivity, in this phase ALT levels are normal and HBV DNA levels are low or undetectable ($<2 \times 10^3$ IU/ml).

1.3.4 Occult hepatitis B infection (OBI)

Occult hepatitis is defined as the presence of HBV DNA in hepatocytes with detectable (< 200 IU/mℓ)/undetectable HBV DNA in the serum of individuals who test negative for the HBsAg using current diagnostic assays (Bell, Makondo et al. 2012, Raimondo, Locarnini et al. 2019). OBI can present itself at various stages of HBV infection and OBI can occur due to structural changes caused by escape mutations within the S gene, leading to the inability of the available serological tests to detect the infection (Bell, Makondo et al. 2012, Kramvis 2018).

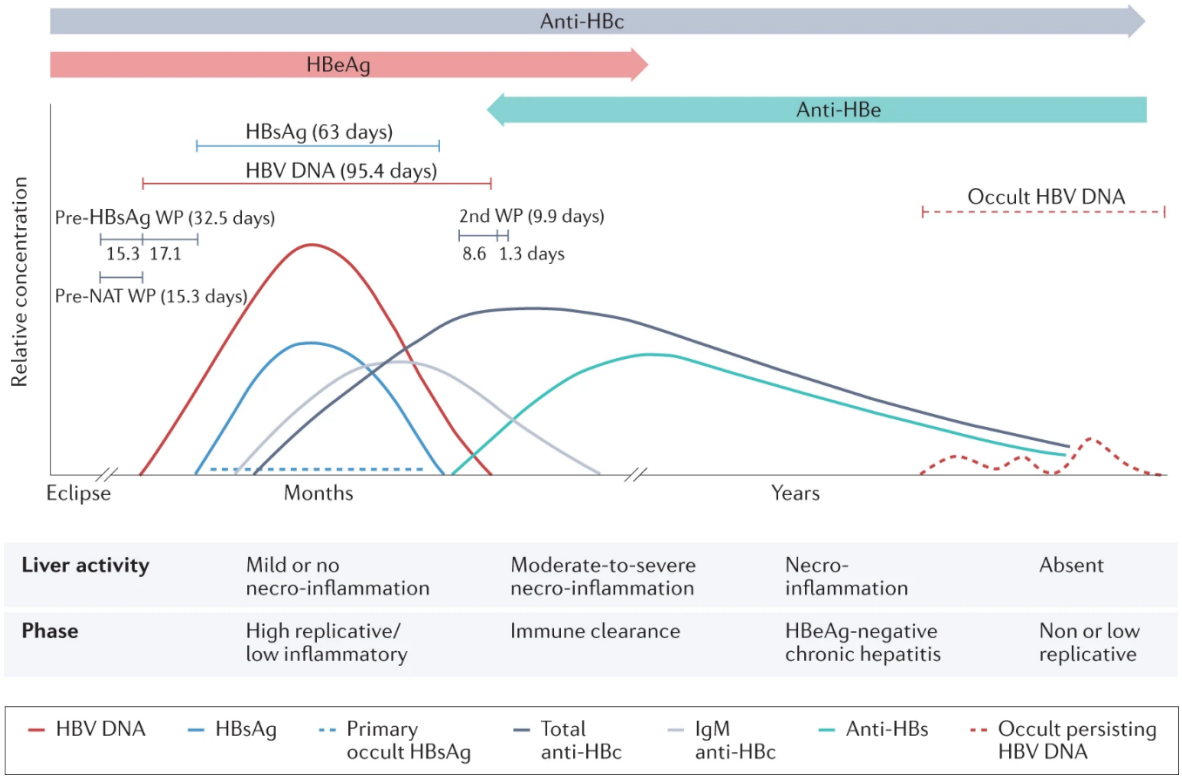


Figure 4: HBV markers during different phases of HBV infection.

Schematic presentation of HBV markers during the various phases of infection. The curves on the y-axis indicate the relative concentration of HBV markers in the natural infection. The curves and lines on the x-axis indicate the time each HBV marker or antibody first appears during infection and the length of the curves indicates the window periods for each marker (with or without detectable HBsAg) together with different activities in the liver in association with various phases of infection. Image used permission from (Kramvis, Chang et al. 2022) (Springer Nature© 5814131215327).

1.4 HBV Epidemiology in Africa

The WHO African region bears a disproportionate burden of chronic HBV infection, with 64 million chronic carriers, 71 000 deaths each year, and an estimated 70% of total new worldwide HBV infections (WHO 2024) (Figure 5). The high endemicity of HBV in Africa is a result of a sustained lack of investment in prevention, diagnosis, treatment, infrastructure, capacity, policy, advocacy, and education (Sonderup and Spearman 2022).

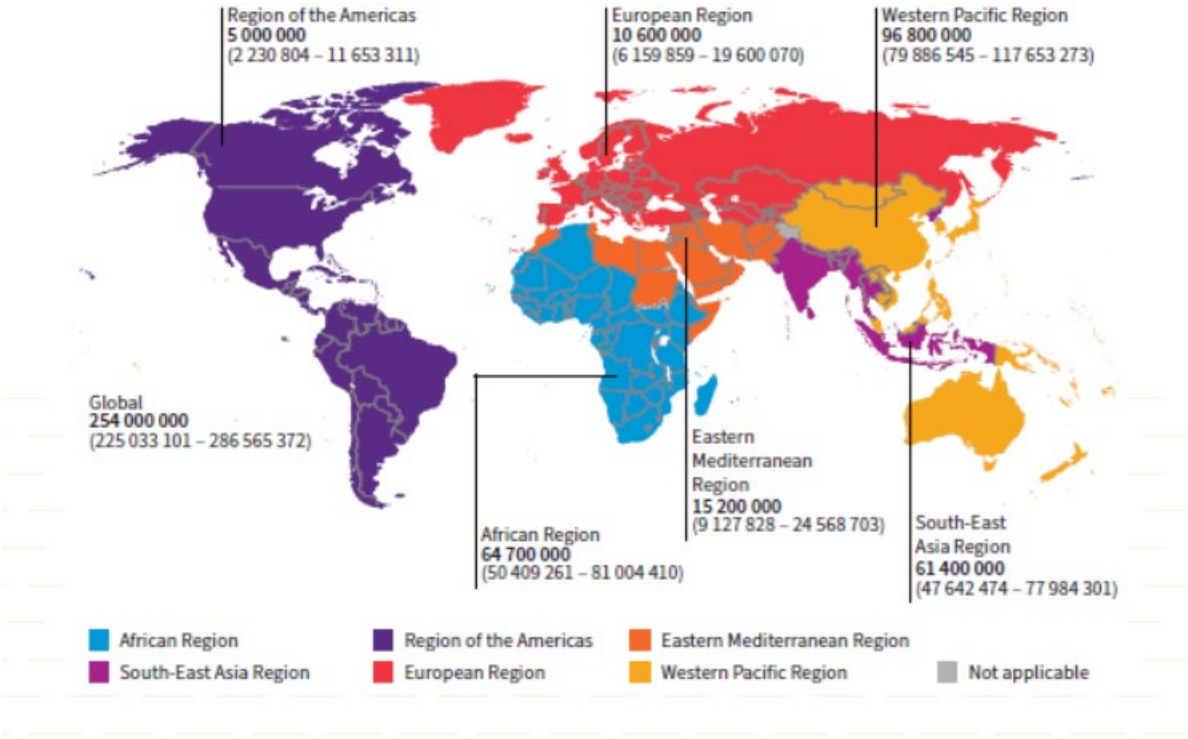


Figure 5: Burden of chronic HBV infection

Schematic map showing the burden of chronic HBV infection across the globe as measured by HBsAg positivity used with permission from (WHO 2024)(CC BY-NC-SA 3.0 IGO). The values in brackets represent the lower and upper limits for that region.

1.5 Mode of transmission

The transmission of HBV is parenteral as it is a blood-borne virus (CDC, 2006; Seeger & Mason, 2015). Before the 1960s, 40% of blood transfusion recipients were infected with HBV, as screening in blood donors was not yet implemented (Blumberg, 1965; İnan & Tabak, 2015; MacCallum, 1946). Nowadays transmission can occur vertically or horizontally. Mother to Child transmission (MTCT) is the most common form of vertical transmission, and there are three possible routes for transmission of HBV from an infected mother to her infant: transplacental transmission of HBV *in utero*, perinatal transmission during delivery, postnatal transmission during care (Hou, Liu et al. 2005). Furthermore, the risk of maternal-infant transmission is related to the presence of HBeAg, as 90% of HBeAg-positive mothers transmit HBV infection to their offspring compared to only 10%–20% of HBeAg-negative mothers (Stevens, Beasley et al. 1975, Navabakhsh, Mehrabi et al. 2011). The high frequency of perinatal transmission in endemic areas is probably related to the high prevalence of HBeAg-

positive women. Studies have shown that the rate of HBeAg seroconversion during the first 20 years of life is relatively slow, leaving many women of childbearing age who have contracted HBV infection in their early childhood still highly infectious to their infants (Mast, Margolis et al. 2005). The virus can survive up to 7 days in bodily fluids at 25°C and therefore be transmitted horizontally through abused intravenous drug needles, and unsterile surgical equipment. Furthermore, the virus can be transmitted through sexual intercourse (Naledi, Spearman, Pillay, & Sonderup, 2019).

In African countries, the majority of infections occur either perinatally or horizontally (Kew, 2013; Naledi et al., 2019). Perinatal infection, from mother to infant is the most frequent form of HBV transmission in children and has a 90% chance of chronicity (Spearman & Sonderup, 2014; WHO 2019). Children who become infected with HBV between 1 year and 5 years of age have a 30-50% chance of chronicity. In adulthood, the chance of chronicity is between 5-10% (Spearman & Sonderup, 2014; Trépo, Chan, & Lok, 2014; WHO 2019). Therefore, the age of the patient plays a huge role in whether or not the virus becomes chronic.

1.6 Vaccination

South Africa was one of the first ten countries in Africa to introduce the universal hepatitis B vaccine in 1995 and has been successful in increasing the immunity of South Africans against hepatitis B (WHO, 2000). However, there are still several improvements to be made such as implementing protocols to screen all pregnant mothers for hepatitis B surface antigen (HBsAg), antenatal testing and ensuring full hepatitis B coverage, especially in rural areas (Mahoney, 1999; Maponga et al., 2020). HBV vaccination can prevent 70%-95% of HBV infections in infants born to HBeAg and HBsAg-positive mothers (Lee, Gong et al. 2006, Navabakhsh, Mehrabi et al. 2011). In most post-exposure prophylaxis studies, the HBV vaccine has been administered to infants within 12-24 hours of birth. The efficacy of vaccines in preventing MTCT declines over time after birth (Harpaz, McMahon et al. 2000, Navabakhsh, Mehrabi et al. 2011). Therefore, to eliminate HBV by 2030, the WHO recommended that all newborn babies receive a hepatitis B birth dose (HepB-BD) vaccine, preferably in the first 24 hours of life (WHO 2019 , Njuguna, Hiebert et al. 2023, WHO 2024). Currently, South Africa has not fully implemented the birth-dose vaccine and it is only available to mothers who are HBsAg positive.

1.7 HBV-induced HCC

HBV is one of the seven known oncogenic viruses. At least 16% of the world's chronic carriers of HBV reside in sub-Saharan Africa (SSA) and this is probably an underestimate because of lack of surveillance. As measured by hepatitis B surface antigen (HBsAg) prevalence, most countries in Africa are of higher-intermediate (5%–7.99%), or high endemicity for HBV (> 8%) (Kramvis and Kew 2007, Kew 2013). This high HBsAg prevalence in SSA corresponds to the high incidence of HCC, being the second leading cancer for men and the third for women, with average age-standardized and incidence rate of 18.9 and 8.0 per 100,000 persons/year, respectively (Kew 2013, Ryerson, Eheman et al. 2016).

HBV infection can promote liver cancer through direct or indirect mechanisms as shown in figure 6. HBV infection can:

- generate genome instability by integrating its viral genome into the host genome or interfering with epigenetic and chromosomal remodelling, which can cause abnormal expression of oncogenes and tumour suppressor genes leading to activation of cancer-related pathways (Thomas, Baker et al. 2007, Guerrieri, Belloni et al. 2013, Jiang, Han et al. 2021).
- affect the liver microenvironment through interference with both the innate and adaptive immunity, and thus help the virus evade immune surveillance, increasing persistence and disease progression to tumour formation (Guerrieri, Belloni et al. 2013, Milich 2019).
- affect the liver microenvironment and increase cell proliferation (Guerrieri, Belloni et al. 2013).
- persist in liver tissue increasing chronic oxidative damage in hepatocytes and hypoxia (Bolukbas, Bolukbas et al. 2005).
- persist in liver tissue leading to immune-mediated inflammation of the liver, and development of HCC (Levrero and Zucman-Rossi 2016).
- interfere with apoptosis signalling to promote viral proliferation and HCC progression (Torresi, Tran et al. 2019, Jiang, Han et al. 2021).

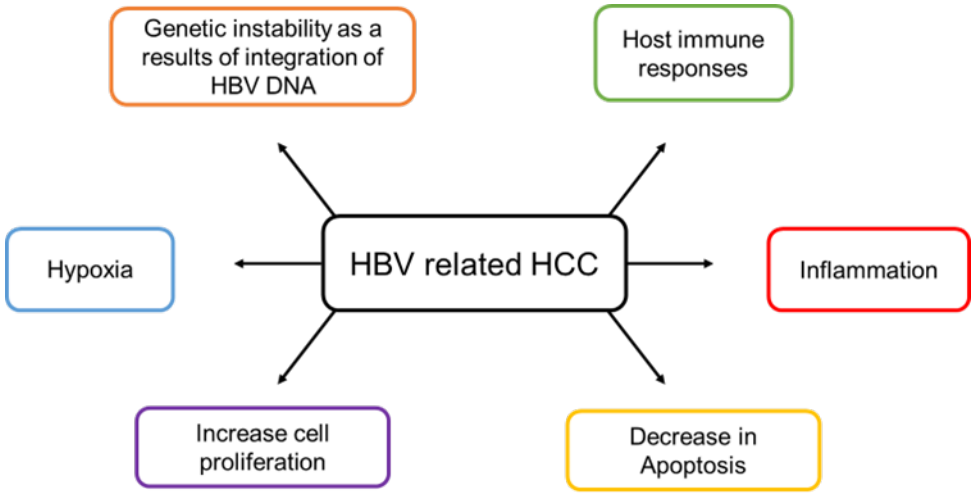


Figure 6: Different mechanisms of HBV-induced HCC

A diagram that shows the direct and indirect roles of HBV in hepatocellular carcinoma (HCC) pathogenesis, adapted with permission from (Guerrieri et al., 2013)

In addition, various proteins of HBV have also been implicated for their hepatocarcinogenic potential. In particular, HBxAg and HBsAg may be potentially oncogenic (Arbuthnot and Kew 2001, Sartorius, An et al. 2021). The investigation into the role of HBeAg and its precursors in carcinogenesis is ongoing. Therefore, Chapter 2 of this thesis explores the mechanisms by which HBeAg and its precursors can be associated with the different hallmarks of cancer.

1.8 HBV genotypes and subgenotypes

Because the HBV reverse transcriptase lacks proofreading ability, the virus accumulates genetic errors over time, leading to sequence heterogeneity (Summers, Smith et al. 1990, Wang and Seeger 1993, Norder, Couroucé et al. 2004, Kay and Zoulim 2007). This genetic diversity has resulted in at least nine genetically distinct HBV genotypes, designated A to I, and a 10th putative, genotype J (Norder, Couroucé et al. 2004, Kramvis, Kew et al. 2005, Tatematsu, Tanaka et al. 2009, Kramvis 2014), with > 7.5% intergroup nucleotide divergence (Okamoto, Tsuda et al. 1988, Norder, Couroucé et al. 1992, Kramvis, Kew et al. 2005, Yu, Yuan et al. 2010). The nine genotypes are further classified into subgenotypes; A1-A7, B1-B9, C1-C16, D1-D9, F1-F4 and I1-I2, based on a nucleotide divergence of 4-8% (Shi, Zhang et al. 2013,

Yousif and Kramvis 2013, Kramvis 2014, Pourkarim, Amini-Bavil-Olyaee et al. 2014). As shown in Figure 7A the different genotypes encode different serological subtypes, which are antigenic determinants of HBsAg, with various amino acid differences in HBsAg (Kramvis, Kew et al. 2005).

Furthermore, the different (sub)genotypes develop different mutations that can affect HBeAg expression at the transcriptional, translational and post-translational levels (Kramvis 2016). The ability to develop mutations, affecting HBeAg expression, can influence the length of the HBeAg-positive phase, which is important in determining both the mode of transmission and the clinical course of HBV infection.

1.8.1 Genotypes prevailing in sub-Saharan Africa

The various HBV genotypes have distinct geographical distributions. In SSA, the most prominent (sub)genotypes are A1, D3, E (Kramvis 2007, Kramvis 2018) (Figure 7B). In this study, we investigated (sub)genotypes A1, D3 and E, in comparison to subgenotype A2, which prevails outside Africa.

Subgenotype A1

Subgenotype A1, which prevails in southern-eastern SSA (Bowyer, van Staden et al. 1997, Kimbi, Kramvis et al. 2004, Kramvis and Kew 2007, Kramvis 2007) (Figure 7B), has genetic variations that affect the expression of HBeAg, at the transcriptional (1762T/1764A), translational (1809-1812 TCAT) and post-translational (G1862T) levels (Parekh, Zoulim et al. 2003, Kimbi, Kramvis et al. 2004, Kramvis, Kew et al. 2005, Kramvis and Kew 2007, Kramvis 2007, Kramvis 2018) (Figure 7C). The 1762T/1764A mutation, has been shown to reduce HBeAg expression in an additive manner and is associated with a higher risk of cancer (Baptista, Kramvis et al. 1999, Kramvis, Kew et al. 2005, Kramvis 2018). Unique to subgenotype A1, the second mutation 1809-1812 TCAT mutation in the Kozak region preceding the precore ORF, converts the region from an optimal to a suboptimal translation context. This causes decreased translation of HBeAg by a ribosomal leaky scanning mechanism (Ahn, Kramvis et al. 2003, Parekh, Zoulim et al. 2003). The third mutation, which is also unique to subgenotype A1, G1862T results in a valine to phenylalanine substitution. The phenylalanine interferes with the signal peptide cleavage and leads to an accumulation of precursor in the ER and decreased expression and secretion of HBeAg (Nielsen, Engelbrecht

et al. 1997, Bhoola and Kramvis 2016). These mutations can lead to viral persistence (Mitra, Wang et al. 2019) and possibly contribute to the higher hepatocarcinogenic potential, of a 4.5-fold increased risk of HCC and earlier age of diagnosis in patients infected with subgenotype A1 compared to other HBV (sub)genotypes (Kramvis, Kew et al. 2005, Kramvis and Kew 2007, Kramvis 2007).

Subgenotype A2

The subgenotype of A found outside Africa is subgenotype A2, and thus, has a different natural history to A1, D3, and E (Kramvis 2016). In a case-control study, comparing individuals infected with subgenotypes A1 and A2, and genotype D, respectively, it was shown that, regardless of the age group, the frequency of HBeAg positivity was higher in individuals infected with subgenotype A2 compared to the other (sub)genotypes(Tanaka, Hasegawa et al. 2004). In subgenotype A2, the only mutations that influence HBeAg expression are 1762T/1764A, which affects transcription decreasing HBeAg levels (Buckwold, Xu et al. 1996). Subgenotype A2 does not develop the G1862T seen in subgenotype A1 nor the classic G1896A seen in genotype D or E (Figure 7C).

Subgenotype D3

Subgenotype D3 is the predominant strain of genotype D in South Africa (Kimbi, Kramvis et al. 2004, Kew, Welschinger et al. 2008, Mayaphi, Martin et al. 2013, Gededzha, Muzeze et al. 2016) (Figure 7B). Genotype D develops 1762T/1764A and the precore stop codon mutation G1896A (Schaefer 2007, Chandra, Biswas et al. 2009)(Figure 7C). Patients infected with genotype D seroconvert from HBeAg to anti-HBe in adolescence or early adulthood, leading to high chronicity (Naoumov, Schneider et al. 1992), which can lead to cirrhosis and HCC (McMahon 2005).

Genotype E

Genotype E is mainly prevalent in Western Africa (Figure 7B). Genotype E is classified by a unique serological subtype *ayw*4 and has a genome of 3212 nucleotides long with a 3-nucleotide deletion in the pre-S1 region (Kramvis 2014). In addition, another unique feature of genotype E is the introduction of another start codon Met83 in the pre-S1 region that may result in the translation of an elongated middle hepatitis B surface protein (Kramvis, Kew et al. 2005, Kramvis 2018). Patients infected with genotype E have significantly higher viral loads and

higher frequency of HBeAg-positivity compared to patients infected with genotype D (Yousif and Kramvis 2013, Yousif, Mukhdawi et al. 2014). This higher HBeAg expression level confers tolerance with milder clinical manifestation and has been linked to higher chronicity (Milich and Liang 2003, Kostaki, Karamitros et al. 2018). Moreover, HBeAg can easily cross the placenta and has led to HBeAg/HBcAg tolerance *in utero* and thus a higher probability of chronicity (Edmunds, Medley et al. 1993, Milich and Liang 2003, Chen, Zeng et al. 2005, Kramvis 2016).

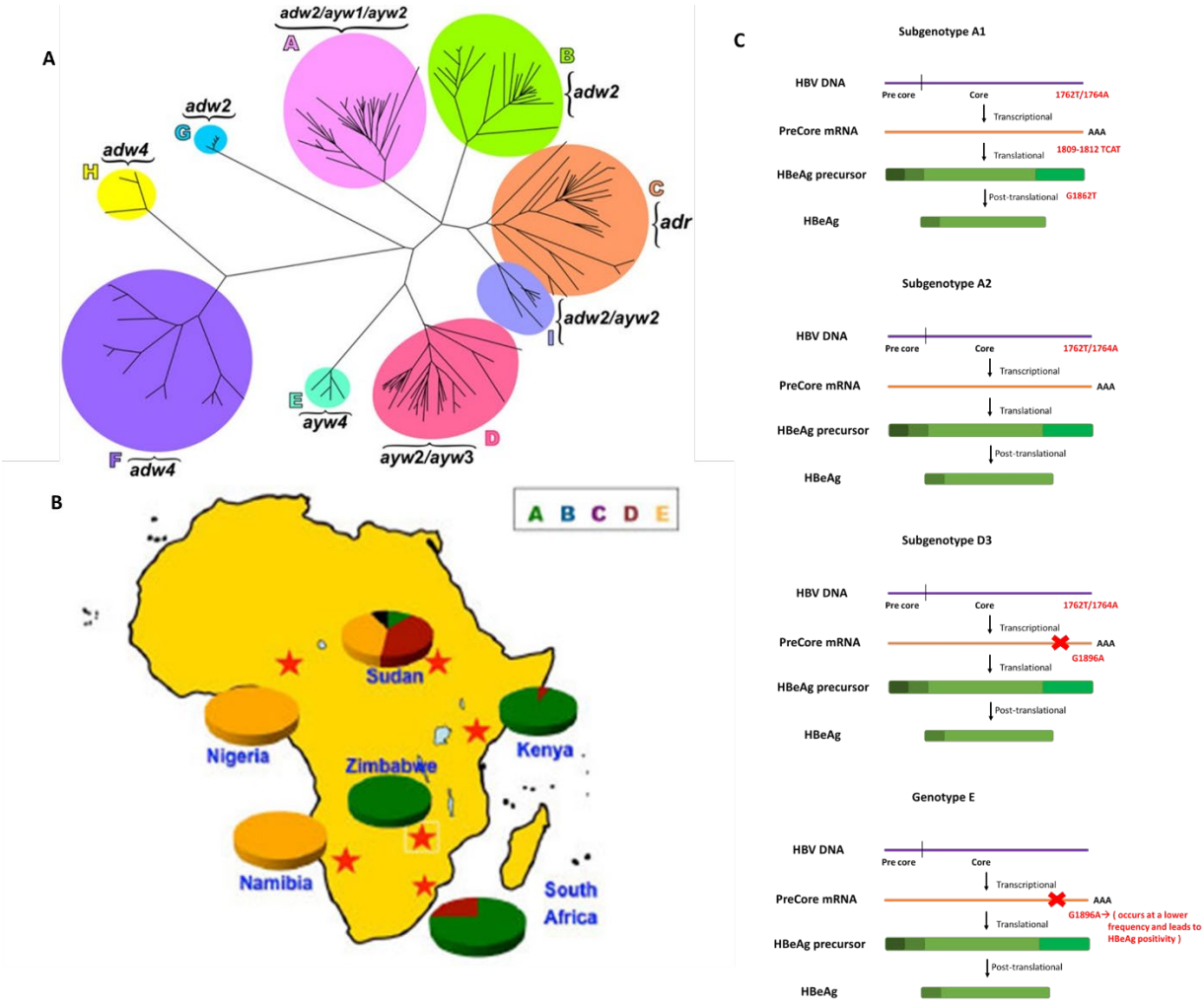


Figure 7: The genotypes of HBV, their distribution in Africa and mutational patterns

(A) An unrooted phylogenetic tree of full genome sequences of hepatitis B virus (HBV) representative of the nine major genotypes, established using neighbour-joining. Letters A to I represent the nine genotypes and the associated serological subtypes are indicated. (B) Map of Africa with the genotype distribution shown in various African countries, focusing on the sSA region. (C) Schematic representation of the different BCP/PC mutations represented in red that affect the expression of HBeAg in hepatitis B virus (HBV) (sub) genotype D3, A1, E and A2.

This image has been adapted and generated used with permission from (Kramvis 2018)(CC BY-NC 4.0) .

The majority of clinical and functional studies on the effect of subgenotypes have been done on HBV isolates prevailing in South East Asia, with fewer studies performed on strains prevailing in SSA (Kim, Revill et al. 2011, Sozzi, Walsh et al. 2016, Kuhnhenn, Jiang et al. 2018, Elizalde, Tadey et al. 2021, Sant'Anna and Araujo 2023). Out of the strains prevailing in SSA, subgenotype A1 has been the most thoroughly studied with genotype E included in studies less frequently (Kimbi, Kramvis et al. 2004, Kramvis, Kew et al. 2005, Kramvis 2007, Kim, Revill et al. 2011, Kramvis 2014, Kramvis 2016, Kramvis 2018, Olubayo, Kinge et al. 2021, Toyé, Loureiro et al. 2023). Very few studies compared the strains circulating in Africa, despite Africa having the greatest burden of disease (Delphin, Mohammed et al. 2024).

1.9 Study of HBV *in vitro*

Characteristics of HBV such as its narrow virus-host range and the strong affinity to hepatocytes (De Meyer, Gong et al. 1997), have led to major challenges in the development of suitable *in vitro* and *in vivo* models that recapitulate the replication cycle of the virus (Wose Kinge, Bhoola et al. 2020). Thus various *in vitro* systems have been developed in order to overcome these challenges.

1.9.1 Background on *in vitro* studies

Primary human hepatocytes (PHH) would be the ideal *in vitro* model to use to study HBV infection. However, PHH have limited availability and are not suitable for long term studies because they are only viable for a few days after culturing, even upon supplementation (Gripon, Diot et al. 1988, Gripon, Diot et al. 1989, Wose Kinge, Bhoola et al. 2020). Furthermore, there is also unpredictable variability between hepatocyte donors. Thus, the most economical *in vitro* models are well-differentiated human hepatic cell lines such as HepG2, Huh-6 and Huh-7. These cell lines have been derived from HCC, are easier to culture and have stable enzyme concentrations compared to PHH (Brandon, Raap et al. 2003, Wose Kinge, Bhoola et al. 2020). One of the major disadvantages of these cell lines is that they cannot be infected with HBV, because of the loss of the surface receptors during de-differentiation of the hepatocyte (Clayton, Rinaldi et al. 2005, Wose Kinge, Bhoola et al. 2020). However, they have been used in various studies using either transfection of recombinant HBV DNA or using viral vector delivery of the HBV genome (Gou et al., 2016; Wose Kinge et al.,2020)

1.9.2 Transfection of Huh7 cells with HBV

The Huh7 cell line (Nakabayashi, Taketa et al. 1982) was used in the present study, as these cells can support HBV replication and transcription, have minimal variability and are relatively cheap (Wose Kinge, Bhoola et al. 2020). The major limitation of using this cell line is that genes expressed in tumour-derived cell lines differ from genes expressed in normal hepatocytes (Verrier, Colpitts et al. 2016, Sperk, van Domselaar et al. 2020, Arzumanian, Pyatnitskiy et al. 2023). Despite this dysregulation, Huh7 cells have been used successfully in the molecular characterisation of HBV, replication and regulation, testing efficiency of novel drugs, drug resistance studies and proteomic studies (Bruix and Llovet 2003, Chen, Yang et al. 2006, Liu 2020)

1.10 Proteome

The proteome is the entire set of proteins that is, or can be, expressed by a genome of a cell, tissue, or organism at a certain time, under defined conditions (Garrels 2001). In this study, the proteome of hepatocytes (Huh7 cells) was analysed following transfection with different HBV (sub)genotypes found in SSA. An annotated workflow on the Mass spectrometry and proteomic analysis can be found in Appendix A.

1.10.1 Methods used to study the proteome

Mass spectrometry (MS) is a commonly used, high-throughput tool used for proteomics and identifying disease biomarkers. The procedure of MS-based protein identification involves digesting proteins into peptides, which are then separated, fragmented, ionised, and captured by mass spectrometers. Proteins are finally identified from the peaks of the captured mass spectra using computational methods, where each peak theoretically represents a peptide fragment ion (McHugh and Arthur 2008). MS can provide qualitative and quantitative analysis of global proteome samples derived from complex mixtures and has had a big impact on the understanding of cellular functions and pathways (Guerrera and Kleiner 2005).

The Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH-MS), a specific variant of data-independent acquisition (DIA) method, was used in the present study. This method supports the quantitative analysis of up to 9000 peptides with high quantitative accuracy and consistency. Using this MS strategy, the fragment ion signals, their relative intensities and the peptide retention time, with additional chromatographic information are

used to identify a given protein on a map uniquely. Furthermore, complete identification and quantitative analysis of virtually all detectable compounds in the sample can be performed simultaneously with a single analysis (Röst, Rosenberger et al. 2014). SWATH-MS has been applied to various viruses and has been successfully used in HIV *in vitro* studies (Sperk, van Domselaar et al. 2020) and protein expression profiles between human livers and hepatic cell lines (Verrier, Colpitts et al. 2016, Arzumanian, Pyatnitskiy et al. 2023).

1.10.2 Bioinformatics programmes used

Bioinformatics is an essential component of proteomics and a powerful data analysis tool. During the post-MS analysis, several bioinformatics tools, and databases were available for the protein pathways. The KEGG, Ingenuity, Pathway Knowledge Base Reactome and BioCarta are some of the pathway databases that include comprehensive data regarding metabolism, signalling and interactions (Croft, O'Kelly et al. 2011, Kanehisa, Goto et al. 2012). In addition to these comprehensive databases, specific databases for signal transduction pathways such as PANTHER have been developed (Mi, Guo et al. 2007, Schaefer, Anthony et al. 2009). Therefore, it is recommended that a combination of bioinformatic tools be used during analysis. The following bioinformatic tools were used in this study, to investigate the role of the HBV (sub)genotypes on host signalling pathways.

PANTHER

PANTHER is a freely available, comprehensive software system for relating protein sequence evolution to the evolution of specific protein functions and biological roles (Mi, Guo et al. 2007). PANTHER pathways provide a detailed, structured representation of protein function in the context of biological reaction networks. The pathway collection currently contains approximately 1500 reactions in 130 pathways, curated by expert biologists with authorship attribution (Mi, Guo et al. 2007).

Reactome

Reactome is a database that was developed in a collaboration among groups at the Ontario Institute for Cancer Research, Cold Spring Harbor Laboratory, New York University School of Medicine and The European Bioinformatics Institute, that developed an open-source curated bioinformatics database of human pathways and reactions (Croft, O'Kelly et al. 2011). This pathway and expression analysis tool enables ID mapping, pathway assignment and overrepresentation analysis of user-supplied data sets (Bruss, Lu et al. 1994).

GSEA

Gene Set Enrichment Analysis (GSEA) is a computational method that determines whether a defined set of genes shows statistically significant, concordant differences between two biological states (Subramanian, Tamayo et al. 2005). The method derives its power by focusing on gene sets (groups of genes) that share common biological function, chromosomal location, or regulation. Compared to Panther and Reactome, GSEA analysis provides more global representation as it analyses the whole proteome and not just selected proteins.

STRING

Search Tool for the Retrieval of Interacting Genes/ Proteins (STRING) is a curated knowledge database used to illustrate predicted interactions of identified proteins and neighbour genes (Franceschini, Szklarczyk et al. 2013). STRING is not only a widely used database for protein interaction data, but it connects to various other resources for literature mining. Furthermore, protein networks can be drawn based on the list of genes provided and the available interactions using STRING database (Schmidt, Forne et al. 2014).

1.10.3 Proteomic studies of HBV infection

High-throughput proteomics technology can be an important tool in the understanding of liver diseases. Some proteomic studies have aimed to describe global changes in host cells following HBV infection or following the expression of individual viral proteins (Kim and Seong 2003, Hannivoort, Hernandez-Gea et al. 2012, Gao, Zhu et al. 2019). Previous studies have mainly focused on the proteome of the host cell in response to HBV infection in a range of applications including HBV-host interactions, how HBV hijacks the host signalling pathway for its gain, and novel drug studies (Wang, Bouwman et al. 2009, Gao, Zhu et al. 2019)

Currently, little is known about the difference in specific biological pathways induced by the different HBV genotypic backgrounds. An Asian study was the first to report on molecular differences in the host proteome between HBV genotype B- and C- induced HCC (Wei, Zeng et al. 2016). The protein expression alterations in the different HBV genotype infected patients were involved in different biological processes, including translation, oxidation-reduction, intracellular transport, establishment of protein localisation, protein transport, and translational elongation, intimating that pathogenesis by genotypes B- and C- induced HCC is associated with different molecular mechanisms (Hannivoort, Hernandez-Gea et al. 2012, Wei, Zeng et al. 2016, Gao, Zhu et al. 2019).

1.11 Rationale

HBeAg is a non-particulate protein of HBV that has a dual role as an immunogen and tolerogen, which allows the virus to establish HBV infection *in vivo*. HBeAg is expressed by both human and non-human hepadnaviruses, with all mammalian-infecting sequences showing high conservation within the amino terminus of the precursor and key immunomodulatory epitopes (Revill et al., 2020; Milich et al., 1987; Belnap et al., 2003; Billaud et al., 2005; Kramvis.2018). Thus, HBeAg-mediated immune regulation may predispose to chronicity and persistence following *in utero* or perinatal transmission and prevent severe liver injury during adult infections (Milich and Liang, 2003). Clinically, HBeAg is an index of viral replication, infectivity, inflammation, severity of disease and response to antiviral therapy (Milich 2019). Several studies have looked at the oncogenic potential of HBeAg therefore, in Chapter 2 of this thesis, the role of HBeAg and its precursors in hepatocarcinogenesis is explored.

The length of the HBeAg-positive phase is important in determining both the mode of transmission and the clinical course of HBV infection. During the natural course of infection, there is seroconversion from an HBeAg-positive phase to an HBeAg-negative, anti-HBe-positive phase. During this seroconversion, there is a loss of tolerance to infection and immune escape-HBeAg-negative mutants can be selected in response to the host immune response (Kramvis 2016, Kramvis 2018). Mother-to-child transmission and subsequent chronic infection in the infant are favoured when mothers are HBeAg-positive (Chen et al., 2012), whereas children born to HBeAg-negative mothers are more likely to develop acute hepatitis B or may be infected horizontally later in life, if not immunized (Hadziyannis and Vassilopoulos, 2001; Kramvis, 2016).

The HBV reverse transcriptase lacks proofreading ability, and the virus accumulates genetic errors over time, leading to sequence heterogeneity (Summers, Smith et al. 1990, Wang and Seeger 1993, Norder, Couroucé et al. 2004, Kay and Zoulim 2007). The sequence variation in the region encoding HBeAg can influence the immunomodulatory role of HBeAg, the ability of HBV to persist and be transmitted, as well as its virulence (Kramvis 2016, Kramvis 2018). Furthermore, the immunomodulation and immune escape of HBeAg can be influenced by the different (sub)genotypes. Understanding the role of genotypic variation in pathogenesis is important and may provide further insight on patient outcomes, chronicity, infectivity, and response to therapeutic approaches, and improve early detection and screening of HCC (Gao et al., 2019). Therefore, Chapter 3 of this thesis provides the first report of the molecular

differences at the proteome level between the SSA (sub)genotypes following functional studies *in vitro*. To achieve this, the proteome of Huh7 cells transfected with replication-competent clones of (sub)genotypes A1, D3 and E relative to subgenotype A2 was analysed by mass spectrometry. Mass spectrometry was used to detect global changes in the proteome of transfected cells to bridge the knowledge gap between virus-host interactions of the different (sub)genotypes and their disease phenotypes

The propensity to develop precore and core promoter mutations that affect HBeAg expression is influenced by (sub)genotype. In SSA, the predominant subgenotype is A1 which has a 4.5-fold higher risk of developing HCC at an earlier age than other HBV genotypes (Kramvis, Kew et al. 2005, Kramvis 2007). Subgenotype A1 has unique BCP/PC mutations that affect the expression of HBeAg at a transcriptional, translational, and post-translational level (Kramvis, Kew et al. 2005, Kramvis 2018). In particular, the G1862T mutation can be found in HBV isolated from both tumour and non-tumorous liver tissues (Kramvis, Kew et al. 1998). Previous studies *in vitro,* have shown mutant HBeAg, as a result of G1862T, accumulated specifically in the endoplasmic reticulum (ER), endoplasmic reticulum-Golgi intermediate compartment (ERGIC) and Golgi (Chen, Crowther et al. 2008). These mutant protein aggregates attracted ubiquitin, heat shock proteins and proteasomes and caused ER stress and the earlier activation of the unfolded protein response (UPR) cascade (Bhoola and Kramvis 2017). ER is a quality-control organelle for proteostasis. ER stress and the UPR can cause disturbances in proteostasis, affecting host signalling pathways involved in epigenetics, mitochondrial regulation, autophagy, and metabolism (Chen, Shi et al. 2023). Furthermore, an increase in ER stress can result in liver damage, which is a contributing factor to hepatocarcinogenesis and may explain why G1862T is frequently found in HCC patients compared to asymptomatic patients (Kramvis, Kew et al. 2005, Kramvis 2018). Therefore, Chapter 4 of this thesis provides the first report of the molecular differences at the proteome level between cells transfected with wildtype subgenotype A1 and subgenotype A1 with G1862T mutation. Understanding the role of G1862T mutation in pathogenesis is important and will provide further insight into patient outcomes, chronicity, and improving early detection and screening of HCC in SSA.

1.13 Aims and objectives

Aim: Comparison of the proteome of Huh7 cells transfected with different (sub)genotypes of HBV prevailing in sub-Saharan Africa, to study the effect on host signalling pathways

Objective 1: To transfect Huh7 cells with replication competent clones of (sub)genotypes A1, A2, D3 and E, to study the proteome using mass spectrometry

Objective 2: To transfect Huh7 cells with replication-competent clones of wild-type and mutant G1862T mutant subgenotype A1 to study the proteome using mass spectrometry

Objective 3: To determine pathways differentially affected by the (sub)genotypes and the G1862T mutant of subgenotype A1, using bioinformatics.

Chapter 2

Paper 1-Literature review: The Complex Role of HBeAg and Its Precursors in the Pathway to Hepatocellular Carcinoma

Journal: Viruses (MDPI), Impact Factor of 4.7

References: The literature cited in the paper below, appears within the reference section of the paper and is not necessarily in the thesis reference section.

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Chapter 2 explores the role of HBeAg and its precursors in hepatocarcinogenesis. HBV is one of the seven oncogenic viruses, and its viral proteins have evolved to take over many host signalling pathways to generate a cellular environment conducive to virus replication (Krump and You 2018). Viral proteins function to stimulate proliferation, delay differentiation, inhibit apoptosis and evade immune detection, which are hallmarks of cancer (Scarth, Patterson et al. 2021). Oncogenic viruses interact and dysregulate the host immune systems throughout persistent infections. Epidemiological evidence suggests that viral mechanisms to evade detection and elimination are adapted to deal with the constant pressure from the host (Thorley-Lawson 2015, Liu, MacDonald et al. 2016). Given that HBeAg has a dual role as an immunogen and tolerogen (Milich 2019), its role in hepatocarcinogenesis has been the focus of numerous studies. The biogenesis of HBeAg, its interaction with immune cells, and the different signalling pathways and mechanisms of HBeAg and its precursors to promote hepatocarcinogenesis through the various hallmarks of cancer are discussed.

Author contributions: Ms Padarath conceptualised the review article, performed the literature review, wrote up the first draft, including figures and tables, and rewrote drafts 6–8 after reading fellow authors' comments.

viruses

MDPI

Review

The Complex Role of HBeAg and Its Precursors in the Pathway to Hepatocellular Carcinoma

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Abstract: Hepatitis B virus (HBV) is one of the seven known human oncogenic viruses and has adapted to coexist with a single host for prolonged periods, requiring continuous manipulation of immunity and cell fate decisions. The persistence of HBV infection is associated with the pathogenesis of hepatocellular carcinoma, and various HBV proteins have been implicated in promoting this persistence. The precursor of hepatitis e antigen (HBeAg), is translated from the precore/core region and is post-translationally modified to yield HBeAg, which is secreted in the serum. HBeAg is a non-particulate protein of HBV and can act as both a tolerogen and an immunogen. HBeAg can protect hepatocytes from apoptosis by interfering with host signalling pathways and acting as a decoy to the immune response. By evading the immune response and interfering with apoptosis, HBeAg has the potential to contribute to the hepatocarcinogenic potential of HBV. In particular, this review summarises the various signalling pathways through which HBeAg and its precursors can promote hepatocarcinogenesis via the various hallmarks of cancer.

Keywords: hepatitis B virus; HBeAg; hepatocellular carcinoma

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1. Introduction

Hepatitis B is caused by the hepatitis B virus (HBV), a hepatrotropic DNA virus belonging to the family *Hepadnaviridae* [1]. Despite the availability of an effective vaccine, hepatitis B continues to be a major health concern, with over 296 million people chronically infected with HBV in 2019 and 1.5 million new infections each year, accounting for 820,000 deaths annually as a result of severe liver disease, including cirrhosis and hepatocellular carcinoma (HCC; liver cancer) [2].

HBV infection is linked to more than 60% of all HCC in developing countries and 40% in developed countries, thus implicating HBV infection as a carcinogen [3]. Carcinogenesis is a multistep process in which normal cells are transformed into cancer cells by acquiring various properties that allow them to form tumours [4]. The acquired properties of cancer cells that distinguish them from normal cells have been classified as a series of hallmarks of cancer [5]. These hallmarks of cancer include: sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, deregulation of cellular energetics, genome instability and mutation, tumour-promoting inflammation, avoiding immune destruction, activating invasion, and metastasis (Figure 1) [6]. HBV infection can promote liver cancer through direct or indirect mechanisms. HBV infection can:

- generate genome instability by integrating its viral DNA into the host genome [7,8]
- cause abnormal expression of oncogenes and tumour suppressor genes leading to activation of cancer-related pathways, interference with epigenetic and chromosomal remodelling through integration [7,8].

- affect the liver microenvironment through interference with both innate and adaptive immunity and thus help the virus evade immune surveillance, increasing persistence and disease progression to tumour formation [7,9].
- interfere with mitochondrial proteins, to change mitochondrial dynamics/signalling, leading to the development of HCC [10,11].
- persist in liver tissue, increasing chronic oxidative damage in hepatocytes, immune-mediated inflammation of the liver, and development of HCC [12].
- interfere with apoptosis signalling to promote viral proliferation and HCC progression [8,13].

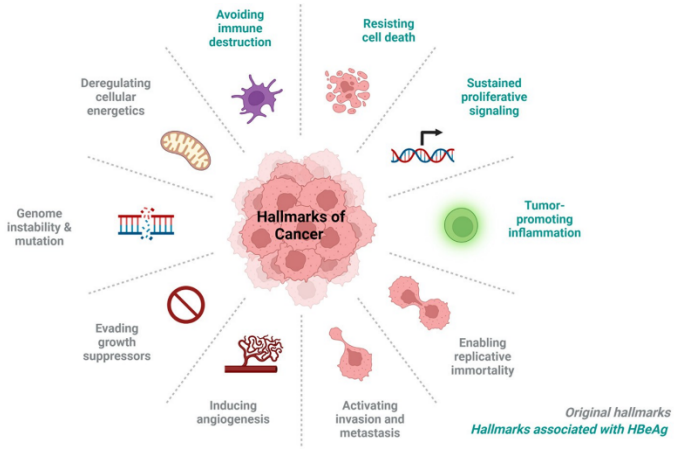


Figure 1. Hallmarks of cancer: HBeAg associated hallmarks of cancer are indicated in blue. Image created using biorender.com.

The 3.2 kb HBV genome has four overlapping reading frames and encodes for the five major RNA transcripts [14–17], which are responsible for the translation of seven viral proteins: HBcAg (capsid), polymerase (pol), envelope proteins, which have three different sizes—small (SHBs), medium (MHBs), and large (LHBs)—and non-particulate proteins HBx and HBeAg [18,19]. HBx and HBsAg have been implicated in the hepatocarcinogenic potential of HBV. The HBx protein has been shown to be involved in the transcriptional activation of cellular growth regulatory genes, modulation of apoptosis, angiogenesis, and metastasis, inhibition of nucleotide excision repair of damaged cellular DNA, interaction with cellular proteins, and activation of cell signalling pathways [20–23]. Accumulation of preS1 large envelope proteins and/or preS2/S mutant proteins in the endoplasmic reticulum (ER) increases ER stress, which has been shown to activate the unfolded protein response and ultimately the development of HCC [24]. Individuals infected with preS mutant HBV have a 13X risk of developing HCC [25].

HBeAg is a non-structural, secreted protein expressed by every member of the family *Hepadnaviridae*, though its expression is not required to maintain infection. The antigen has been used clinically as an index of viral replication, infectivity, the severity of disease, and response to treatment [9,26,27]. The presence of HBeAg can delineate the different phases of chronic hepatitis B virus infection [28]. Although the majority of the protein is secreted, approximately 20–30% is retained in the cytoplasm [9,26]. HBeAg has the dual role of being a tolerogen and an immunogen [9]. The exact mechanisms by which HBeAg achieves these opposing functions is not fully understood. Its immune-modulatory role allows for humoral and cell-mediated immune evasion of HBV [26,27]. In addition, studies have shown that HBeAg can protect hepatocytes from apoptosis, leading to the survival of infected hepatocytes and contributing towards the development of HBV chronicity [29–31].

By evading the immune response and interfering with apoptosis, HBeAg has the potential to contribute to the hepatocarcinogenic potential of HBV. In this review, we discuss the biogenesis of HBeAg, its interaction with immune cells, and the different signalling pathways. We propose ways in which this protein and its precursors can promote hepatocarcinogenesis through the various hallmarks of cancer (Figure 1).

2. Biogenesis of HBeAg

The core protein, HBcAg, is the structural component of the viral capsids, found in mature and incomplete virions. HBeAg, a non-structural HBV protein, is the N-terminal processed product of the precore/core protein, with different forms of HBeAg precursors. The 25 kDa precursor of HBeAg (p25) is translated from the preCore/Core open reading frame (ORF) on the precore mRNA, whilst HBcAg is translated from the Core ORF on the pregenomic RNA (pgRNA) (Figure 2) [32–34]. The translation of the HBeAg precursor p25 from precore mRNA starts at the first ATG site, while the translation of the core protein on pgRNA starts at the second ATG site [7,35,36]. p25 is translated and targeted to the ER membrane for post-translational modifications [37–39]. In the ER, the 29 amino acid N terminal signal of p25 is proteolytically removed to create the precursor HBeAg protein p22 [40,41]. Due to the presence of a nuclear localisation signal (NLS) on the arginine-rich C-terminal domain (CTD) of p22, this protein can also be transported into the nucleus [39,42]. In vitro studies using subgenomic p22 confirmed the protein's nuclear and cytoplasmic localisation, but the role of p22 in the nucleus remains unknown [43]. The p22 protein undergoes further modification in the ER through the cleavage of the CTD by a furin endoprotease to give rise to the mature HBeAg, which is also known as p17 [44–47]. The mature HBeAg is secreted in a soluble form in the blood serum [48,49].

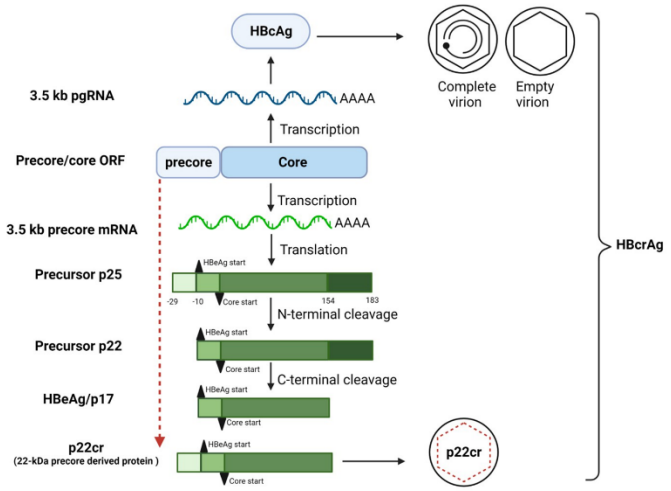


Figure 2. Biogenesis of HBeAg and its precursors. HBeAg and HBcAg translations from the pre-Core/Core ORF, adapted with permission from [50], © 2021 American Society for Microbiology. Image created using biorender.com.

HBeAg can form part of a collection of three species of proteins known as HB core related antigen (HBcrAg), which consists of related proteins sharing an identical 149 amino acid sequence: HBcAg, HBeAg, and a truncated 22 kDa precore protein (p22Cr). Like HBeAg, p22cr is also a processed product of the precore protein, but with protein processing only occurring at the C-terminal and not the amino end [50] (Figure 2). Recent studies have shown that the detection of HBcrAg levels is a useful marker for disease monitoring,

predicting treatment response and disease outcome in chronic hepatitis B (CHB), and can also predict the risk of HCC [28,51,52].

3. Effect of BCP/Precore Mutations on the Expression of HBeAg

There are multiple mutations in the basal core promoter (BCP) or precore region that affect the expression of HBeAg at transcriptional, translational, and post-translational levels [53]. The tendency of the HBV genome to develop these mutations is dependent on the HBV (sub)genotypes, as the sequence of the preC/C region differs in the different (sub)genotypes [54,55]. For example, 1858C in the preCore/Core(PreC/C) region is positively associated with genotypes A, F, and H and 1858T with genotypes B, D, and E [55]. Subgenotype C2 has 1858T as opposed to C1 that has 1858C, and F1/F4 can be differentiated from F2/F3 by having 1858T instead of 1858C, while 1888A is positively associated with subgenotype A1 [55]. It has been suggested that the different mutations in preC/C can account for the different clinical manifestations and duration of HBV infection in individuals infected with the different (sub)genotypes [53,54].

Genotypes with T at position 1858 are frequently associated with the 1896A mutation, which converts the codon for tryptophan to a stop codon. This premature stop codon leads to the truncation of the HBeAg precursor and abrogation of HBeAg expression, which is responsible for HBeAg to anti-HBeAg seroconversion as a result of the truncation of the precore/core fusion protein [56]. The G1896A mutation has been reported in patients with chronic liver disease and severe liver damage in Africa and Asia [57,58]. The G1896A mutation occurs in up to 30% of all HBV sequences and is frequent in (sub)genotypes C1, D, E, and F [59,60].

The BCP double mutation 1762T/1764A can affect transcription of precore mRNA and result in decreased levels of HBeAg [61,62], without switching off HBeAg expression. This mutation occurs in approximately 25% of all HBV sequences [63] and is significantly more frequent in genotypes A and C compared to D and B [64,65]. A high prevalence of 1762T/1764A has been detected in HBV strains from HCC patients compared to asymptomatic carriers of HBV [61]. Genotype D develops 1762T/1764A and the precore stop codon mutation G1896A [66,67]. Patients infected with genotype D seroconvert from HBeAg to anti-HBe in adolescence or early adulthood, leading to high chronicity [68,69], which can lead to cirrhosis and HCC [70].

Subgenotype A1 has unique BCP/precore mutations that affect the expression of HBeAg at a transcriptional, translational, and post-translational level [32,71]. The first is the presence of the 1762T/1764A mutation; this mutation has been shown to reduce HBeAg expression in an additive manner and is associated with a higher risk of cancer [32,61,71]. The second mutation is the 1809–1812 TCAT mutation in the Kozak region preceding the precore ORF, which converts the region from an optimal to a suboptimal translation context. This mutation causes decreased translation of HBeAg by a ribosomal leaky scanning mechanism [72,73]. Lastly, in the precore region there is a G to T transversion at position 1862. This mutation results in a valine to phenylalanine substitution. Phenylalanine interferes with the signal peptide cleavage, resulting in decreased expression and secretion of HBeAg, as well as the accumulation of its precursor (p22) in the cytoplasm of the hepatocyte [29,74,75]. Thus, in subgenotype A1, HBeAg loss and immune evasion are the result of a number of mutations that HBV has evolved to survive, persist, and infect [53,76]. Furthermore, subgenotype A1 has been associated with severe liver disease and a 4.5-fold increased risk of developing HCC at an earlier age compared to other HBV genotypes [32,53].

The BCP/precore mutations can be found in 94.4% of HBV isolates from HCC cases; therefore, it is extremely important to understand and investigate the downstream effects of these mutations on hepatocarcinogenesis [77]. The expression of HBeAg is affected at two levels by these mutations: reduction or abrogation of HBeAg expression, removing the tolerogenic effect of HBeAg and change of expression and localisation of HBeAg and/or its precursors in the cellular compartments, which can promote endoplasmic reticulum (ER) stress and/or apoptosis. These changes can promote carcinogenesis [78].

4. HBeAg-Associated Hallmarks of Cancer

The HBeAg-associated hallmarks of cancer, depicted in blue in Figure 1, will be discussed.

1. Immune Evasion, Leading to Persistence

The dual roles of HBeAg as a tolerogen and immunogen and its ability to suppress or activate the immune response reveal the complexity of the interactions between HBeAg and the host [9]. Numerous studies have shown that HBeAg can impact both the innate and adaptive immune responses, in order to promote HBV persistence.

Macrophages, which exhibit phenotypic plasticity, form the first line of defence against HBV. They can release either pro-inflammatory cytokines (M1 polarisation), or anti-inflammatory cytokines (M2 polarisation) [79]. Kupffer cells, found in the sinusoids of the liver, form the largest macrophage population in the human body and play an important role in liver inflammation. Previous exposure to HBeAg can affect the response of Kupffer cells to HBV and play a key role in the induction of macrophage activation, which may exacerbate liver injury by increasing the production of inflammatory cytokines [80,81]. Macrophages from mice that have previously been exposed to maternal HBeAg, undergo M2 polarisation, with increased programmed Death Ligand 1 (PD-L1) expression in both Kupffer and cytotoxic T-cells. Increased PD-L1 expression plays an important role in T cell exhaustion and impairment of the cytotoxic T lymphocyte (CTL) response in chronic HBV (CHB) patients [82]. In the absence of maternal HBeAg in the mice, Kupffer cells undergo M1 polarisation and can inhibit viral replication by the activation of CD8+ T cells [83]. These experiments demonstrate the importance of HBeAg as a tolerogen because both M2 macrophage polarisation and increased PD1 expression lead to HBV persistence [83]. Lower levels of baseline soluble PD1 (sPD1) are associated with HBeAg seroclearance following two years of antiviral treatment in HBeAg-positive CHB patients [84] (Figure 3).

HBeAg can activate the mitogen-activated protein kinase (MAPK) pathway, including activation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 in macrophages, and influence the production of interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) [85]. Overexpression of miR-212-3p, located on chromosome 17q13.3, which has been shown to exert tumour-suppressive roles by targeting MAPK pathways in prostate cancer [86], leads to decreased mRNA and protein expression of pro-inflammatory cytokines IL-6 and TNF-α [85,86]. In HBeAg-stimulated human and mouse Kupffer cells, miR-212-3p is also upregulated [85]. However, this process is not uncontrolled, and HBeAg-induced macrophages can regulate the expression of miR-212-3p via the MAPK pathway; specifically, ERK acts as an inhibitor and blocks the expression of miR-212-3p, whereas p38 and JNK increase its expression [85]. In addition, cAMP response element-binding protein (CREB), an important transcription factor, is activated in HBeAg-induced macrophages and inhibits the expression of miR-212-3p via the ERK/CREB pathways [85]. Thus, although HBeAg promotes the expression of miR-212-3p in macrophages, miR-212-3p expression can be regulated via the ERK/CREB signalling pathways by negative modulation of inflammatory cytokine production (Figure 3) [85].

Alternatively, HBeAg can regulate cytokine production in macrophages by modulating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and P13K signalling pathways. In macrophages, HBeAg induces expression of miR-155, located on chromosome 21q21.3 and associated with inflammation and immunity [87]. miR-155 is induced by the activation of Toll-like receptor (TLR) ligands, in particular TLR4 and TLR3 [88,89]. The increased miR-155 represses the expression of SH-2 containing inositol 5′ polyphosphatase 1 (SHIP-1), leading to *Akt* phosphorylation upon lipopolysaccharide (LPS) stimulation [90] and inflammatory response to LPS through targeting SOCS-1 [91]. Thus, HBeAg-induced miR-155 functions as a positive regulator in HBeAg-induced expression of inflammatory cytokines by targeting BCL-6, SHIP-1, and SOCS-1 in macrophages [87]. miR-155 also directly represses the expression of BCL-6, a transcription factor that downregulates NF-κB signalling in mouse macrophages [87,91,92]. Thus, by inducing miR-155, HBeAg can downregulate interleukin1 (IL-1) and reduce the activation of NF-κB [87]. In fact, increased

NF-κB levels were found in liver biopsies of HBeAg-negative patients compared to HBeAg-positive patients, and HBeAg-activation of macrophages via the TLR-2/NF-κB signal pathways, where HBeAg binds to the TLR2, has been found in patients with exacerbated hepatic fibrosis [87,93]. HBeAg has also been shown to directly downregulate NK cell-mediated gamma interferon (IFN-γ) production and interleukin-18 (IL-18) signalling [94] (Figure 3).

In addition, studies have shown that HBeAg could suppress both Toll-like receptor 2 (TLR2) and IL-1β-induced activation of NF-κB in cells and clinical samples. A study by Wang and colleagues identified that HBeAg can associate with NF-κB essential modulator (NEMO) [95]. NEMO is a scaffolding component of the IκB kinase complex required for NF-κB activation in vitro [96]. HBeAg can associate with NEMO, and interfere with the activation of NF-κB, thus reducing inflammation and increasing virus replication. Viral mutations such as G1862T and G1896A, which reduce or abrogate HBeAg expression, respectively, can lead to the removal of the tolerogenic effect of HBeAg, leading to the activation of the immune response [97]. As a result, loss of HBeAg in patients infected with HBeAg negative mutants can induce higher levels of NF-κB activity compared to HBeAg positive patients [95].

HBeAg can also bind to peripheral blood monocytes (PBMC), neutrophils, and B lymphocytes but not to T lymphocytes [98]. The interaction between HBeAg and monocytes and neutrophils was shown to be dose-dependent, leading to the inhibition of both cell types [98]. Monocytic myeloid-derived suppressor cells (mMDSCs) are derived from myeloid progenitor cells and comprise of only ~0.5% of PBMCs in healthy individuals. The mMDSC population expands during infection, inflammation, and cancer [99]. HBeAg plays an important role in mMDSC population expansion and induction of immune tolerance [100]. Compared to HBeAg negative patients, HBeAg-positive patients were shown to have significantly higher levels of mMDSCs [100]. When PBMCs from healthy patients were exposed to HBeAg, there was an increase in mMDSCs and the expression of IL-6 and IL-1β. In addition, mMDSCs from HBeAg-positive patients reduced the proliferation of CD4+ and CD8+ T cells [100]. This may be a possible mechanism by which HBeAg modulates the host immune response during CHB by physically depleting or weakening virus-specific CD4 and CD8 T cells. These cells are thus unable to proliferate in response to the viral antigens or produce important antiviral and immunostimulatory cytokines, which are critical for the control of the virus in CHB patients [101] (Figure 3).

The TLR family is part of the pattern recognition receptor (PRR) family and is responsible for recognising diverse molecules derived from pathogens and damaged host cells, also called pathogen-associated molecular patterns (PAMPs) [102]. HBeAg down-regulates the expression of the TLR family within Kupffer cells and blood monocytes [103,104]. When peripheral blood monocyte cells were pre-treated with HBeAg, they displayed an impaired TLR signalling response [104]. TLR-2 has been shown to play an important role in the recognition of HBV and, once activated, induces the expression of INF and proinflammatory cytokines by macrophages [104]. HBeAg positive patients have lower levels of TLR-2 than HBeAg negative patients [104]. Patients who lost HBeAg after interferon therapy had increased TLR-2 levels and increased expression of IL-6 [105]. In addition, the HBeAg precursor, p22, has been shown to bind to toll/IL-receptor (TIR) and disrupt TLR signalling [106]. These results indicate that HBeAg has a negative role in the regulation of TRL2, which may be beneficial in HBV replication and persistence. Furthermore, HBeAg-containing conditioned medium but not hepatitis B core antigen (HBcAg)-containing conditioned medium suppresses the p38 MAPK phosphorylation induced by TLR2- or TLR4- agonists in blood CD14+ monocytes [104]. In another study, the cytosolic-form HBeAg suppressed TLR-mediated NF-κB activation by targeting the Toll/IL-receptor containing proteins TRAM and Mal [106]. These findings imply that HBeAg may have a potential to reduce TLR signalling in monocytes [98] and promote viral persistence.

Although the precise mechanism is unknown, HBeAg may regulate T cell-mediated immune responses. Circulating HBeAg preferentially activates Th2 cells and has the

potential to deplete HBeAg- and HBcAg-specific Th1 cells, which are required for viral clearance. This depletion can lead to the persistence of HBV [29,30,107].

JAK-STAT Pathway

Alpha interferon (IFN-α) is used clinically to treat CHB patients; however, only approximately one-third of patients respond to IFN-α treatment [108]. Patients with low HBeAg levels prior to treatment are more likely to respond to IFN-α therapy, and HBeAg negative patients with core promoter mutations respond better to IFN-α therapy than HBeAg positive patients [109,110], suggesting that HBeAg or its intracellular precursors may interfere with the IFN signalling representing a viral strategy to persist in the host through induction of immune tolerance.

Two mechanisms have been proposed to explain the reason for the reduced response to IFN treatment in the presence of HBeAg (Figure 3).

- Mitra and colleagues hypothesised that the reduced antiviral activity of IFN-α against HBV could be due to blockade of the IFN-elicited JAK-STAT pathway by a HBV protein [111]. In their study HBeAg-positive patients exhibited weaker induction of interferon stimulated genes (ISGs) in their livers than HBeAg-negative patients upon IFN-α therapy. Furthermore, the cytosolic HBeAg precursor p22 protein significantly reduced interferon-stimulated response element (ISRE) activity and the expression of ISGs upon IFN-α stimulation in cell culture [111]. p22 did not alter the total STAT1 or pSTAT1 levels in cells treated with IFN-α, but instead blocked the nuclear translocation of pSTAT1 by interacting with karyopherin α1 (Kα1) through its CTD domain, thus impeding JAK-STAT signalling, resulting in host innate immune response evasion and causing resistance to IFN therapy [111].
- Yu and colleagues hypothesised that HBeAg reduced IFN effectiveness and enhanced HBV infection by hijacking the IFN/JAK/STAT pathway [112]. Members of the intracellular suppressor of cytokine signalling (SOCS) family are regulators of cytokine signalling pathways [113,114] that are induced by cytokines and act in a classic negative-feedback loop to inhibit cytokine signalling [115]. SOCS1 and SOCS3 inhibit interferon-mediated antiviral and antiproliferative activities [116,117]. In their study, Yu and colleagues revealed that HBeAg initially activates SOCS2 through the ERK pathway. HBeAg-activated SOCS2 subsequently reduces tyrosine kinase 2 (TYK2) stability, down-regulates IFN receptor expression, represses STAT1 phosphorylation, and finally attenuates ISGs production [112]. Thus, revealing a novel mechanism by which HBeAg is coordinated to enhance HBV replication by hijacking the IFN/JAK/STAT pathway and antiviral action.

HBeAg interaction with the innate and adaptive immunity is multifaceted and complex. The main role of HBeAg is to direct the immune system away from HBV hepatocytes to ensure immune evasion, which is a hallmark of cancer. HBeAg can lead to the down-regulation of pro-inflammatory transcription factors such as NF-κB, JAK/TYK/STAT, and P13K. These activated transcription factors mediate the expression of key cytokines and chemokines as well as inflammatory enzymes during HBV infection, leading to persistence.

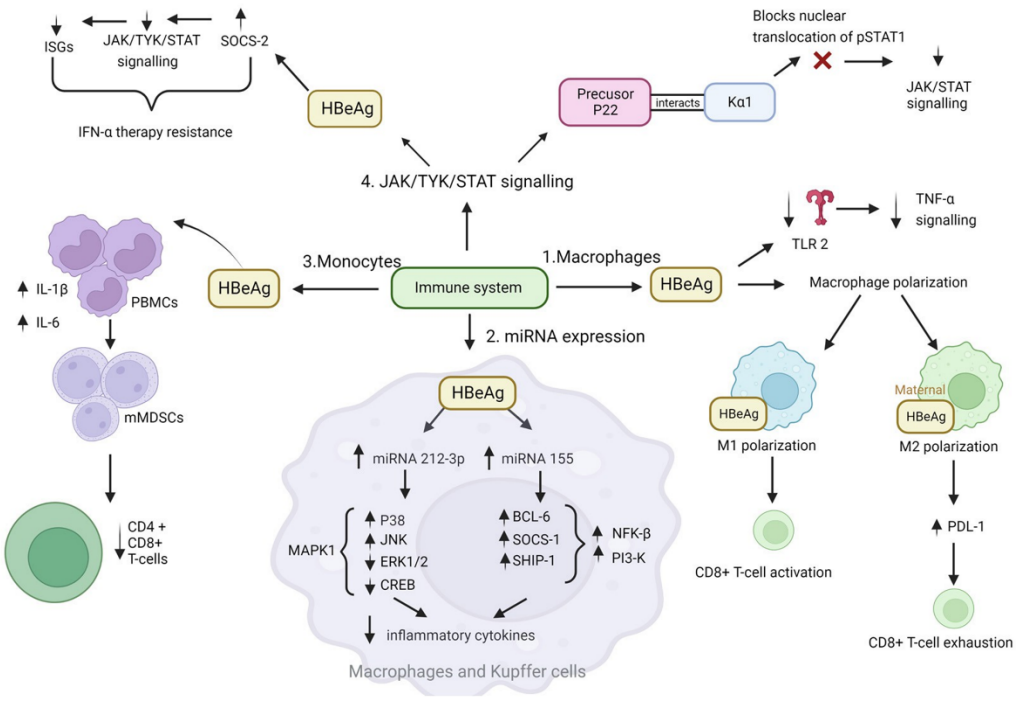


Figure 3. The role of HBeAg in immune evasion: influence of HBeAg on different components of the immune system that HBeAg can influence: macrophages, monocytes, miRNA expression, and JAK/TYK/STAT signalling. 1. Macrophages—exposure of macrophages to HBeAg can influence the type of macrophage polarisation Kupffer cells undergo [84]. In addition, HBeAg can influence macrophages by decreasing TLR 2 [104]. 2. miRNA expression—HBeAg leads to an increase in miRNA-212-3p and miRNA-155, which leads to a decrease in inflammatory cytokines and immune evasion [85,87]. 3. Exposure of PBMCs monocytes to HBeAg resulted in an increase in mMDSCs, the expression of IL-6 and IL-1β, and reduced proliferation of CD4+ and CD8+ T cells [100]. 4. JAK/TYK/STAT pathway—HBeAg can influence the JAK/TYK/STAT pathway via two mechanisms to decrease response to INF therapy. In the first mechanism, the HBeAg precursor p22 blocked the nuclear translocation of pSTAT1 by interacting with Kα1, thus impeding JAK/TYK/STAT signalling [111]. In the second mechanism, mature HBeAg activates, which represses STAT1 phosphorylation, thus impeding JAK/TYK/STAT signalling [112]. Image created using biorender.com.

4.2. Tumour Promoting Inflammation

Hanahan and Weinberg included tumour-promoting inflammation as one of the enabling characteristics of cancer [5]. There are several types of inflammation that can promote cancer development and progression, differing by cause, mechanism, outcome, and intensity [118]. Approximately 20% of human cancers may be related to chronic inflammation caused by infections, exposure to irritants, or autoimmune disease [119]. HBV infection can persist in liver tissue, increasing immune-mediated inflammation of the liver and the development of HCC [120]. The main role of HBeAg is tolerogenic, to promote persistence and decrease the expression of inflammatory cytokines. During HBV infection, viral diversity increases during the course of infection, with the emergence of strains defective in HBeAg expression [78]. Various BCP/precore mutations can affect the

expression of HBeAg, resulting in decreased HBeAg expression, mislocalisation of HBeAg and its precursors, or complete loss of HBeAg. As a result, during HBV infection, there is a progression from the HBeAg positive phase, which is associated with high virus replication and low inflammation, to the HBeAg negative phase, which is associated with chronic infection and more inflammation in the liver [78,121]. Defective expression of HBeAg results in the immune system and inflammatory cytokines being directed towards HBV infected hepatocytes, which may explain why BCP/precore mutations are more frequently associated with HCC [53,78,122].

3. Resisting Cell Death

Apoptosis is the cell's natural mechanism for programmed cell death [123] and plays a critical role in development as well as homeostasis [124]. There are two different pathways that lead to apoptosis: the intrinsic and extrinsic pathways. Intrinsic apoptosis occurs when an injury, such as direct DNA damage, hypoxia, or deprivation of growth factors, occurs within the cell. Whilst extrinsic apoptosis occurs, the cell uses extracellular signals, such as cytotoxic stress and receptor-mediated pathways, to induce apoptosis [125].

1. Intrinsic Mechanisms

- p53 driven apoptosis

p53 is a critical activator of the intrinsic apoptotic pathway. p53, a tumour suppressor located in the nucleus, plays a critical role in determining whether the DNA will be repaired or if the damaged cell will undergo apoptosis. MDM2 (the murine double minute 2) is a protein that regulates p53 function by degrading it and inhibiting its location in the nucleus [126]. Regions in MDM2 and p53 are sensitive to exposure to environmental carcinogens and can lead to the development of HCC. Mutations in the MDM2-p53 axis and chronic HBV infection have been shown to trigger the development of HCC [127]. A study by Liu and colleagues identified a previously unidentified mechanism in which HBeAg and its precursor p22 may play an important role in tumour growth and hepatocellular carcinogenesis [128]. This study showed that NUMB Endocytic Adaptor Protein (NUMB), a cell fate determinant, antagonises the activity of the plasma membrane receptor of the NOTCH family [129]. NUMB positively regulates p53 by forming a tricomplex with p53 and the E3 ubiquitin ligase HDM2 (also known as MDM2) and thus preventing the ubiquitination and degradation of p53 [129]. One recognised function for NUMB is its involvement in inhibition of the NOTCH pathway, which plays a role in carcinogenesis. NUMB inhibits Notch signalling through interaction with the Notch intracellular domain and promotion of its ubiquitination and degradation [130]. NUMB was also shown to control the intracellular trafficking of the Notch to suppress its function. Studies also indicated that the expression of NUMB correlates with the suppression of hedgehog signalling (Shh) by downregulating the activated Notch1 protein and thereby inhibiting the mRNA expression of Shh [131]. The NUMB protein was shown to interact with the precursor p22 and mature HBeAg at the 150–165 amino acid regions (CTD), with no interaction observed between HBcAg and NUMB [128]. HBeAg/precore could associate with NUMB and impair the stability and transcriptional activity of p53, thereby enhancing carcinogenesis. This carcinogenic effect was as a result of HBeAg's antiapoptotic effect caused by compromised p53 activity rather than deregulated NOTCH activity [128]. Besides compromising p53 activity, it has been shown that HBeAg/precore can inhibit NF-κB activity and expression of its downstream inflammatory cytokines and chemokines, whereas HBV variants causing reduced HBeAg expression lead to a higher level of inflammation compared to the wild-type [106]. Analysis of the clinical samples from HCC patients with the G1896A mutation had lower p53 activity than those with the wild-type [128]. HBeAg-deficient HBV variants may cause a greater inflammatory response compared with the wild type, and can worsen liver diseases with increased possibility of developing HCC. This is a possible explanation for why HBeAg-negative mutations can also have a higher risk of HCC [26,132] (Figure 4).

- p25 regulation of apoptosis via UPR

HBV precore variants, such as the G1862T mutation, result in HBeAg accumulation in the cytoplasm of Huh7 cells. During post-translational modifications of the p25 precursor of HBeAg, the G1862T mutation prevents signal peptide cleavage of the amino end, causing accumulation in the ER leading to the prevention of synthesis and secretion of HBeAg [29,133]. Furthermore, the retro-transport of the misfolded G1862T preC/C precursor protein from the ER to the cytosol for degradation results in increased expression of ubiquitin and proteasomes and the formation of ubiquitin-rich mini-aggregates along the microtubules in the cytosol [29,74,134].

The ER quality control (QC) mechanism can thus be compromised when unfolded or misfolded viral proteins accumulate and aggregate in the lumen of the ER, resulting in ER stress [135,136]. In order to overcome this ER stress, the cell activates the unfolded protein response (UPR) or ER stress response [136–138]. Once initiated, it results in activation of signalling cascades in a sequential order by three ER-localised transmembrane transducers, which include: (1) double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK), (2) activating transcription factor (ATF) 6, and (3) inositol-requiring enzyme (IRE1) [137,138].

These signalling cascades alter the cell's transcriptional and translational programmes to cope with stressful conditions and resolve protein-folding defects, thus ensuring cell survival. If the UPR fails, cells initiate lethal programmes such as autophagy or cell death by either apoptosis or necrosis [136,139,140]. In a study by Bhoola and colleagues, the activity of the PERK and IRE1/XBP1 pathways appeared to be decreased, whereas the ATF6 pathway appeared to be increased, while apoptosis was unchanged for cells transfected with the G1862T mutant when compared to the wild-type counterpart. Therefore, HBeAg in patients infected with the G1862T mutant leads to increased ER stress, which can result in liver damage, which has been shown to be a contributing factor to HCC [122]. A higher frequency of G1862T has been found in both serum and liver tissue of HCC patients compared to asymptomatic carriers [141] (Figure 4).

4.3.2. Extrinsic Pathways

- HBeAg regulation of apoptosis via Fas/FasL

Hepatic apoptosis during HBV infection is mostly mediated by death signalling from members of the tumour necrosis factor (TNF) protein family, including TNF-α, Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL) [142]. Fas, a glycoprotein, is present in the liver cell membrane and triggers apoptosis signals to the hepatocytes when Fas ligand (FasL) binds with it [143]. FasL can induce massive hepatic apoptosis in most types of liver disease and is increased in patients with chronic and acute hepatitis B or HBV-related hepatocellular carcinoma [144,145]. The Fas/FasL apoptosis signal is strictly regulated at both receptor and intracellular apoptotic signalling events. Previous studies suggest that Fas transcription is activated by p53 in hepatocytes, and the cross talk between the Fas signalling and p53 in controlling apoptosis is of clinical importance [146,147].

As shown previously, HBeAg and its precursors may function as a significant enhancer of HCC through many mechanisms, including repressing p53 activity through interacting with NUMB [128,148]. A study by Liu and colleagues showed that HBeAg serves to inhibit p53-dependent FasL- and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated hepatic apoptosis by down-regulating both membrane (m) Fas death receptors DR4 and DR5 expression at a transcriptional level and enhancing the expression of soluble (s) Fas by increasing Fas alternative splicing [148]. The results indicate that HBeAg can protect hepatocytes from apoptosis induced by the Fas/FasL and TRAIL/DR systems [148]. Therefore, HBeAg likely possesses negative immunomodulatory roles [30,31]. These findings show that inhibition of the Fas and TRAIL apoptotic signals by HBeAg can lead to the survival of infected hepatocytes and contribute towards the development of HBV chronicity and hepatocarcinogenesis (Figure 4).

The loss of apoptotic control is a hallmark of cancer because it allows cancer cells to survive longer and allows for the accumulation of mutations, which can increase invasiveness during tumour progression, stimulate angiogenesis, deregulate cell proliferation, and interfere with differentiation [149]. Although through different mechanisms, the role of HBeAg in these apoptotic pathways helps promote the survival of hepatocytes infected with HBV.

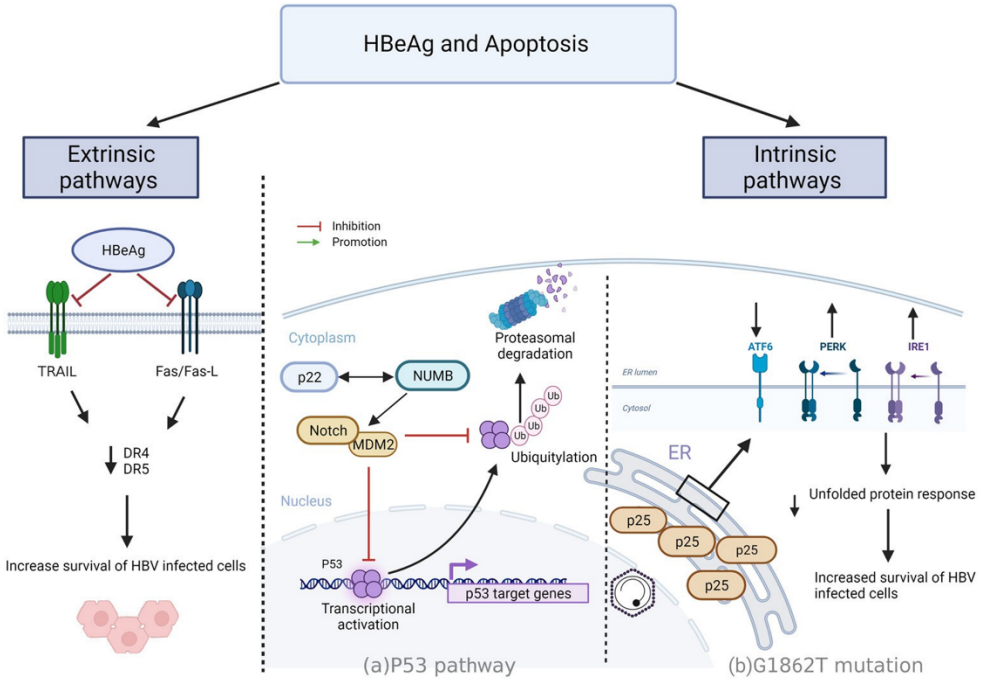


Figure 4. The role of HBeAg in Apoptosis: the role of HBeAg and its precursors in extrinsic and intrinsic apoptotic pathways. Extrinsic pathway: HBeAg inhibits Fas/FasL and TRAIL/DR systems which leads to the down-regulation of Fas death receptors, DR4 and DR5, expression to promote the survival of HBV infected hepatocytes [148]. Intrinsic pathways: (**a**) P53 pathway—p22 interacts with NUMB, which regulates Notch and MDM2 preventing ubiquitination and degradation of p53 and impairing the stability and transcriptional activity of p53 [128]; (**b**) G1862T mutation—G1862T mutation results in the accumulation of p25 in the ER, decreasing the activity of the PERK and IRE1/XBP1 while increasing the activity of the ATF6 receptor pathways. This results in a decrease in UPR to promote survival of HBV infected hepatocytes [122]. Image created using biorender.com.

4.4. Promote Sustained Proliferative Signalling

Cancer cells can proliferate indefinitely. Cells have a receptor at their surface which, when receiving a growth factor signal from neighbouring cells, initiates an intracellular cascade of signalling resulting in cell growth and division [150]. Cells normally need this feedback from other cells to know when to divide, ensuring that proliferation happens in a coordinated manner.

1. HBeAg Interferes with Wnt/β-catenin Signalling

The *Wnt* signalling pathway had been identified and studied extensively in carcinogenesis and is regulated via multiple mechanisms [151]. The most common occurrence has been investigated in colon cancer, where it results in constitutively active *Wnt* signalling downregulation by naturally occurring inhibitors. This leads to increased or decreased transcription of *T-cell factor (TCF)/β-catenin* target genes downstream [152,153].

Liver cancers, including HCC, display similar *Wnt*-related mechanisms to colon and gastric cancer [153]. Studies have shown that constitutively active *Wnt* signalling in HCC is primarily via mutations to the *CTNNB1* gene that remove the regulatory phosphorylation sites from the N-terminus of β-catenin [154]. A recent study by Tran and colleagues screened various HBV proteins for their impact on *Wnt* signalling. The study showed that HBeAg precursor p22 had a strong correlation with *Wnt* signalling. The p22 protein was more potent than the HBx protein, which is a known and well-studied carcinogen. Other pre-core/core proteins (p25, p21, or p17) did not activate TCF/β-catenin transcription despite significant amino acid sequence overlap with p22 [155].

The study also demonstrated that Frizzled-7 (Fzd7) and Glutamate-Ammonia Ligase (GLUL), which have a known association with liver cancer and interact with TCF/β-catenin, are induced by p22 in vivo [155]. Furthermore, they demonstrated that p22 can increase *TCF/β-catenin* transcription on its own and in conjunction with ectopically expressed wild-type or mutant β-catenin. Activation of *TCF/β-catenin* transcription by p22 was blocked by dnTCF4, confirming its impact specifically on *Wnt* signalling [155] (Figure 5).

Selective regulation of *Wnt* signalling is central to identifying possible therapeutic interventions for cancer. Further investigations need to be done to determine if hyperactive *Wnt* signalling is due to loss of transcriptional repression or activation of transcription [155].

2. HBeAg Interferes with the Regulation of the Cell Cycle

Increasing evidence is emerging that the expression of cellular miRNA and their gene targets play a prominent role in HBV pathogenesis [21,156]. miRNA are small non-coding RNAs of 19–23 nucleotides that play key roles in the regulation of almost every cellular process in all multicellular eukaryotes [157]. Oncogenic miRNAs may facilitate cell cycle entry and progression by targeting CDK inhibitors or transcriptional repressors of the retinoblastoma family. On the other hand, tumour suppressor miRNAs induce cell cycle arrest by downregulating multiple components of the cell cycle machinery [158]. Viruses can be affected by these miRNAs, whilst other viruses, such as herpesvirus, encode their own miRNA to increase replication and evade the immune system [159]. HBV can modulate cellular miRNA to achieve the same results [21].

HBx, is an oncoprotein that is a major regulator of both gene expression and miRNA expression in the host [21], and it has been well documented to interact with various miRNA. HBeAg could also influence miRNA expression in a manner similar to HBx. Indeed, clinical studies have indicated differences in miRNA profiles between HBeAg positive and negative patients. miRNAs identified with disrupted plasma expression specifically in HBeAg-positive patients with chronic hepatitis and with liver-specific target genes are biomarkers for disease progression and might impact the development of HCC [160]. This has paved the way for extending research into the association of HBeAg and miRNA regulation.

Cell-cycle progression is stimulated by protein kinase complexes, each of which consists of a cyclin and a cyclin-dependent kinase (CDK), to ensure faithful replication of the genome before cell division [161]. A study by Samal and colleagues showed that HBeAg is an oncoprotein that regulates the cell cycle through the induction of miR-106b. MicroRNA-106b is a member of the miR-106b-25 cluster and is known to act as an oncogene that is associated with poor prognosis among HCC patients [94]. Samal and colleagues used anti-miR-106b (anti-sense oligonucleotides that inhibit miR-106b) to show an increase in retinoblastoma gene (Rb) gene expression in Huh7 cells, suggesting that the *Rb* gene is a target of miR-106b at 3^tUTR (3^t untranslated region) of the *Rb* gene. The *Rb* gene is a tumour suppressor gene with a well-documented role in HCC, including the modulation

of cell proliferation and cell cycle progression [94]. Anti-miR-106b inhibits cell proliferation in HBeAg expressing Huh7 cells but not in control Huh7 cells and these findings clearly highlight the link between HBeAg-induced miR-106b [162]. By targeting the *Rb* genes, this leads to enhanced cell proliferation and promotes progression from the G0/G1 phase into the S phase of the cell cycle in Huh7 cells [162]. This suggests that HBeAg is an oncoprotein that inhibits the *Rb* gene expression via induction of an oncogenic miRNA, miR-106b (Figure 5).

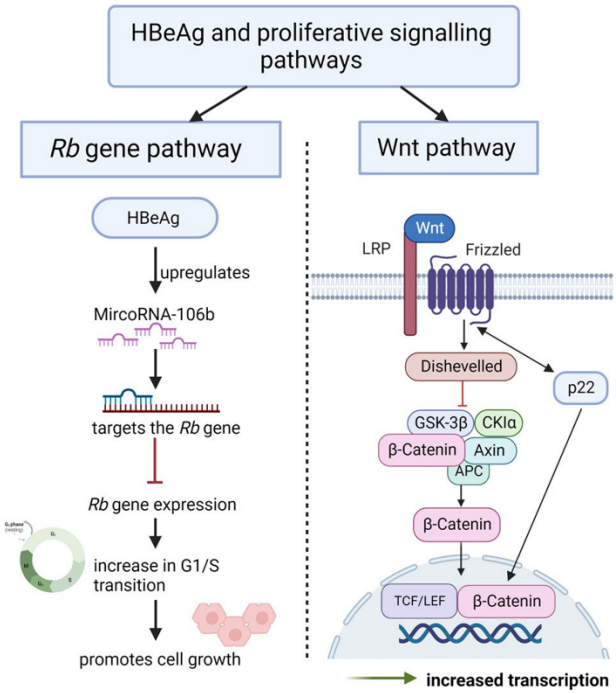


Figure 5. Role of HBeAg in proliferative signalling pathways: the role of the HBeAg *Rb* gene pathway and the wnt signalling pathway. *Rb* gene pathway—HBeAg upregulates miR-106b, which targets the Rb gene. Thus, reducing *Rb* gene expression, resulting in a promotion of progression from the G0/G1 phase into the S phase of the cell cycle in Huh7 cells, adapted with permission from [162], Scientific reports (http://creativecommons.org/licenses/by/4.0/, accessed on 23 March 2023). Wnt signalling pathway—p22 induces Fzd7 and GLUL and is able to increase TCF/β-catenin transcription on its own to promote proliferative signalling [155]. Image created using biorender.com.

5. Discussion

HBV is one of the seven known human oncogenic viruses and has adapted to coexist with a single host for prolonged periods, requiring continuous manipulation of immunity and cell fate decisions [163,164]. Oncogenic viruses promote tumorigenesis using relatively few multifunctional viral oncoproteins [165,166].

HBx protein is the most well-researched HBV oncoprotein. As shown for human papillomavirus (HPV) and simian vacuolating virus 40 (SV40), a virus can have several viral oncoproteins [166]. Thus, the complex interlinking roles of HBeAg and its precursors, which we have reviewed, may implicate HBeAg and its precursors as potential HBV

oncoproteins. However, this would need to be confirmed by studies in in vivo animal models, as has been done for HBx and HBsAg [20,21,167].

In summary, mutations of the BCP/precore region that result in loss of extracellular HBeAg and accumulation of various HBeAg precursors in the cytoplasm of HBV infected cells [29,53,78]. HBeAg and its precursors can lead to the downregulation of pro-inflammatory transcription factors such as NF-κB, JAK/TYK/STAT, and P13K [132,164]. These activated transcription factors mediate the expression of key cytokines and chemokines to direct the immune system away from HBV-infected hepatocytes and ensure immune evasion [111]. Secondly, defective expression of HBeAg results in the immune system and inflammatory cytokines being directed towards HBV-infected hepatocytes, which cause an increase in inflammation [74,78]. Thirdly, HBeAg and its precursors interfere with both intrinsic and extrinsic apoptotic pathways to ensure the survival of HBV-infected hepatocytes [122,128,148]. Lastly, HBeAg and its precursors, can initiate an intracellular signalling cascade, which leads to cell growth and division [155,162]. Therefore, this review has demonstrated that the HBeAg and its precursors can play roles in at least four hallmarks of cancer: avoiding immune destruction; tumour-promoting inflammation; resisting cell death; and sustained proliferative signalling (Figure 1). Thus, these roles of HBeAg and its precursors implicate them as potential oncoproteins.

Further research on the role of HBeAg and its precursors in carcinogenesis is ongoing. These studies could discover mechanisms by which HBeAg and its precursors can be associated with additional hallmarks of cancer. For example, it has been shown that p22 and p25 can translocate to the nucleus [43], where they could possibly interact with the host genome, leading to genomic instability and mutation and possibly enabling the replication of immortal liver cells. Fifty years after its discovery [49], HBeAg continues to intrigue, with its unique properties, and keep researchers active in elucidating its intricate role in the molecular biology and pathogenesis of HBV infection, in particular its role in hepatocarcinogenesis.

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Chapter 3

Paper 2- Article: Comparative proteomic analysis of Huh7 cells transfected with sub-Saharan African Hepatitis B Virus (sub)genotypes reveals potential oncogenic factors

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The genetic variation in HBV (sub)genotypes plays a significant role in the natural history of infection, the clinical outcome, and the response to antiviral treatment (Kramvis, Kew et al. 2005, Kramvis 2007). In addition, differences in the mode of expression of viral proteins such as HBeAg (marker for infectivity) in different (sub)genotypes distinguish between acute and chronic infections (Coleman 2006) and may account for the differences in disease progression (Kramvis 2016). As demonstrated in the previous chapter, HBeAg plays a significant role in host signalling pathways through the various hallmarks of cancer and can influence the hepatocarcinogenic potential of the (sub)genotype. Currently, little is known about the difference in specific biological pathways induced by the different HBV genotypic backgrounds, found in sub-Saharan Africa (SSA). Therefore, Chapter 3 of this thesis, provides the first report of the molecular differences at the proteome level between Huh7 cells transfected with the SSA (sub)genotypes A1, D3 and E and subgenotype A2 which prevails outside of Africa.

Author contribution: Ms Padarath performed all the investigations, methodology and analysis, validated the results, and mined the data using various bioinformatical software. She prepared the original draft including figures and tables, and rewrote 5 drafts after reading fellow authors' comments.

viruses

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Article

Comparative Proteomic Analysis of Huh7 Cells Transfected with Sub-Saharan African Hepatitis B Virus (Sub)genotypes Reveals Potential Oncogenic Factors

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Abstract: In sub-Saharan Africa (SSA), the (sub)genotypes A1, D3, and E of the hepatitis B virus (HBV) prevail. Individuals infected with subgenotype A1 have a 4.5-fold increased risk of HCC compared to those infected with other (sub)genotypes. The effect of (sub)genotypes on protein expression and host signalling has not been studied. Mass spectrometry was used to analyse the proteome of Huh7 cells transfected with replication-competent clones. Proteomic analysis revealed significantly differentially expressed proteins between SSA (sub)genotypes. Different (sub)genotypes have the propensity to dysregulate specific host signalling pathways. Subgenotype A1 resulted in dysregulation within the Ras pathway. Ras-associated protein, RhoC, was significantly upregulated in cells transfected with subgenotype A1 compared to those transfected with other (sub)genotypes, on both a proteomic (>1.5-fold) and mRNA level ($p < 0.05$). Two of the main cellular signalling pathways involving RHOC, MAPK and PI3K/Akt/mTOR, regulate cell growth, motility, and survival. Downstream signalling products of these pathways have been shown to increase MMP2 and MMP9 expression. An extracellular MMP2 and MMP9 ELISA revealed a non-significant increase in MMP2 and MMP9 in the cells transfected with A1 compared to the other (sub)genotypes ($p < 0.05$). The upregulated Ras-associated proteins have been implicated as oncoproteins in various cancers and could contribute to the increased hepatocarcinogenic potential of A1.

Keywords: hepatitis B virus; (sub)genotypes; hepatocellular carcinoma; viral oncogenesis; oncogenic pathways

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1. Introduction

Despite the availability of an effective vaccine, hepatitis B virus infections remain hyperendemic in the sub-Saharan Africa (SSA) region. HBV, one of the seven known oncogenic viruses, is responsible for the high incidence rate of hepatocellular carcinoma (HCC) in SSA. In 2020, SSA had the fourth-highest number of diagnosed primary liver cancer (PLC) cases worldwide, of which 77% were HCCs (varying from 67% to 88% by region) [1]. This cancer accounts for 8.3% of the deaths caused by all cancers globally, being the third leading cause of worldwide cancer death [2,3]. It is estimated that, by 2025, more than one million individuals will develop liver cancer annually [4]. In less developed countries, HCC occurs in chronic viral hepatitis patients more frequently than in more

developed Western countries (e.g., 53% of the cases in South Africa [5] compared to only 9.5% in the U.S. [6]).

In addition to the hyperendemic occurrence of HBV in SSA, genetic variation in HBV can influence the clinical manifestation of infection. HBV reverse transcriptase lacks a proofreading function, which allows the virus to accumulate genetic errors over time, leading to sequence heterogeneity [7–10]. This genetic diversity has resulted in at least nine genetically distinct HBV genotypes, designated A to I, and a tenth putative, genotype J [8,11–13], with >7.5% intergroup nucleotide divergence [12,14–16]. The nine genotypes are further classified into at least 35 subgenotypes, based on a nucleotide divergence of 4–8% [11,17–19]. The genotypes and certain subgenotypes have distinct geographical distributions and are important in both the clinical manifestation of infection, response to antiviral therapy, and the development of HCC. In SSA, the most prominent (sub)genotypes are A1, D3, and E [20,21].

Subgenotype A1, which prevails in Southeastern SSA, has a higher hepatocarcinogenic potential, with a 4.5-fold increased risk of HCC development and an earlier age of diagnosis in patients infected with subgenotype A1 compared to other HBV genotypes [12,21,22]. Subgenotype D3 is associated with high chronicity, which can lead to cirrhosis and HCC, whilst genotype E is associated with significantly higher viral loads and a higher frequency of HBeAg positivity [8,19,23–26]. Subgenotype A2, which prevails outside SSA and has been used as a comparison for (sub)genotypes prevailing in SSA, has a different natural history to A1, D3, and E [27]. In a case–control study, comparing individuals infected with subgenotypes A1 and A2, and genotype D, respectively, it was shown that, regardless of the age group, the frequency of HBeAg positivity was higher in individuals infected with subgenotype A2 compared to the other (sub)genotypes [28]. This difference reached statistical significance in individuals younger than 30 years of age [22,28]. The reason for this is that the only mutations that can influence HBeAg expression in strains isolated from individuals infected with subgenotype A2, which has 1858C in the precore region, are 1762T/1764A in the basic core promoter (BCP). This mutation only decreases HBeAg levels [29]. On the other hand, subgenotype A1 strains, which also have 1858C, can develop additional mutations in the Kozak sequence within the BCP (1809–1812) [30] and the G1862T in the precore [31,32], which further decreases HBeAg levels [22] and thus accounts for the higher frequency of HBeAg negativity observed in individuals infected with subgenotype A1. Subgenotype D3 and genotype E, which both have 1858T, can develop the classic G1896A mutation, which abrogates HBeAg expression as a result of a stop codon [33].

Proteomic studies have aimed to describe global changes in host cells following HBV infection [34–36]. Previous studies have shown that the protein expression alterations between HBV genotypes B and C are involved in several different biological processes, including translation, oxidation–reduction, intracellular transport, the establishment of protein localisation, protein transport, and translational elongation, the pathogenesis of HBV-induced HCCs associated with different molecular mechanisms [34,37,38]. Currently, little is known about the differences in the specific biological pathways induced by the different HBV genotypic backgrounds found in SSA. Therefore, this study provides the first report of the molecular differences at the proteome level between Huh7 cells transfected with the SSA (sub)genotypes A1, D3, and E, and subgenotype A2, which prevails outside of Africa. Understanding the role of genotypic variation in pathogenesis is important and will provide further insight into patient outcomes, chronicity, infectivity, response to therapeutic approaches, and improve the early detection and screening of HCCs in SSA.

2. Materials and Methods

1. *Plasmid Constructs*

A collection of 1.28-mer replication-competent plasmids for wild-type (sub)genotypes A1 (KM519453), A2 (KM519454), D3 (KM519455), and E (PP790594) were

constructed and were able to replicate and express proteins in vitro [39,40]. Ethics approval to perform this study was obtained from the University of the Witwatersrand Research Ethics Committee (Medical), Johannesburg, South Africa (HREC: W-CBP-210416-01).

2. *Cell Line and Transfections*

Huh7 cells were transfected as previously described [39]. Twenty-four hours before transfection, 3×10^6 Huh7 cells were plated into each of the 10 cm culture dishes for mass spectrometry, whilst 2×10^5 Huh7 cells were plated in each of the 12 well plates containing either coverslips for indirect immunofluorescence or no coverslips for the real-time polymerase chain reaction (RT-PCR) (viral load/RNA expression) or enzyme-linked immunosorbent assay, ELISA, and then incubated overnight at 37 °C in a humidified incubator containing 5% (*w*/*v*) CO_2. For each transfection experiment, an eGFP control was included in triplicate to measure the transfection efficiency 1 day after transfection by determining the number of cells that had been successfully transfected. This was measured using the FLoid™ Cell Imaging Station (Thermofisher, Waltham, MA, USA).

3. *Mass Spectrometry Analysis*

1. Cell Lysis and Protein Preparation

Five days post-transfection, the cells were washed 3 times with PBS. Aliquots of 0.5 million cells were prepared and pelleted at 300× *g* for 10 min. The supernatants were removed and cell pellets were stored at −80 °C until further use. For protein preparation, the cell pellets were thawed on ice and resuspended in 200 µL of lysis buffer (1% SDS, 50 mM Tris-HCl pH 8.0) per pellet. Protein was extracted from the cell pellets using a PIXUL multi-sample sonicator (Active Motif, Carlsbad, CA, USA). The sonication settings were as follows: Pulse = 50; PRF = 1; Process Time = 30; and Burst = 20. Thereafter, the lysates were incubated with 25 units of benzonase, and $MgCl_2$ to a final concentration of 2 mM, at 37 °C for 30 min. Next, the samples were centrifuged at 15,000× *g* to clear cell debris. The supernatants were then collected, and the concentrations were determined using a BCA assay. Protein solutions were reduced using DTT to a final concentration of 10 mM for 30 min at 37 °C, and then alkylated using IAA to a final concentration of 40 mM for 30 min in the dark.

2. Sample Clean-Up and Digestion

All experiments were performed with a KingFisher™ Flex (Thermo Fisher Scientific, Waltham, MA, USA) magnetic particle processing robot, as previously described [41]. The KingFisher™ Flex system was configured for automated HILIC protein clean-up and on-bead Trypsin digestion. In brief, deep 96 well plates were loaded in each carousel position, with each plate filled as follows: (1) 96 well tip heads (Thermo Fisher Scientific, Waltham, MA, USA); (2) 10 µL of 20 mg/mL hyper porous magnetic MagReSyn HILIC microspheres (ReSyn Biosciences, Pretoria, South Africa) in 20% EtOH and 180 µL of Equilibration Buffer (100 mM NH_4Ac, 15% MeCN pH 4.5); (3) Equilibration Buffer (500 µL); (4) Protein extract mixed 1:1 with Binding buffer (200 mM NH_4Ac, 30% MeCN pH 4.5) to a final volume of 100 µL; (5) 500 µL of 95% MeCN (wash 1); (6) 500 µL of 95% MeCN (wash 2); (7) 200 µL of 50 mM Ammonium Bicarbonate (pH 8.0); and (8) Promega Sequencing Grade Trypsin for an enzyme/protein ratio of 1:10. The Bindit programme was then run, with the magnetic pins transferring the magnetic HILIC beads from position 2 to 8 and, in the process, binding proteins, washing off SDS and other contaminants, and finally generating peptides ready for LC–MS analysis following the on-bead Trypsin digest.

3. LC–MS Data Acquisition

Following HILIC, the peptide samples were vacuum dried and then resuspended in 2% MeCN/0.2% FA. Analysis was performed on a Dionex Ultimate 3000 RSLC system

coupled to an AB Sciex 5600 TripleTOF mass spectrometer (Sciex, Toronto, ON, Canada). The injected peptides were inline de-salted using an Acclaim PepMap C18 trap column (Thermofisher, Waltham, MA, USA) (75 µm × 2 cm; for 2 min at 5 µL/min, using 2% ACN/0.2% FA). The trapped peptides were gradient eluted and separated using a Waters Acquity CSH C18 NanoEase column (Waters™, Milford, MA, USA) (75 µm × 25 cm, 1.7 µm particle size) at a flowrate of 0.3 µL/min with a gradient of 6–40% B over 60 min (A: 0.1% FA; B: 80% ACN/0.1% FA). For Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH), precursor scans were acquired from 400 to 1100 *m*/*z* with an accumulation time of 50 milliseconds and fragment ions acquired from 200 to 1800 *m*/*z* for 48 variable-width precursor windows, with a 0.5 Da overlap between the windows and an accumulation time of 20 milliseconds per window.

4. SWATH–MS Data Processing

SWATH data was processed using Spectronaut v17 software (Biognosys, Zurich, Switzerland). The default directDIA identification and quantification settings were used for data processing. Carbamidomethylation was added as a fixed modification, and N-terminal acetylation and methionine oxidation were added as variable modifications. Swiss-Prot Human sequences (downloaded on 12 December 2022 from www.uniprot.org) and common contaminating proteins was used as the search database. A q-value ≤ 0.01 cut-off was applied at the precursor and protein levels. Quantification was performed at the MS1 and MS2 levels. Label-free cross-run normalisation was employed using a global normalisation strategy. Candidate dysregulated proteins were filtered at a q-value ≤ 0.05, absolute fold change (FC) ≥ 1.5, and a minimum of two unique peptides identified.

5. Mass Spectrometry Bioinformatics

Data Visualisation

Volcano plots and heat maps were generated using https://www.bioinformatics.com.cn/, accessed on 12 March 2024. All Venn diagrams were drawn using Venny (Oliveros, J.C. (2007–2015)), an interactive tool was used for comparing lists with the Venn diagrams (https://bioinfogp.cnb.csic.es/tools/venny/index.html, accessed on 24 March 2023).

Pathway Analysis

A list of significantly differentially expressed proteins (p-value ≤ 0.05) was analysed using the Protein Analysis Through Evolutionary Relationships (PANTHER online: http://pantherdb.org, accessed on 23 March 2023) classification system [42]. PANTHER is a comprehensive system which combines gene function, ontology, pathways, and statistical analysis tools to allow for the analysis of a large volume of data [43]. The top 10 signalling pathways of subgenotype A1 compared to the untransfected cells, vector control, and each of the other (sub)genotypes A2, D3, and E, were categorised.

Gene-Set Enrichment Analysis (GSEA)

Gene-set enrichment analysis (GSEA) allows for the detection of modest but coordinated changes in the expression of protein-encoding genes involved in a common biological function. To determine the top exclusive oncogenic pathways amongst the various (sub)genotypes compared to the vector control, GSEA was performed by the R package clusterProfiler (version 4.3.3). The t-statistic mean of the genes was computed in Hallmark's pathway using 4 replications per condition. The upregulated pathways were defined by a normalised enrichment score (NES) > 0. Gene sets with a p-value less than 0.05 were considered to be significantly enriched or depleted.

STRING Analysis

The STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) is a curated knowledge database used for the illustration of predicted interactions of identified proteins and neighbour genes. The proteins that were significantly differentially expressed in subgenotype A1, relative to each of the other (sub)genotypes and the vector control, were processed in STRING version 12.0 (https://string-db.org/, accessed on 12 March 2024) to obtain medium-confidence interaction data (score ≥ 0.4). The PPI network was visualised using the Cytoscape 3.2.1 software (https://cytoscape.org/, accessed on 12 March 2024).

2.4. RNA Expression Levels

Total RNA was isolated from the Huh7 cells transfected with the various (sub)genotypes and controls (untransfected and vector controls) using the RNAeasy mini extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. An aliquot of 500 ng of RNA was reverse transcribed to generate cDNA using the SuperScript™ VI cDNA First-Strand Synthesis System synthesis kit (Invitrogen, Boston, MA, USA), as per the manufacturer's instructions. A total of 5 µL of cDNA (1:10 dilution) was amplified using the SYBR®Green Realtime PCR Master Mix (Thermofisher, Waltham, MA, USA) and performed on a MyCycler Thermocycler (Bio-Rad Laboratories, Hercules, CA, USA). Thermal cycling conditions were as follows: Initial denaturation at 95 °C for 2 min, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 1 min, and a final elongation at 72 °C for 10 min. Three relative expression levels of the target genes were calculated using the ΔCt method, with GAPDH as the control housekeeping gene. Primers for the qPCR are listed in the following Table 1.

Table 1. Primers use for reverse transcriptase qPCR.

Gene	Direction	Sequence
RHOC	F	5′-TCCTCATCGTCTTCAGCAAG-3
	R	5′-CTGCAATCCGAAAGAAGCTG-3′
Rap2B	F	5′-TTACCGCAAGGAGATTGAG-3′
	R	5′-GGCTGTAGACCAGGATGAAG-3′
GNB1	F	5′-TGCTTGGAGAGTGTGGGCTTCT-3′
	R	5′-TGGAGGGCATCTCCAGAATCTG-3′
GAPDH	F	5′-GAAGGTGAAGGTCGGAGT-3′
	R	5′-GAAGATGGTGATGGGATTTC-3′

2.5. Viral Loads

Analysis of the viral loads was performed using the protocol described by Bhoola et al. [39]. On day 5 post-transfection, the total DNA was extracted from the harvested transfected Huh7 cells using the NucleoSpin® Tissue kit (Macherey–Nagel, Nordrhein-Westfalen, Germany). Meanwhile, an aliquot of 5 mL of transfected medium that had been collected on day 3 or 5 was centrifuged at 22,000× *g* for 5 min at 4 °C, after which a 100 µL aliquot of the sample was adjusted with 10 µL of 10× Incubation Buffer (Roche Diagnostics, Basel, Switzerland), treated with 1 µL of 10 U/µL DNase I (Roche Diagnostics) and 10 µL 100 mg/mL RNase A (Fermentas Molecular Biology Tools, Waltham, MA, USA), and then incubated at 37 °C for 20 min. Subsequently, the reaction was terminated by adding 5 µL of 0.2 M EDTA, pH 8.0, and then incubating at 75 °C for 10 min. Thereafter, extracellular total DNA and intracellular total DNA were extracted using the NucleoSpin® Tissue kit (Macherey–Nagel, Nordrhein-Westfalen, Germany). The PCR primers, HBV DNA F (5′-CGTGTGTCTTGGCCAAAATTCG-3′) and HBV DNA R (5′-CATCCAGCGATAACCAGGACAA-3′), with a FAM/NFQ labelled TaqMan® MGB probe (5′-FAM-TCACTCACCAACCTCC-NFQ-3′), were used to quantify HBV DNA in the ABI 7500 Real-Time PCR system (Applied Biosystems by Life Technologies, Waltham, MA, USA), using the PCR

thermal cycling conditions previously described [44]. A serial dilution of plasmid DNA containing a single genome of subgenotype A1, ranging from 1×10^4 to 1×10^8 copies/mL, was used as a template to generate the standard curve. The second WHO International Standard for Hepatitis B Virus DNA (National Institute for Biological Standards and Controls (NIBSC), Hertfordshire, UK), which has a final concentration of 1×10^6 IU/mL, was used as the positive control and for calibration of the standard curve.

2.6. ELISA

After 5 days post-transfection, the expression of MMP9, MMP2, and RHOC were analysed with various ELISA kits according to the manufacturer's protocol. Intracellular proteins were harvested by sonication. The concentrations were determined using a standard curve generated using the manufacturer's protocol.

1. MMP9—Human MMP-9 ELISA Kit (RK00217) (ABclonal, Woburn, MA, USA) https://abclonal.com/elisa-kits/HumanMMP-9ELISAKit/RK00217, accessed on 12 December 2023
2. MMP2—Human Matrix Metalloproteinase 2 ELISA Kit (MMP2) (RK00309) (ABclonal, Woburn, MA, USA) https://abclonal.com/elisa-kits/HumanMMP-2ELISAKit/RK00309, accessed on 12 December 2023
3. RHOC—Human Rho-related GTP-binding protein RhoC (RHOC) ELISA Kit (RK11965) (ABclonal, Woburn, MA, USA) https://ap.abclonal.com/elisa-kits/HumanRhorelatedGTPbindingproteinRhoCRHOCELISAKit/RK11965, accessed on 12 December 2023.

2.7. Immunofluorescence

The following protocols were adapted from Deroubaix et al. [45]. Cells cultured on coverslips in 12 well plates were washed 3 times with PBS and then fixed with 3.7% formaldehyde in 1x PBS for 10 min at room temperature. The fixed cells were again washed 3 times with 1x PBS and permeabilised with 0.1% Triton–PBS for 7 min, before being washed 3 more times with PBS. The cells were then incubated for 1 h at room temperature with 1% bovine serum albumin (BSA, Fraction V, Roche Diagnostics GmbH by Roche Applied Science, Basel, Switzerland) (diluted in PBS 1x). Next, the cells were incubated with the respective primary antibodies (Transfected cells: anti-HBV core protein rabbit DAKO [46], Agilent Technologies (Santa Clara, CA, USA), 1/1000, anti-RHOA/RHOC mouse, Invitrogen, 1/250) for 1 h at 37 °C. Then the cells were washed 8 times with PBS 1X and incubated with the corresponding secondary antibodies (Alexa-Fluor 488 labelled anti-mouse (1:1000, Invitrogen) and Alexa Fluor 546 labelled anti-rabbit (1:1000, Invitrogen)) for 1 h at 37 °C. Cells were washed 8 more times with 1x PBS. The DNA was stained with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, 1 mg/mL, 1/2000, Sigma-Aldrich, St. Louis, MO, USA) for 10 min at room temperature in the dark. The coverslips were then mounted using Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich), sealed using a clear nail polish after 3 h, and then analysed by fluorescent microscopy the following day.

2.8. Microscopy and Image Analysis

Microscopy was performed using a Leica Microsystems THUNDER Imager Tissue Microscope (Wetzlar, Germany) equipped with a 63x objective (HC PL FLUOTAR 63x/1.30 OIL by Leica Microsystems, Wetzlar, Germany) and Leica Application Suite X (LAS X) software (version 1.4.6 28433). The exposure time for each channel (Alexa Fluor 488 (325.05 ms), Alexa Fluor 546 (475.95 ms), and DAPI (11.25 ms)) was kept constant for each condition ((sub) genotypes, untransfected cells, and controls) to obtain comparable results between the different experimental slides. The exposure time was determined by removing any background staining from the negative control for Alexa Fluor 448 and 546. After capturing the image, the Leica deconvolution software was used to process the images.

Quantification of fluorescence was performed using ImageJ software (Fiji/Image J (version: ij153), accessed on https://imagej.net/Fiji/Downloads), as described by Deroubaix et al. [45]. Briefly, the intensity of fluorescence for RHOA/RHOC across the cytoplasm was measured, averaged across a minimum of 30 transfected cells per (sub)genotype, and then compared to the untransfected control.

2.9. Data Statistical Analysis

Statistics were calculated using GraphPad Prism version 10.2.2.

3. Results

1. Comparison of the Proteome of Huh7 Cells Transfected with the SSA HBV Genotypes

Following transfection of the Huh7 cells with replication-competent clones and a vector control, the proteome was analysed 5 days post-transfection. Using SWATH–MS, a specific variant of the data-independent acquisition (DIA) method, 3906 proteins and 36 107 peptides were identified in the Huh7 cells transfected with the different HBV (sub)genotypes (Figure S1). Principal component analysis (PCA) was able to differentiate the proteome of the cells transfected with individual (sub)genotypes (Figure S1).

Volcano plots were generated to identify differentially expressed proteins in the transfected and untransfected cells (Figure 1A). Significantly differentially expressed proteins (DEPs) were classified into upregulated and downregulated proteins. A minimum fold change ≥1.5 and maximum false discovery rate (FDR)-adjusted *p*-value (q-value) of ≤0.05 were used to filter proteins that were significantly different between the various HBV (sub)genotypes and the vector control group. Firstly, the DEPs of the different HBV (sub)genotypes to the vector control were compared (Figure 1A). Compared to the vector control, the different (sub)genotypes showed different numbers of DEPs, which did not differ in the AVG $\log_2$ expression levels and range (Table 2). Subgenotype A1 had the least number of DEPs (323), whereas subgenotype A2 had the greatest number of DEPs (1025) (Figure 1B). However, subgenotype A1 showed the highest percentage of upregulated DEPs (72%) (Table 1). When comparing the commonly expressed proteins for all the (sub)genotypes, the F-box only protein (FBXO2) and Arginase type II (ARG2) were upregulated in subgenotype A1 compared to the remaining (sub)genotypes where they were downregulated. Compared to subgenotypes A2 and D3, the vitamin K epoxide reductase complex (VKORC1) and normal mucosa of oesophagus-specific gene-1 (NMES1) were upregulated in subgenotype A1. Splicing Factor-SWAP (SFSWAP) showed downregulation in subgenotype A1, while the other (sub)genotypes showed upregulation (Figure S3A,B).

For simplification, the DEPs were classified according to their corresponding pathways using the PANTHER bioinformatic tool, accessed 23 March 2023. The pathways were ranked according to the number of DEPs, and pathways with fewer than two proteins allocated were excluded from the analysis. When comparing the top 10 signalling pathways between (sub)genotypes A1, A2, D3, and E to the vector control, it was observed that the different (sub)genotypes favoured the dysregulation of specific host signalling pathways (Figure 2A). It should be noted that pathways marked with a (-) were not found in the top 10 signalling pathways but could still be dysregulated. The top 10 pathways shared between (sub)genotypes A1, A2, D3, and E when compared to the vector control were apoptosis, angiogenesis, cytoskeletal regulation by Rho GTPase, inflammation-mediated signalling, fibroblast growth factor (FGF) signalling, the wingless-related integration site (Wnt) signalling pathway, tumour p53 pathways, the rat sarcoma (RAS) pathway, the integrin signalling pathway, and the cholecystokinin receptor (CCKR) signalling pathway (Figure 2B). Interestingly, only the CCKR signalling and Ras pathways appeared in the top 10 categories amongst all the HBV genotypes. Subgenotype A1 caused dysregulation in seven proteins involved in the Ras signalling pathway (Figure 2C), whereas subgenotype A2 caused the most dysregulation in the Wnt pathway (13 DEPs), and subgenotype D3 caused the most dysregulation in the Integrin signalling pathway (12 DEPs).

Interestingly, Genotype E did not favour a dysregulation of proteins in any of the top 10 signalling pathways (Figure 2C). The p53 pathway was not in the top 10 pathways dysregulated by subgenotype A1 relative to the vector control because only one protein was dysregulated in that pathway and the cut off used was >2 proteins. However, subgenotype A1 also showed dysregulation of a single protein in an alternate p53 pathway, the p53 pathway by glucose deprivation. No genes were dysregulated in the latter pathways by (sub)genotypes A2, D3, and E.

Table 2. AVG $\log_2$ expression of differentially expressed proteins found in the different HBV (sub)genotypes A1 compared to the vector control.

Expression	(Sub)genotype	Mean AVG $\log_2$ Expression	AVG $\log_2$ Range	Mean *p*-Value	% of DEPs
Upregulated	A1	0.90	0.58–1.98	0.0073764	72
	A2	0.91	0.58–2.26	0.0027466	63
	D3	0.85	0.58–2.11	0.0032396	63
	E	0.87	0.58–2.71	0.0030094	62
Downregulated	A1	−0.89	−0.58—2.18	0.0150298	28
	A2	−0.92	−0.58—2.30	0.0040270	37
	D3	−0.84	−0.58—2.21	0.0070993	37
	E	−0.86	−0.58—2.45	0.0099477	38

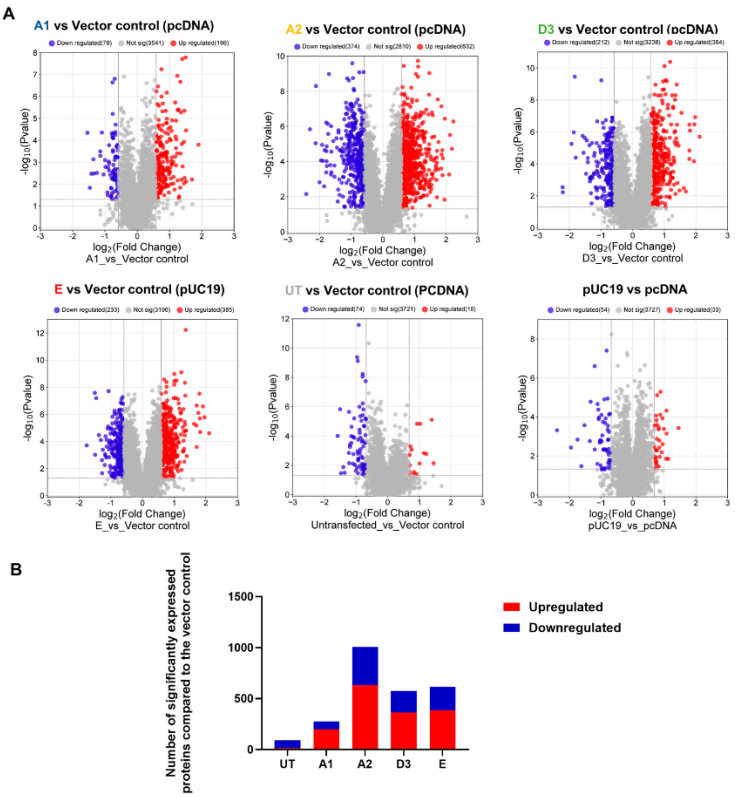
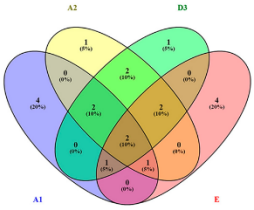


Figure 1. Mass spectrometry analysis of the HBV genotypes: (**A**) Volcano plots of the differentially expressed proteins in Huh7 cells transfected with replication-competent A1, A2, D3, E, and untransfected control against the vector-only control, using ©Spectronaut software. The negative *x*-axis represents downregulation (blue) in the vector control group, and the positive axis represents upregulated (red) proteins in the vector control group. (**B**) A bar graph showing the number of significantly upregulated (red) and downregulated (blue) proteins in each of the HBV (sub)genotypes, and the untransfected cells, compared to the vector control.

A



B

Top 10 Signalling pathways	A1	A2	D3	E
Adrenaline & noradrenaline biosythesis	-	-	-	↓
Angiogenesis	-	↑↓	↑↓	↑↓
Apoptosis signalling pathway	-	↑↓	↑↓	↑↓
CCKR signalling map	↑↓	↑↓	↑↓	↑↓
Cytoskeletal regulation by RHO GTPase	↑	↑↓	-	↑↓
DNA replication	↑↓	-	-	-
EGF receptor signalling pathway	-	↑↓	-	-
FGF signaling pathway	↑↓	↑↓	↑↓	-
Glycolysis	-	-	↑	-
Inflammation mediated signalling pathway	↑↓	↑↓	↑↓	-
Integrin signalling pathway	↑↓	-	↑↓	↑↓
p38 MAPK pathway	↑↓	-	-	-
p53 pathway	-	↑↓	↑↓	↑↓
PDGF signalling pathway	-	-	-	↑↓
PI3 Kinase pathway	↑	-	-	-
Ras Pathway	↑	↑↓	↑↓	↑↓
T cell activation	↑↓	-	-	-
Toll receptor signalling pathway	-	-	-	↑↓
Wnt signaling pathway	-	↑	↑↓	-

Legend	
↓	Proteins in this pathway are downregulated
↑	Proteins in this pathway are upregulated
↑↓	Proteins from this pathway are both up and downregulated
-	Pathway is not included in the top 10 signalling pathways

C

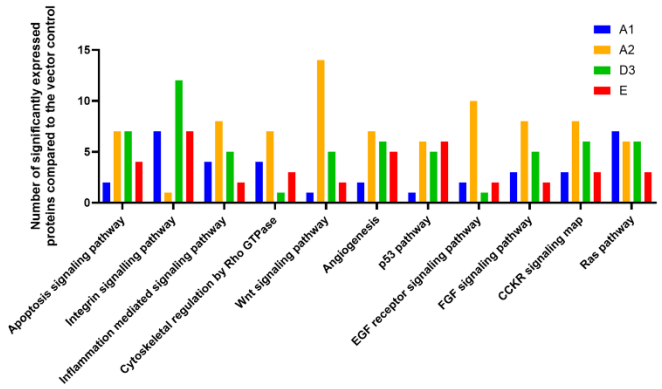


Figure 2. Proteomic analysis of the top 10 signalling pathways amongst the HBV genotypes. (**A**) Venn diagram indicating the pathways associated with differentially expressed proteins from A1, A2, D3, and E. Proteins were normalised against both the untransfected and vector-only control. (**B**) Comparison table demonstrating the top 10 pathways across the various genotypes. Proteomic analysis revealed significantly differently expressed proteins ($p < 0.05$) between the various (sub)genotypes These differentially expressed proteins were further classified into pathways. (**C**) Comparison table of the number of proteins amongst the top 10 pathways in (sub)genotypes A1, A2, D3, and E, which were present in more than one (sub)genotype.

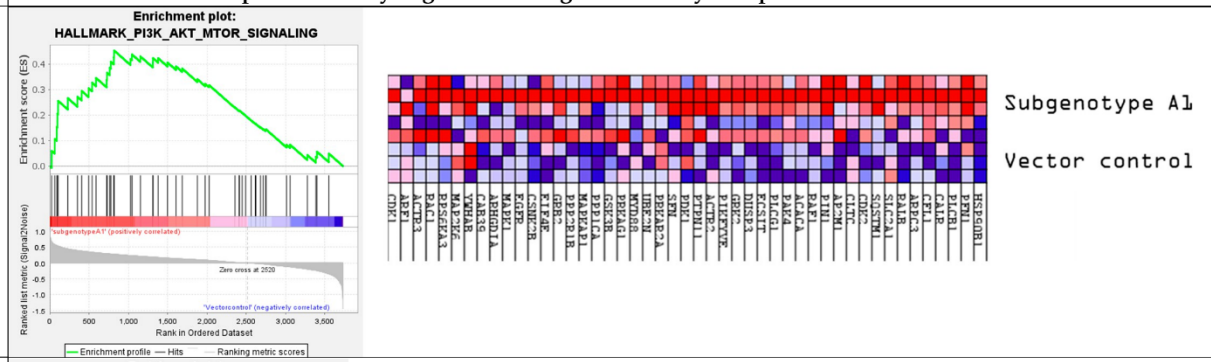
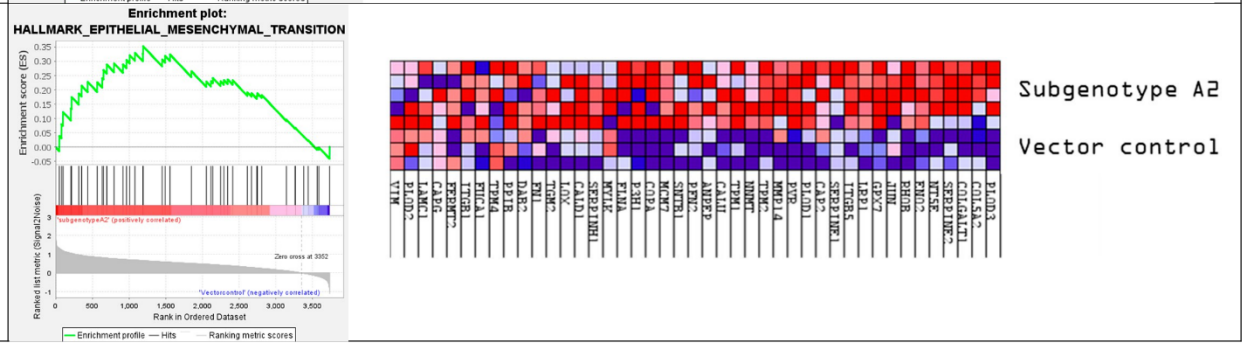
Out of the top 10 pathways dysregulated in the transfected cells, several were dysregulated only by a single subgenotype; exclusively by each of the different HBV (sub)genotypes as follows: T cell activation, PI3K pathway, DNA replication and P38 MAPK pathway by A1; EGF receptor signalling pathway by A2; glycolysis by D3; and PDGF signalling, adrenaline and noradrenaline receptor, and toll receptor signalling pathways by genotype E (Figure 2B). It should be noted that these pathways were exclusively found in the top 10 signalling pathways dysregulated by the different HBV (sub)genotypes, and this does not imply that these pathways were not dysregulated by other HBV (sub)genotype but were not in the 10 pathways dysregulated.

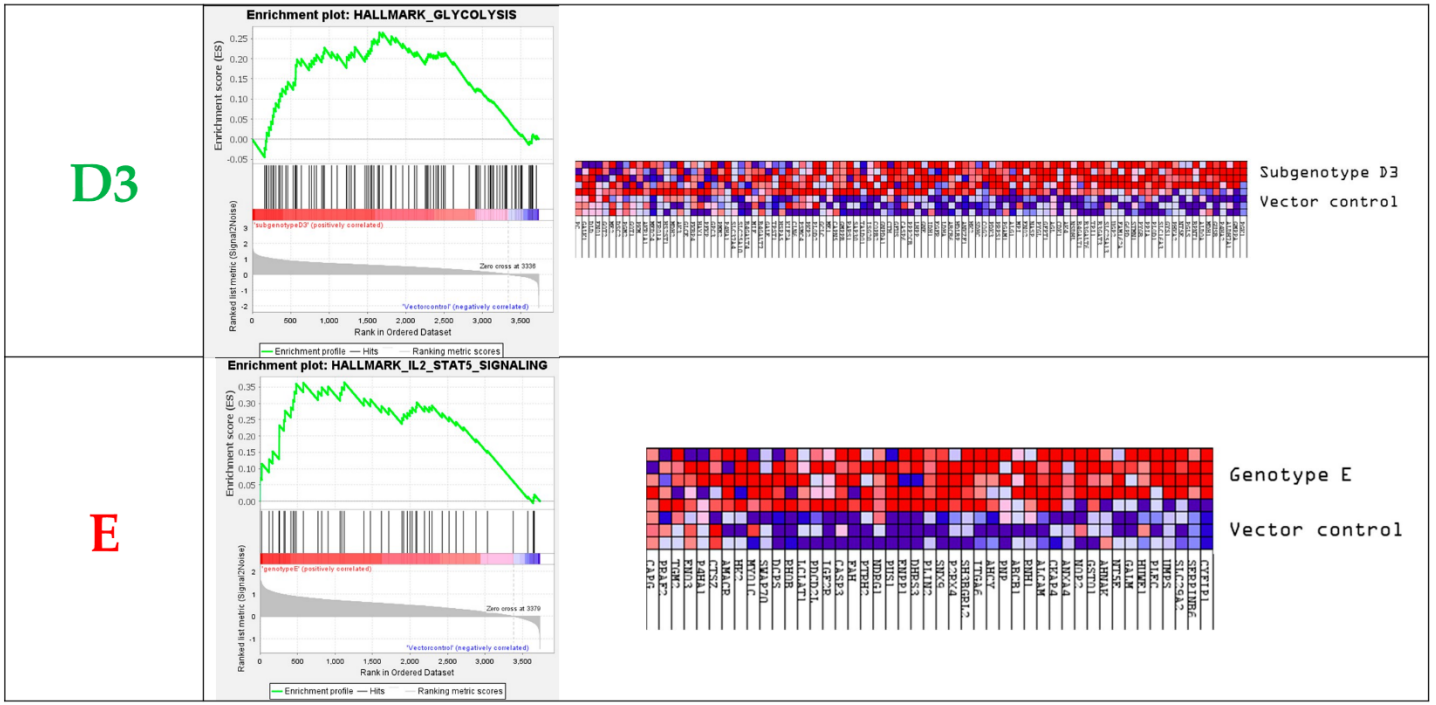
These results were confirmed using Gene Set Enrichment Analysis (GSEA), which is a computational method that determines whether a defined set of genes shows statistically significant, concordant differences between two biological states [47]. The proteome of Huh7 cells transfected with the various HBV (sub)genotypes was analysed by GSEA compared to the vector control. The significantly enriched genes were classified according to their oncogenic pathways. GSEA revealed the top exclusive oncogenic pathways for each (sub)genotype (Table 3). The hallmarks of cancer pathway PI3K_AKT_MTOR and epithelial_mesenchymal_ transition were significantly enriched for A1 and A2, respectively, whereas the glycolysis and IL2_STAT5_oncogenic pathways were enriched for subgenotype D3 and genotype E, respectively. Collectively, these results indicate that different HBV (sub)genotypes have a propensity to favour dysregulation in a specific host signalling pathway. No significant differences were observed in both the intracellular and extracellular viral load expression between the different (sub)genotypes 5 days post-transfection (Figure S2). However, differences in the expression of other viral proteins (e.g., HBsAg, HBeAg, etc.) which were not evaluated in this study may influence the proteome.

3.2. Hepatocarcinogenic Potential of Subgenotype A1 May Be Attributed to the Dysregulation of Ras-Associated Proteins

Previous studies have shown that subgenotype A1 has a higher hepatocarcinogenic potential compared to other (sub)genotypes [12]. The proteomic expression of the different (sub)genotypes was compared to determine whether different protein expressions may be responsible for differences that may contribute to the hepatocarcinogenic potential. Proteomic data suggest that subgenotype A1 favours the dysregulation of proteins involved in the Ras pathways compared to the other genotypes in the vector control background (Figure 2C). The dysregulated Ras proteins have been shown to affect downstream signalling in the P13K/mTOR/Akt signalling pathways, which was confirmed by the GSEA analysis between subgenotype A1 and the vector control (Table 3). The DIA SWATH–MS data analysis was repeated and used to compare quantifiable proteins in subgenotype A1 relative to the different HBV (sub)genotypes, using the same parameters as the previous analysis. A minimum fold change ≥1.5 and maximum FDR-adjusted *p*-value (q-value) ≤ 0.05 were used to filter proteins that were significantly different between subgenotype A1 and the various HBV (sub)genotypes (Figure 3A). Compared to subgenotype A2, subgenotype A1 showed the most DEPs (856), whereas subgenotype D3 (541) and genotype E (545) had a similar number of DEPs (Figure 3B).

Table 3. GSEA analysis of the top exclusive oncogenic pathway and the associated proteins dysregulated in Huh7 cells amongst the different HBV (sub)genotypes.

(Sub)genotypes	Top Exclusive Dysregulated Oncogenic Pathway Compared to the Vector Control
A1	
A2	


D3
Enrichment plot: HALLMARK_GLYCOLYSIS
Enrichment score (ES)
Ranked list metric (Signal2Noise)
'subgenotypeD3' (positively correlated)
Zero cross at 3336
'Vectorcontrol' (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
Subgenotype D3
Vector control
E
Enrichment plot: HALLMARK_IL2_STAT5_SIGNALING
Enrichment score (ES)
Ranked list metric (Signal2Noise)
'genotypeE' (positively correlated)
Zero cross at 3379
'Vectorcontrol' (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
Genotype E
Vector control

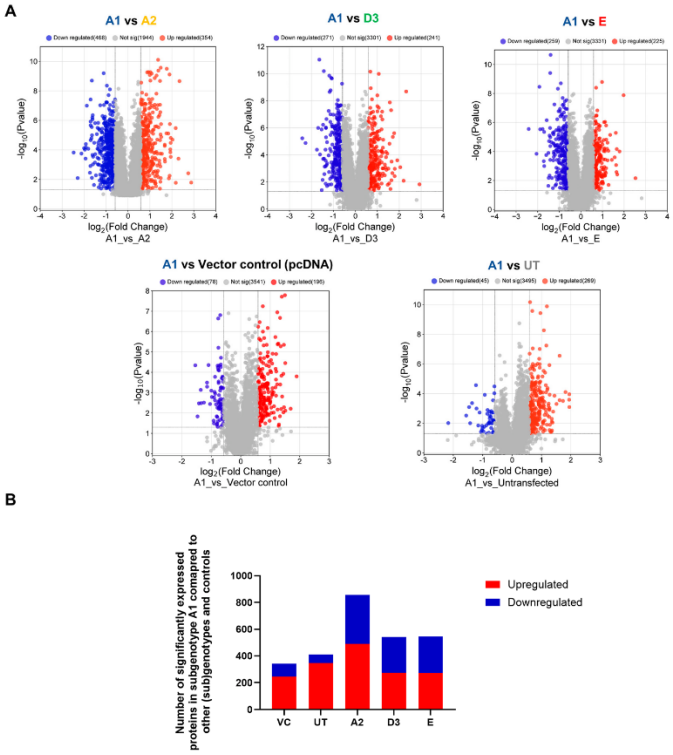
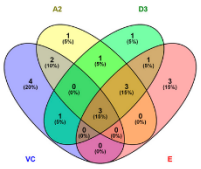


Figure 3. Mass spectrometry analysis of subgenotype A1 compared to different (sub)genotypes and controls. (**A**) Volcano plots of the differentially expressed proteins in Huh7 cells transfected with replication-competent A2, D3, E, and untransfected control, and vector control against subgenotype A1 using ©Spectronaut software. The negative *x*-axis represents downregulation (blue) in subgenotype A1, and the positive axis represents upregulated (red) proteins in subgenotype A1. (**B**) A bar graph showing the number of significantly upregulated (red) and downregulated (blue) proteins in each of the HBV (sub)genotypes, untransfected, and vector control, compared to subgenotype A1.

For simplification, the DEPs were classified according to their corresponding pathways using the PANTHER bioinformatic tool, accessed 23 March 2023. The pathways were ranked according to the number of DEPs, and pathways with fewer than two proteins allocated were excluded from the analysis. The top 10 signalling pathways present in subgenotypeA1 compared to the other HBV (sub)genotypes and the vector control were as follows: apoptosis, blood coagulation, CCKR signalling map pathway, cytoskeletal regulation by RHO GTPases, FGF signalling, inflammation-mediated signalling pathway, Integrin signalling pathway, p53 signalling pathway, Ras signalling pathway, and Wnt signalling pathway (Figure 4A). The CCKR signalling, cytoskeletal regulation by RHO GTPases, and integrin signalling pathways were dysregulated in A1 compared to each of the other (sub)genotypes and the vector control. Interestingly, only the CCKR signalling, RHO GTPases, and Integrin signalling pathways were common in the top 10 pathways involving subgenotype A1 compared to the other (sub)genotypes and vector control. With the exception of the blood coagulation pathway, subgenotype A2 had the highest number of DEPs in all of the pathways when compared to A1 (Figure 4B). Collectively, these

results indicate that the greatest difference occurred between subgenotypes A1 and A2, revealing that even within the same genotype, the different subgenotypes can have varying effects on the Huh7 proteome.

A



B

Top 10 Signalling pathways in subgenotype A1 compared to other HBV (sub)genotypes and vector control	VC	A2	D3	E
Adrenaline and noradrenaline biosynthesis	-	-	-	↓
Angiogenesis	-	↑↓	↑↓	-
Apoptosis signalling pathway	-	↑↓	↑↓	↑↓
Blood coagulation	-	-	↓	↓
CCKR signalling map	↑↓	↑↓	↑↓	↑↓
Cytoskeletal regulation by RHO GTPase	↑	↑	↑	↑↓
DNA replication	↑	-	-	-
Dopamine receptor mediated signaling pathway	-	-	-	↓
EGF receptor signalling pathway	-	↑↓	-	-
FGF signaling pathway	↑↓	↑↓	-	-
Heterotrimeric G-protein signaling pathway	-	-	↑↓	-
Inflammation mediated signalling pathway	↑↓	↑↓	-	-
Integrin signalling pathway	↑	↑	↑↓	↑↓
p38 MAPK pathway	↑↓	-	-	-
p53 pathway	-	↑↓	↑↓	↑↓
PI3 Kinase pathway	↑	-	-	-
Ras Pathway	↑	-	↑	-
T cell activation	↑↓	-	-	-
Ubiquitin proteasome pathway	-	-	-	↓
Wnt signaling pathway	-	↓	↓	↓

Legend	
↓	Proteins in this pathway are downregulated in subgenotype A1
↑	Proteins in this pathway are upregulated in subgenotype A1
↑↓	Proteins from this pathway are both up and downregulated in subgenotype A1
-	Pathway is not included in the top 10 signalling pathways

C

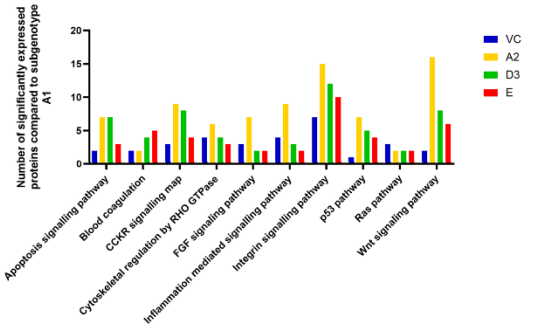


Figure 4. Proteomic analysis of the top 10 signalling pathways amongst the HBV genotypes: (**A**) Venn diagram indicating the pathways associated with differentially expressed proteins from A2, D3, E, and the vector control (VC). Proteomic analysis revealed significantly differently expressed proteins ($p < 0.05$) between the various (sub)genotypes These differentially expressed proteins were further classified into pathways. (**B**) Comparison table of the number of proteins amongst the top 10 pathways in subgenotype A1 compared to (sub)genotypes A2, D3, E, and the VC. (**C**) Comparison table of the number of proteins amongst the top 10 pathways in subgenotype A1 compared to (sub)genotypes A2, D3 and E, and vector control, which were present in more than one sample.

Next, common upregulated (32) and downregulated (31) DEPs in subgenotype A1 compared to the various HBV (sub)genotypes were identified, as shown in Figures 5 and

6, respectively. ShinyGO Gene Ontology enrichment analysis [48] was performed using the differentially expressed common up- and downregulated proteins in subgenotype A1 compared to the other HBV (sub)genotypes and vector control. The average (AVG) log_2 expression in A1 relative to vector control and other genotypes is represented in Table 4 below.

Table 4. AVG log_2 expression of the common differentially expressed proteins found subgenotypes A1 compared to the other (sub)genotypes.

Expression	(Sub)genotype/Control	Mean AVG log_2 Expression	Mean AVG log_2 Range	Mean *p*-Value
Upregulated	VC	1.10	0.59–1.66	0.0074896
	A2	1.40	0.64–2.92	0.0038629
	D3	1.40	0.61–2.45	0.0045624
	E	1.73	0.60–2.77	0.0083334
Downregulated	VC	−0.82	−0.60—1.62	0.0159729
	A2	−1.18	−0.59—2.12	0.0044853
	D3	−1.00	−0.61—2.26	0.0042862
	E	−1.11	−0.60—1.92	0.0066141

FBOX2 was the only protein upregulated in subgenotype A1, relative to A2, D3 and E, at an AVG log_2 expression > 2, whereas ARG2 was upregulated at an AVG log_2 expression > 2, relative to A2 and D3. Three proteins, lactase dehydrogenase B (LDBH), cytochrome c oxidase copper chaperone (COX11), and NMES1, were upregulated at an AVG log_2 expression > 2, only relative to A2.

The enrichment of 11 similar upregulated pathways (Figure 5C) and 20 similar downregulated pathways was observed (Figure 6C). Furthermore, the data confirmed the enrichment of activated pathways through several interconnecting networks using STRING analysis (Figures 5E and 6E). The common upregulated proteins were involved in cell proliferation, apoptotic resistance, evasion, and metastasis processes (Figure 5C). These included the following Reactome pathways: RHO GTPases activating formins, G alpha 12/13 signalling events, G Beta—Gamma signalling through PI3K signalling, RHO GTPases activating ROCKS, and G protein gamma signalling pathways, etc. The common downregulated proteins were involved in immunosuppression and carbohydrate metabolism (Figure 6C). These included the following Reactome pathways: Extracellular matrix organisation, metabolism of carbohydrates, disease of metabolism, disease of immune system and disease associated with the TLR signalling cascade, etc. and others.

Amongst the 32 commonly upregulated proteins, 3 proteins of interest were identified, namely guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 (GNB1), Rho-related GTP-binding protein (RHOC), and Ras-Associated Protein 2b (Rap2b), for their involvement in the Ras pathway (Figure 5). Each of these proteins was significantly upregulated (>1.5 fold change increase) in subgenotype A1 compared to other (sub)genotypes and the vector control (Figure 5B).

The increased expression of the Ras-associated proteins in subgenotype A1 compared to other HBV (sub)genotypes was verified on an RNA level, using RT-qPCR. mRNA expression levels of the Ras-associated proteins 5 days post-transfection revealed that RHOC and Rap2B were significantly upregulated in A1 compared to the other HBV genotypes, vector control, and the untransfected control, validating the results from the SWATH–MS (Figure 7A,C) using a one-way ANOVA statistical test. However, mRNA expression levels of the Ras-associated protein GNB1 only revealed a significant increase in subgenotype A1 compared to subgenotype A2, the vector control, and the untransfected control, using a one-way ANOVA statistical test (Figure 7C).

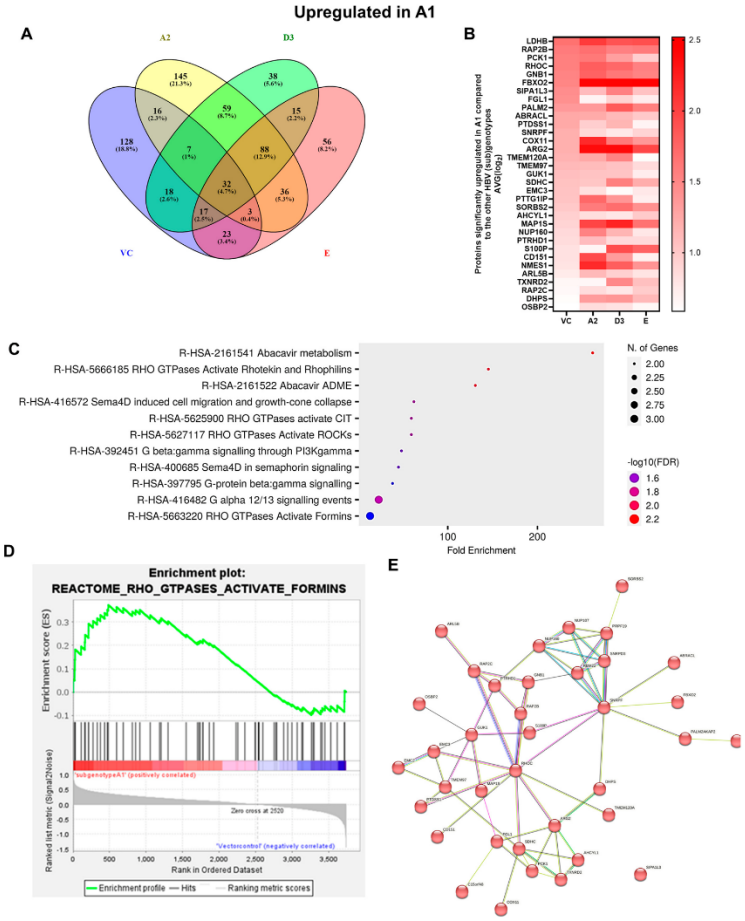


Figure 5. Upregulated common proteins found in subgenotype A1 in comparison to the other HBV (sub)genotypes. (**A**) Venn diagram illustrating the 32 significantly differentially expressed upregulated proteins found in A1 compared to the other HBV (sub)genotypes and the vector control. (**B**) The heatmaps show the upregulated (red) average $\log_2$ expression of the 32 significantly expressed common proteins in A1 compared to other HBV (sub)genotypes and the vector control. (**C**) A dot plot generated using the upregulated potential protein in ShinyGO analysis, with Reactome pathway enrichment and fold enrichment based on the number of genes present in each pathway. The FDR cut-off was set at 0.05, and the number of pathways was set to 10. (**D**) GSEA enrichment plot between subgenotype A1 and the vector control, showing the upregulated enrichment of Reactome pathway of RHO GTPases activating formins. (E) Network analysis of upregulated dysregulated proteins using STRING network (Fold cut-off set to 0.4). Lines of different colors represent seven types of evidence used in predicting associations. Red line: fusion evidence; green line: neighborhood evidence; blue line: co-occurrence evidence; purple line: experimental evidence; yellow line: text mining evidence; light blue line: database evidence; black line: co-expression evidence.

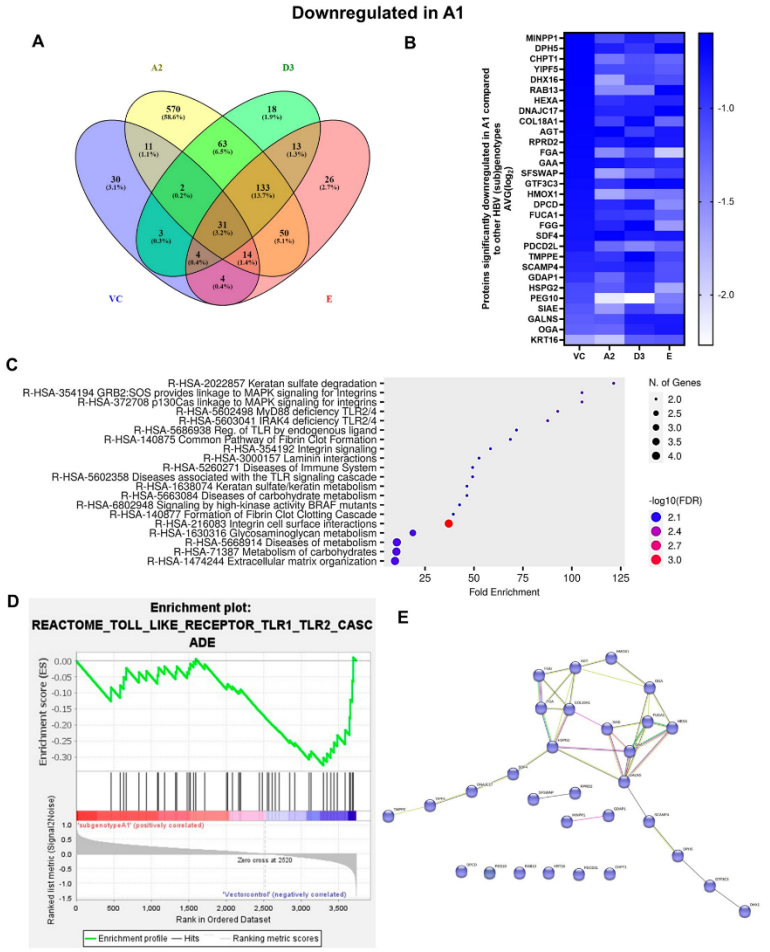


Figure 6. Downregulated common proteins found in subgenotype A1 in comparison to the other HBV (sub)genotypes. (**A**) Venn diagram illustrating the 31 significantly differentially expressed downregulated proteins found in A1 compared to the other HBV (sub)genotypes and the vector control. (**B**) The heatmaps show the downregulated (blue) average $\log_2$ expression of the 32 significantly expressed common proteins in A1 compared to other HBV (sub)genotypes and the vector control. (**C**) A dot plot generated using the downregulated potential protein in ShinyGO analysis, with Reactome pathway enrichment and fold enrichment based on the number of genes present in each pathway. The FDR cut-off was set at 0.05, and the number of pathways was set to 10. (**D**) GSEA enrichment plot between subgenotype A1 and the vector control, showing the downregulated enrichment of the Reactome pathway of Toll-like receptor TLR1–TLR2 cascade. (**E**) Network analysis of downregulated dysregulated proteins using STRING network (Fold cut-off set to 0.4). Lines of different colors represent seven types of evidence used in predicting associations. Red line: fusion evidence; green line: neighborhood evidence; blue line: co-occurrence evidence; purple line: experimental evidence; yellow line: text mining evidence; light blue line: database evidence; black line: co-expression evidence.

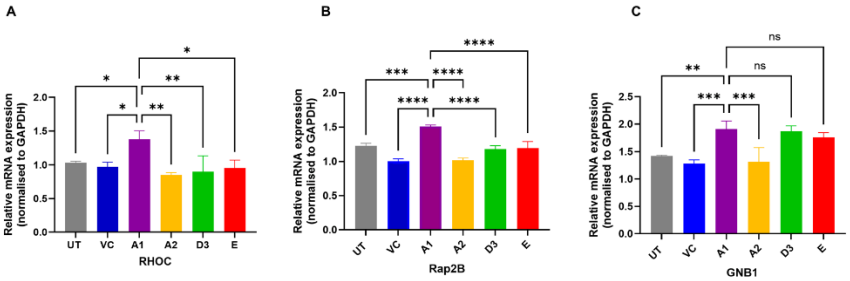


Figure 7. Verification of the potential Ras-associated proteins. Ras-associated proteins RHOC (**A**), Rap2B (**B**), and GNB1 (**C**) were verified as potential proteins by measuring the mRNA expression normalised to the GADPH housekeeping control 5 days post-transfection. Results are expressed as a ratio between the mRNA expression of the Ras-associated proteins and GAPDH. Significant differences were analysed using a one-way ANOVA statistical test and compared to the expression in subgenotype A1. * = *p*-value < 0.05, ** = *p*-value < 0.01, *** = *p*-value < 0.001, **** = *p*-value < 0.0001 and ns = not significant.

3.3. Subgenotype A1 Influence on RHOC and Its Downstream Signalling Products

Gene enrichment analysis using both ShinyGO 8.0 and GSEA between subgenotype A1 and the vector control (Figure 5C,D) indicated that the top common pathway amongst the 32 upregulated proteins in subgenotype A1 compared to the other HBV (sub)genotypes and vector control was RHO GTPases activating formins using the Reactome pathways. Recently, mounting evidence has demonstrated that the Rho GTPase family plays an important role in mediating HCC progression [49]. Therefore, overexpression of RHOC was explored as a protein of interest for subgenotype A1. The overexpression of RHOC in subgenotype A1, compared to the other HBV (sub)genotypes and vector control was further verified on a proteomic level by ELISA. We examined both the intercellular and extracellular expression of RHOC 5 days post transfection and found that intracellular RHOC expression was significantly higher in subgenotype A1 compared to other HBV (sub)genotypes and controls (untransfected and vector control), validating the results from the DIA SWATH-MS using a one-way ANOVA statistical test (Figure 8A). These results agreed with mass spectrometry and mRNA expression results. However, as expected, there was no difference in the extracellular expression of RHOC, as it is only expressed intracellularly [50].

Next, downstream extracellular signalling products activated by RHOC overexpression matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9) were tested. The extracellular MMP2 and MMP9 ELISA revealed a non-significant increase in MMP2 and MMP9 in the supernatant of the subgenotype A1-transfected cells compared to those transfected with the other (sub)genotypes 5 days post-transfection (Figure 8B).

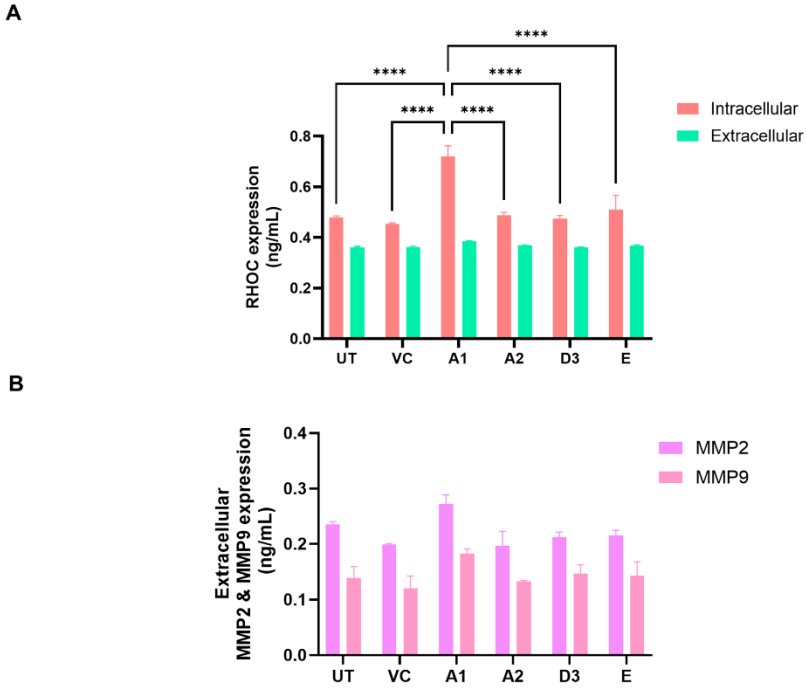


Figure 8. The expression of RHOC and its downstream signalling products. (**A**) Intracellular (red) and extracellular (green) expression of RHOC in Huh7 cells transfected with the various HBV (sub)genotypes after 5 days. Significant differences were analysed using a one-way ANOVA statistical test and compared to the expression in subgenotype A1 (**** = *p*-value < 0.0001). (**B**) The extracellular supernatant was harvested from HBV (sub)genotypes and controls, 5 days post-transfection, and analysed with MMP2 and MMP9 ELISA kit. Results indicated that MMP2 (purple) extracellular expression levels were significantly higher than MMP9 expression levels (pink) using a two-way ANOVA statistical test. Subgenotype A1 showed a non-significant increase in both MMP2 and MMP9 expression levels compared to the other HBV (sub)genotypes and controls.

Using the RHOA/RHOC antibody, mean fluorescent intensity was significantly higher in the subgenotype A1-transfected cells compared to the other (sub)genotypes and the untransfected control (Figure 9A). The majority of Huh7 cells transfected with the HBV (sub)genotypes displayed a diffused gradient staining of the cytoplasm throughout the cell. Co-localisation of the RHOA/RHOC antibody and the HBV core protein (HBc) was observed in 35% of the Huh7 cells transfected with subgenotype A1, compared to the other HBV genotypes and untransfected control, which showed a lower range of 15–20% (Figure 9B,C). This localisation occurred mainly at the periphery of the cells, intimating the role of RHOC as a regulator of focal cell adhesion and in lobopodial migration [51]. It should be noted the anti-HBc (Dako), which is used to detect viral proteins in transfected cells, does not differentiate between core and precore proteins.

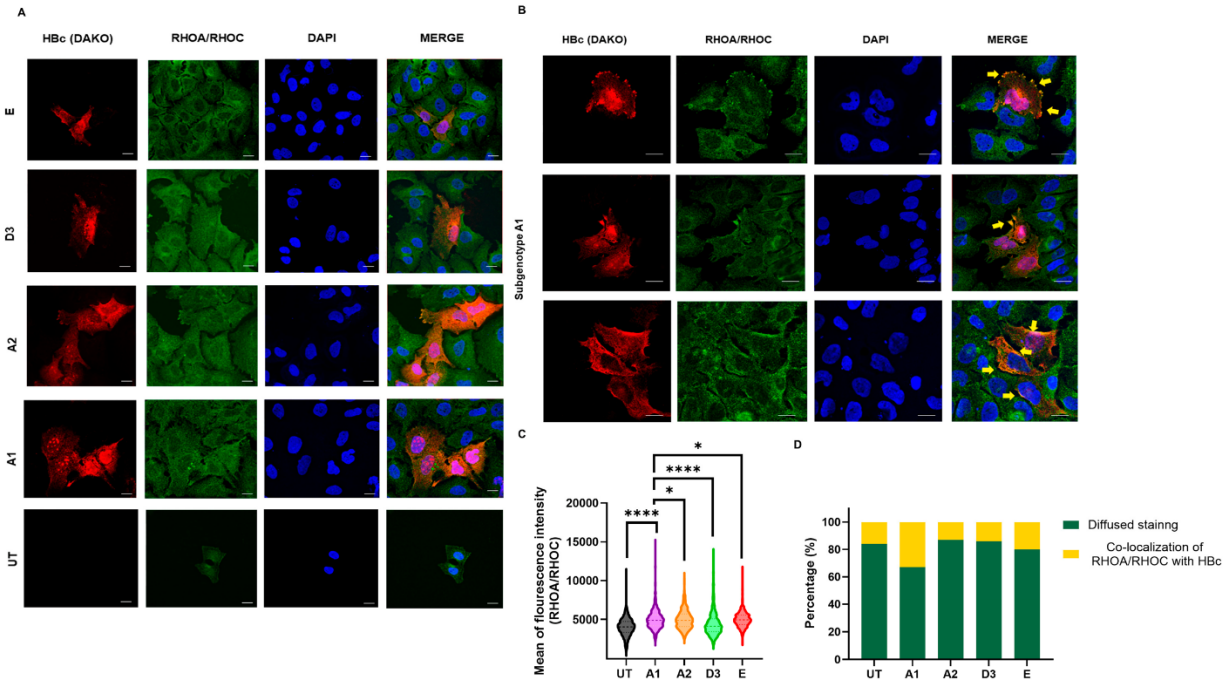


Figure 9. The localisation of RHOC expression in the various (sub)genotypes. (**A**) Microscopy of transfected Huh7 cells with the different HBV (sub)genotypes after 5 days, viewed with a fluorescent microscope. Cells were immunostained with a polyclonal rabbit anti-HBc antibody (DAKO) and monoclonal mouse anti-RHOA/RHOC antibody. The nuclei were visualised by DAPI staining. (**B**) Selected microscopy of transfected Huh7 cells with subgenotype A1 after 5 days, viewed with a fluorescent microscope. Cells were immunostained with a polyclonal rabbit anti-HBc antibody (DAKO) and monoclonal mouse anti-RHOA/RHOC antibody. The nuclei were visualised by DAPI staining. Yellow arrows indicate the areas of focal adhesion where RHOA/RHOC is co-localised with the HBV core protein. (**C**) A volcano plot indicating the mean fluorescence intensity of the RHOA/RHOA expression in cells transfected with subgenotype A1 compared to other HBV (sub)genotypes and the untransfected (UT) control. Significant differences were analysed using a one-way ANOVA statistical test and compared to the expression in subgenotype A1. * = p-value < 0.05 and **** = p-value < 0.0001. (**D**) Comparative bar chart showing the percentage of Huh7 cells that have displayed diffused RHOA/RHOC staining or focal adhesion staining, amongst the various HBV genotypes.

4. Discussion

HBV has a significant pathogenic potential, which is associated with both the ability to establish chronic infection, persist, and ultimately lead to the clinical manifestation of cirrhosis and/or HCC [52]. The course and outcomes of the infection depend on several virus–host interactions [53]. Although the incidence of chronic HBV infection (CHB) is declining globally, its incidence and associated HCC morbidity and mortality has increased in SSA [3,54]. Genetic variation in the HBV prevailing in SSA may play an important role in the virologic and clinical characteristics of infection, impacting disease progression and the response to antiviral therapy [20].

Using a DIA SWATH–MS-based quantitative proteomic approach, a comparative analysis of the whole proteome of Huh7 cells transfected with the different SSA HBV (sub)genotypes relative to subgenotype A2 was performed. Huh7 cells were used instead of HepG2 cells because the former are less sensitive to stress and serum deprivation [55]. To the best of our knowledge, this study represents the first of its kind. The different SSA HBV (sub)genotypes, compared to the vector control (Figure 2) or to each other (Figure 4), dysregulated specific host signalling pathways. For the generation of the plasmids, the sequence closest to the consensus sequence of each (sub)genotype was used to ensure the representation of the (sub)genotypes [39]. Although subgenotypes A1 and A2 belong to the same genotype, they demonstrate a higher divergence in the BCP/precore region between each other than between subgenotype A1 and (sub)genotypes D3 and E, respectively [20]. This could be the reason why the greatest difference in the proteome was between two subgenotypes of A, A1 and A2, and not between two different genotypes. Even though subgenotype A2 had the highest number of DEPs compared to subgenotype A1, the latter had a higher percentage of upregulated DEPs (Table 1).

Subgenotypes A1 and A2 have different clinical manifestations [11,20,27]. Subgenotype A1 develops different mutations in the BCP and pre-core region, whereas subgenotype A2 only develops mutations in the BCP, decreasing precore mRNA levels, which affect the expression level of HBeAg [11,20,27], resulting in significantly higher HBeAg negativity in subgenotype A1-infected patients than subgenotype A2-infected patients [21,22]. Accumulation of viral proteins such as HBeAg and HBsAg can lead to ER stress [31,56,57]. An in vitro study showed that Huh7 cells transfected with subgenotype A1 had increased ER stress and higher PERK, ATF6, and IRE1/XBP1 activity than cells transfected with subgenotype A2 and D3 [31]. In addition, FBXO2, which is an ubiquitin ligase, was the only protein upregulated in subgenotype A1 relative to A2, D3, and E, at an AVG $\log_2$ expression >2 (Figure 5B). The persistent activation of the unfolded protein response (UPR) when the cells were transfected with subgenotype A1 may explain why even though it had the least number of DEPs, it still had a higher hepatocarcinogenic potential. Additional research will be beneficial in elucidating this paradox.

1. Dysregulated Signalling Pathways

Four pathways, p53, ERK/MAPK, Wnt/β-catenin, and PI3K/Akt/mTOR were identified as part of the top 10 signalling pathways across the different (sub)genotypes when compared to the vector control (Figure 2) and were also dysregulated in subgenotype A1 when compared to the other HBV (sub)genotypes and vector control (Figure 4). Previous studies on the mechanism of HBV-induced HCCs have identified five main signalling pathways which are dysregulated in the host as follows: the p53, oxidative stress, ERK/MAPK, PI3K/Akt, and Wnt/β-catenin signalling pathways [34,37,52,58]. The oxidative stress pathway was the only pathway not in the top 10 dysregulated pathways identified in this study (Figures 2 and 4).

1. p53 Pathway

The p53 signalling pathway was dysregulated in subgenotype A1 compared to the different HBV (sub)genotypes but not relative to the vector control, as only one protein

was dysregulated in vector control and the cut-off used was >2 proteins. However, subgenotype A1 also showed dysregulation of a single protein in an alternate p53 pathway, the p53 pathway by glucose deprivation. The p53 by glucose deprivation pathway is activated in ER stress [59], which is increased in subgenotype A1 compared to the other (sub)genotypes [31]. It is possible that subgenotype A1 dysregulates p53 by alternative pathways compared to the other subgenotypes. Proteins involved in the p53 pathway were both upregulated and downregulated in subgenotype A1 compared to the other HBV (sub)genotypes (Figure 4A,B). This could be the result of the multiple feedback loops associated with this signalling pathway [60]. Mutations in the p53 tumour suppressor gene are found in 12–48% HBV-associated HCC cases [58,61–63]. These mutations result in the inactivation of p53 and cell cycle pathway activation and they are associated with tumour aggressiveness and poor prognosis [64]. It would be interesting to determine if these mutations occur more frequently in HCC patients infected with subgenotype A1.

2. MAPK Pathway

The p38 MAPK signalling pathway was identified as one of the top signalling pathways dysregulated between cells transfected with subgenotype A1 and the vector control. Furthermore, gene enrichment analysis between the common downregulated proteins in subgenotype A1 compared to the other HBV (sub)genotypes or vector control showed enrichment in the immune system pathways (Figure 6C,D), thereby allowing for the persistence of subgenotype A1. MAPK signalling has been shown to play an important role in immune escape as well as HBV replication, the promotion of tumour cell survival and drug resistance [52]. In fact, p38 MAPK has been demonstrated to play a crucial role in HBsAg/HBeAg secretion, viral replication, and the formation of HBV cccDNA in HBV-infected cells [65,66]. This downregulation in MAPK would allow subgenotype A1 to evade immune surveillance and thus persist. In SSA, subgenotype A1 is transmitted before the age of one year [67,68], when 90% of infections subsequently lead to chronic infection and can be associated with the early development of HCCs [22].

The coagulation cascade signalling pathway has been shown to mediate both host innate and adaptive immunity against viral infection [69]. As one of the top ten pathways, the blood coagulation pathway was downregulated in Huh7 cells transfected with subgenotype A1, but not in cells transfected with either of the (sub)genotypes of D3 or E (Figure 4). Dysregulation of the complement cascade was also demonstrated following proteomic analyses of the plasma derived from patients infected with either genotypes B or C [38]. Thus, the downregulation in the coagulation signalling pathway may be an additional mechanism for immune evasion by subgenotype A1, which requires further investigation.

3. Wnt/β-Catenin Pathway

The Wnt signalling pathway was downregulated in subgenotype A1 compared to the other HBV (sub)genotypes. On the other hand, subgenotype A2 showed an upregulation in 13 DEPs, leading to the dysregulation of the Wnt signalling pathways (Figure 2C). An abnormal activation of the Wnt/β-catenin signalling pathway has been observed in approximately 66% of patients with HCC, and the activation of this pathway is closely related to the occurrence, development, stemness, and drug resistance of HCC [70–72]. Similarly, the CTNNB1 mutation found in HCC tumour cells but not in adjacent non-tumour cells has been demonstrated to activate the Wnt pathway [34].

Additionally, the presence of viral proteins HBx and HBsAg drives Wnt signalling in HBV-infected patients. HBx dysregulates the Wnt pathway through several mechanisms, including the regulation of GSK3β, β-catenin, Frizzled receptor, and URG7 [72]. Considering that the HBx of subgenotype A1 differs from that of other (sub)genotypes [73] and its effect on the Wnt pathway has not been studied, the effect of A1 HBx on Wnt may also be different. Furthermore, HBsAg modulates the expression of lymphoid enhancer-binding factor 1(LEF-1), a downstream mediator and transcription factor of the Wnt signalling

pathway [74]. Studies have shown that A2 has the highest intracellular expression levels of HBsAg compared to subgenotypes A1, B1, D1, C1, and F1b [75,76]. This increase in intracellular HBsAg could explain why subgenotype A2 favours dysregulation of the Wnt signalling pathway (13 DEPs).

4.1.4. PI3K/Akt/mTOR Pathway

Proteomic analysis using GSEA revealed that the top dysregulated pathway was PI3K/Akt/mTOR pathway (Table 1). Furthermore, gene enrichment analysis between the common upregulated proteins in cells transfected with subgenotype A1 compared to the other HBV (sub)genotypes or vector control showed enrichment in genes, which were upregulated in the PI3K signalling (Figure 5). The PI3K/Akt/mTOR pathway is overexpressed in nearly 50% of HCCs, and the dysregulated activation of this pathway affects a wide range of processes, including cell proliferation, metabolism, tumour cell differentiation, lipid metabolism, autophagy, and epithelial to mesenchymal transition (EMT) [77,78]. Furthermore, previous studies have shown that the interaction of the mTOR signalling pathway and HBV life cycle is complex. The mTOR signalling pathway can regulate HBV replication and gene expression [79–81], whereas HBV infection can activate the PI3K/Akt/mTOR pathway. This occurs following the synthesis of a large quantity of viral proteins, which leads to the accumulation of HBsAg/HBeAg in the endoplasmic reticulum (ER) lumen that then triggers ER stress [31,82]. A previous study using the same plasmid construct showed that subgenotype A1 exhibited a greater amount of ER stress compared to subgenotypes A2 and D3 [31], which could possibly lead to the activation of the PI3K/Akt/mTOR pathway.

4.2. Dysregulated Proteins

Further analysis was performed to identify proteins that were commonly upregulated and affected in more than one of the four dysregulated pathways in the cells transfected with subgenotype A1, compared to the other (sub)genotypes or vector control. Three significantly upregulated Ras-associated proteins were identified. These included Ras homolog gene family, member C (RHOC), Ras-related protein-2b (Rap2B), and GNB1, which are upstream regulators of the MAPK and PI3K/Akt/mTOR pathways at the protein level (Figure 10). Both the MAPK and PI3K signalling pathways were also exclusively found in the top 10 signalling pathways with dysregulation from subgenotype A1 when compared to the vector control (Figure 2B), whilst the PI3K pathway was found to be the top exclusive oncogenic pathway in subgenotype A1 compared to the vector control using GSEA analysis (Table 1). These pathways regulate cell growth, motility, survival, and metabolism [83–86] (Figure 10).

Quantitative reverse transcription PCR was performed to confirm this upregulation. The mRNA levels of RHOC and Rap2B were significantly upregulated in subgenotype A1 relative to the other HBV (sub)genotypes or vector control (Figure 7A,B), whereas GNB1 mRNA expression was not significantly upregulated relative to D3 and E (Figure 7C), which could be a result of the post-translation steps required for the maturation and expression of GNB1 [87,88].

Proteomic analyses in the cells transfected with subgenotype A1 compared to the different HBV (sub)genotypes or vector control showed enrichment in multiple pathways where RHO GTPases activity is upregulated (Figure 5C,D). Thus, RHOC expression was further analysed. The significant upregulation in RHOC in the subgenotype A1-transfected cells was confirmed using a RHOC-specific ELISA, as a secondary proteomic assay (Figure 7B).

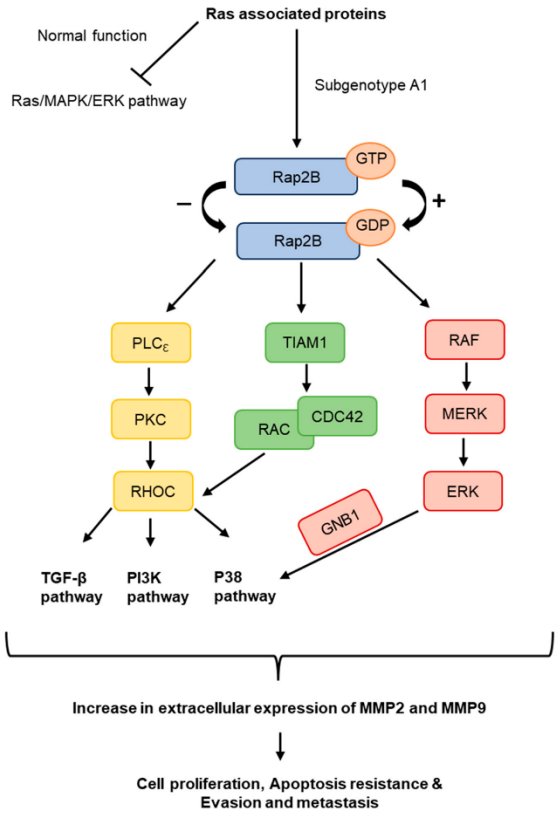


Figure 10. The role of potential Ras-associated proteins upregulated in A1. The predicted overexpressed or activated signalling Ras pathway that subgenotype A1 favours. Potential Ras-associated proteins, Rap2B, RhoC and GNB1, are seen throughout the pathway and can affect downstream signalling pathways, TGF-β, PI3K/Akt, and p38 signalling pathways. These downstream signalling pathways have been shown to increase cell proliferation, apoptosis resistance, evasion, and metastasis in HCCs by increasing extracellular MMP2 and MMP9 expression.

Next, because RHOC is an important regulator of cell migration [89] and involved in cancer progression, especially metastasis in various cancers including HCC [49,89], the cellular localisation of RHOC in the Huh7 cells transfected with subgenotype A1 was compared to the other (sub)genotypes or untransfected cells. Furthermore, overexpression of RHOC has been implicated in poor clinical prognosis because of its association with metastatic and aggressive features of HCC [49]. To avoid cross-reactivity between the primary antibodies used to detect the RHOC and the Dako (anti-HBc), we used a combination RHOA/RHOC antibody. RHOA and RHOC are both located in the cytoplasm; however, their functionality differs [90]. RHOA plays a key role in regulating actomyosin contractility and cell proliferation, whereas RHOC is more important in cell movement [91]. In cells transfected with subgenotype A1, increased co-localisation of RHOA/RHOC and HBc was observed at the periphery of a majority of the cells (Figure 9). This observation agrees with a study by Xie et al. which showed that the stable transfection of the RHOC expression vector into a normal hepatocyte cell line imparted tumour phenotypes, including proliferation, anchorage-independent growth, migration, and invasion [92]. The increased focal adhesion staining of RHO proteins in the subgenotype A1-transfected cells

compared to cells transfected with other (sub)genotypes may be related to higher hepatocarcinogenic potential of this subgenotype. More in-depth studies are necessary.

Downstream signalling products of RHOC, MMP2, and MMP9 have been shown to be increased [93,94]. Therefore, extracellular MMP2 and MMP9 levels were compared using ELISA. Although not statistically significant, the increased extracellular expression of MMP2 and MMP9 was detected 5 days post-transfection in the supernatant of the subgenotype A1-transfected cells compared to those transfected with the other (sub)genotypes (Figure 8B). RHOC has been shown to promote the malignant progression of cholangiocellular carcinoma cells via the regulation of MMP 2, 3, and 9 expression levels [95,96]. Moreover, Liao et al. reported that HCC cell invasion and migration are modulated by the genes RHOC, MMP2, and MMP9 [97].

5. Conclusions

There were a number of limitations present in this study, including the use of Huh7 cells derived from a hepatoma, the lack of a secondary proteomic assay for GNB1, and the use of RHOA/RHOC antibody instead of a RHOC-specific antibody. Despite these limitations, using proteomic analysis, subgenotype A1 was demonstrated to dysregulate four of the five HBV-associated hepatocarcinogenic pathways—p53, MAPK, Wnt/β-catenin, and PI3K/Akt/mTOR. The upstream regulator proteins (RHOC, Rap2B, and GNB1) of the MAPK and PI3K/Akt/mTOR pathways were also shown to be upregulated in the cells transfected with subgenotype A1. Furthermore, the increased expression and localisation of RHO proteins in the subgenotype A1-transfected cells compared to cells transfected with other (sub)genotypes may be related to the higher hepatocarcinogenic potential of this subgenotype. As an in vitro system, transfection of the Huh7 cells with HBV plasmid constructs provides us with a widely used model system that allows us to follow HBV infection and dissect complex biological processes [98]. However, to further elucidate the mechanisms of the pathogenesis of subgenotype A1-associated HCCs, further in vivo studies using patient tissues and/or sera will be required.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v16071052/s1, Figure S1: Internal validation of mass spectrometry data; Figure S2: Intracellular and extracellular viral load expression of the various HBV (sub)genotypes; Figure S3: Commonly expressed protein in HBV (sub)genotypes compared to the vector control.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors upon request.

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Conflicts of Interest: S.S. has a commercial interest in ReSyn Biosciences which supplied the magnetic microspheres used during mass spectrometry sample preparation. The authors have no additional conflicts of interest to declare.

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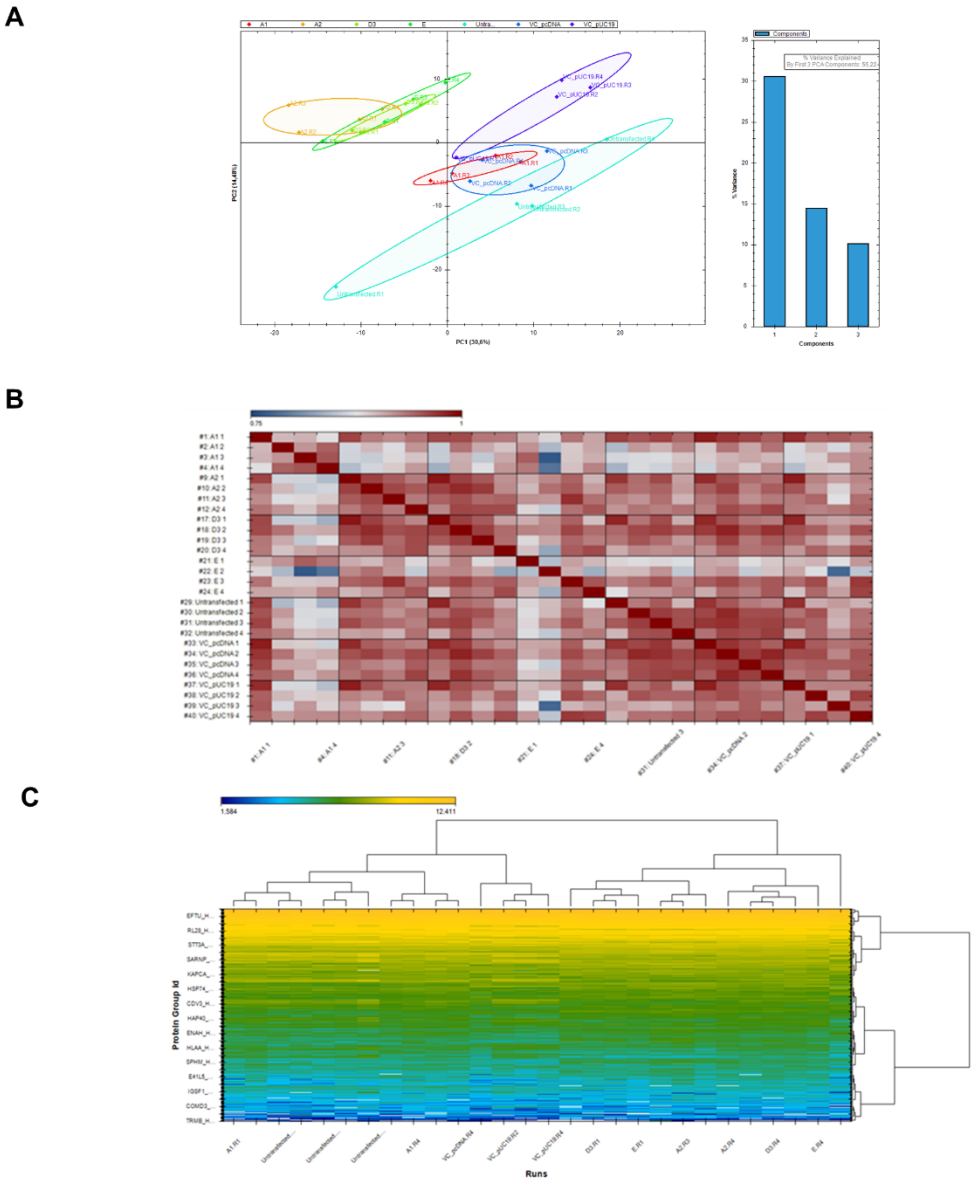
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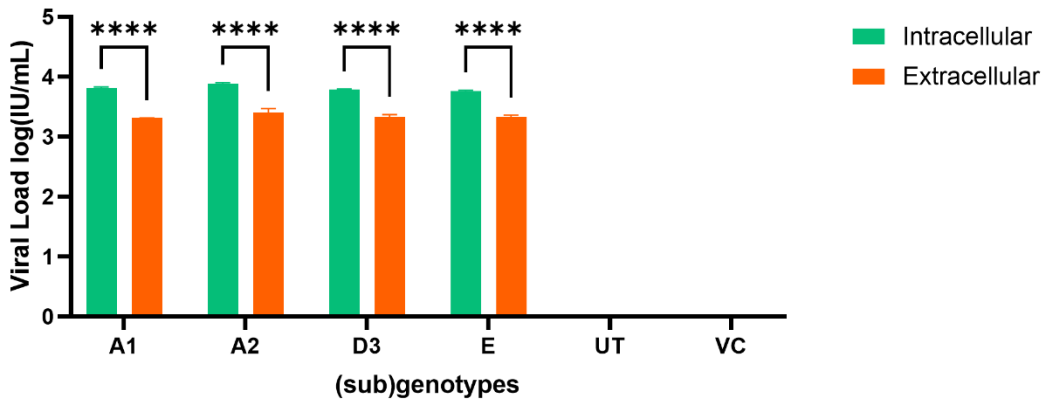
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Supplementary figure 1



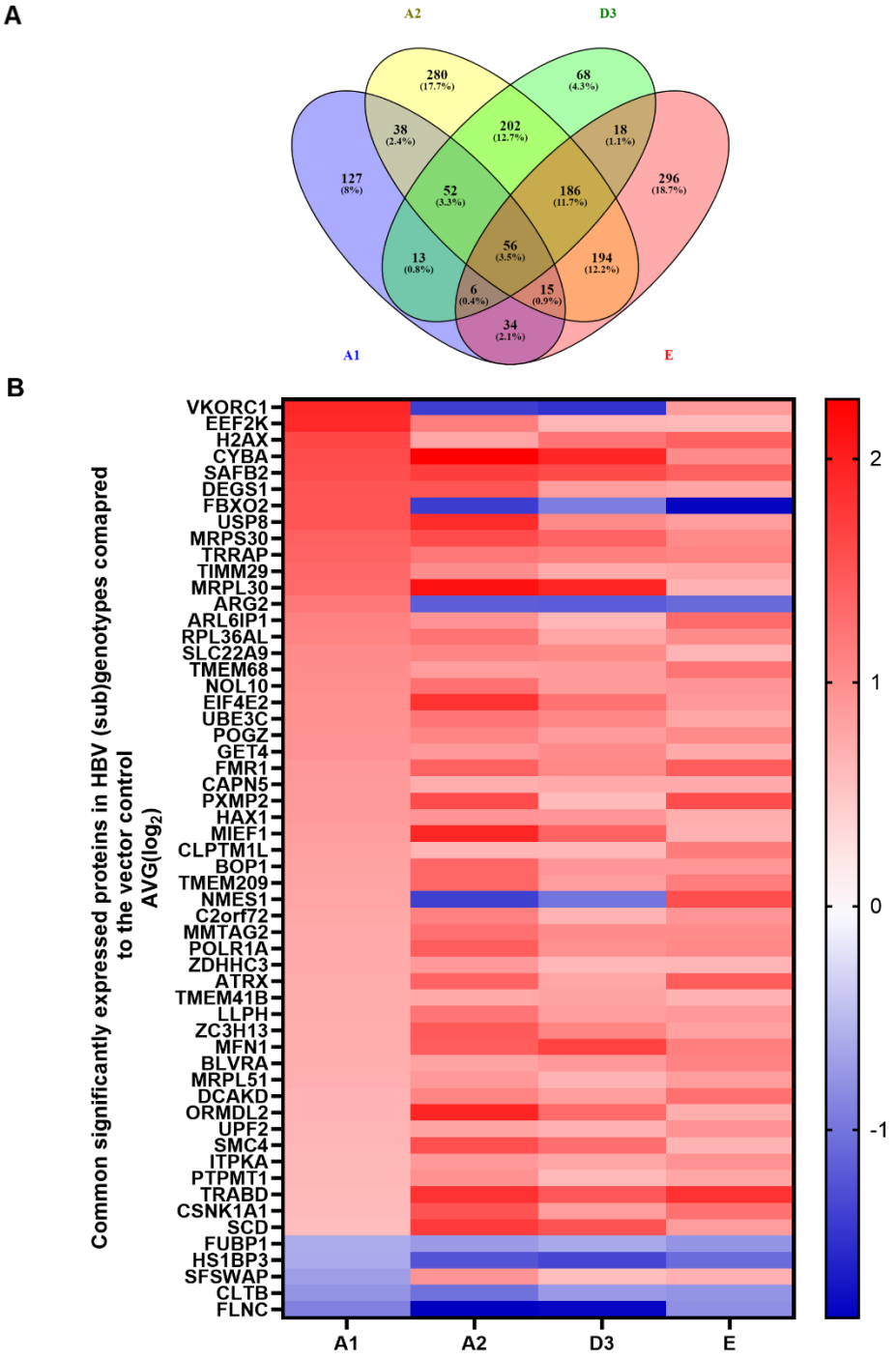
S1 Fig. Internal validation of mass spectrometry data
(A)Principal component analysis (PCA) showing the various HBV (sub)genotypes, untransfected control, and vector control (pcDNA and pUC19) as different circles. Subgentype A1 is represented in red , subgenotype A2 in yellow, subgenotype D3 in light green, genotype E in darker green, untransfected in light blue, vector control (pcDNA) in blue and vector control (pUC19) in dark blue. The PCA plot was generated using peptide abundance data of all peptides analysed per (sub)genotype with 4 replicates. (B) Sample correlation analysis between different HBV (sub)genotypes, untransfected control and vector control. (C) A comparative heatmap of all the replicates per (sub)genotype and the different protein groups identified

Supplementary figure 2



S2 Fig. Intracellular and extracellular viral load expression of the various HBV (sub)genotypes
The intracellular and extracellular HBV DNA level was determined after 5 days post –transfection from Huh7 cells that were transfected with the different HBV (sub)genotypes. There was a no significance between the intracellular and extracellular viral loads of the different (sub)genotypes, only significance difference ($p<0.05$) between the extracellular and intracellular viral loads per (sub)genotype, using a two-way ANOVA statistical test.

Supplementary figure 3



S3 Fig. Commonly expressed protein in HBV (sub)genotypes compared to the vector control
(A) Venn diagram illustrating the 56 significantly differentially expressed pressed proteins found in different HBV (sub)genotypes compared to the vector control. (B)-The heatmaps shows the upregulated(red) and downregulated(blue) average log2 expression of the 56 significantly expressed common proteins in the different HBV (sub)genotypes compared to the vector control.

Chapter 4

Paper 3- Article: Proteomic analyses of Huh7 cells transfected with replication-competent clones of subgenotype A1, with and without the G1862T mutation

Journal: Current Issues in Molecular Biology (CIMB) (MDPI), Impact Factor of 2.8

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The propensity to develop precore and core promoter mutations that affect HBeAg expression is influenced by (sub)genotype. Subgenotype A1 has unique BCP/PC mutations that affect the expression of HBeAg at a transcriptional, translational, and post-translational level (Kramvis, Kew et al. 2005, Kramvis 2018). In the previous chapter subgenotype A1 was shown to favour dysregulation of PI3K/Akt/mTOR pathways as a possible mechanism for its increased hepatocarcinogenic potential (Kramvis, Kew et al. 2005, Kramvis 2007). The G1862T mutation, found in subgenotype A1, causes ER stress and can increase the hepatocarcinogenic potential of this subgenotype (Chen, Crowther et al. 2008). ER stress can cause disturbances in proteostasis, affecting host signalling pathways involved in epigenetics, mitochondrial regulation, autophagy, and metabolism (Bhoola and Kramvis 2017, Chen, Shi et al. 2023) . Therefore, Chapter 4 of this thesis provides the first report of the molecular differences at the proteome level between cells transfected with wildtype subgenotype A1 and subgenotype A1 with G1862T mutation.

Author contribution: Ms Padarath performed all the investigations, methodology and analysis, validated the results, and mined the data using various bioinformatical software. She prepared the original draft including figures and tables, and rewrote 5 drafts after reading fellow authors' comments.

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Article

Comparison of the Proteome of Huh7 Cells Transfected with Hepatitis B Virus Subgenotype A1, with or without G1862T

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Abstract: HBeAg is a non-structural, secreted protein of hepatitis B virus (HBV). Its p25 precursor is post-translationally modified in the endoplasmic reticulum. The G1862T precore mutation leads to the accumulation of P25 in the endoplasmic reticulum and activation of unfolded protein response. Using mass spectrometry, comparative proteome profiling of Huh-7 cells transfected with wildtype (WT) or G1862T revealed significantly differentially expressed proteins resulting in 12 dysregulated pathways unique to WT-transfected cells and 7 shared between cells transfected with either WT or G1862T. Except for the p38 MAPK signalling pathway, WT showed a higher number of DEPs than G1862T-transfected cells in all remaining six shared pathways. Two signalling pathways: oxidative stress and cell cycle signalling were differentially expressed only in cells transfected with G1862T. Fifteen pathways were dysregulated in G1862T-transfected cells compared to WT. The 15 dysregulated pathways were involved in the following processes: MAPK signalling, DNA synthesis and methylation, and extracellular matrix organization. Moreover, proteins involved in DNA synthesis signalling (replication protein A (RPA) and DNA primase (PRIM2)) were significantly upregulated in G1862T compared to WT. This upregulation was confirmed by mRNA quantification of both genes and immunofluorescent confocal microscopy for RPA only. The dysregulation of the pathways involved in these processes may lead to immune evasion, persistence, and uncontrolled proliferation, which are hallmarks of cancer.

Keywords: G1862T; HBV; subgenotype A1; p38 MAPK; replication protein A (RPA); DNA primase (PRIM2)

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1. Introduction

In Africa, there are an estimated 64.7 million people chronically infected with hepatitis B virus (HBV). This is an underestimate due to inadequate screening and disease surveillance [1]. During chronic hepatitis B (CHB), hepatitis B e antigen (HBeAg) is used as a biomarker for viral infection and patient outcomes [2,3]. HBeAg is a non-structural protein, that has a dual role as a tolerogen and immunogen [4]. The loss of HBeAg with reduced viral replication is considered a favourable sign for patients with CHB [2,5]. HBeAg loss associated with persistently elevated HBV DNA levels can lead to cirrhosis and hepatocellular carcinoma (HCC) [6]. Therefore, HBeAg-negative CHB represents a clinical challenge [7,8].

Several basic core promoter (BCP) and precore (PC) gene mutations in HBV can affect HBeAg expression leading to HBeAg-negative CHB [9,10]. In southeastern sub-Saharan Africa (SSA), the predominant subgenotype is A1. Subgenotype A1 has a 4.5-fold higher risk of developing HCC at an earlier age than other HBV genotypes [11]. Subgenotype A1 has unique BCP/PC mutations that affect the expression of HBeAg at a transcriptional, translational, and post-translational level [12]. In particular, the G1862T mutation can be found in HBV isolated from both tumour and non-tumorous liver tissues but is more prevalent in tumorous tissue [13]. G1862T is a missense mutation resulting in a G to T transversion at amino acid 18 of precore precursor p25, which converts valine to phenylalanine and results in a bulky aromatic ring that interferes with the signal peptide cleavage of p25, between positions 18 and 19 [13]. Therefore, G1862T leads to the accumulation of the HBeAg precursor (p25) in the cytoplasm of the hepatocyte and decreased secretion of mature HBeAg [14–16].

Previous studies have shown that G1862T mutant precursor accumulates in the endoplasmic reticulum (ER), endoplasmic reticulum–Golgi intermediate compartment (ERGIC) and Golgi [16–18]. These aggregates of mutant protein attract ubiquitin, heat shock proteins and proteasomes and cause ER stress and the earlier activation of the unfolded protein response (UPR) cascade [17]. As the ER is a quality-control organelle for proteostasis, this accumulation of proteins in the ER, ER stress and the activation of UPR can disturb proteostasis, affecting host signalling pathways involved in epigenetics, mitochondrial regulation, autophagy, and metabolism [19]. Furthermore, an increase in ER stress can result in liver damage, which is a contributing factor to hepatocarcinogenesis and may explain why G1862T is frequently found in HCC patients compared to asymptomatic carriers of HBV [12].

Proteomic studies have been used to describe global changes in liver cells from patients with and without HBV-associated liver disease [20–22]. Lu et al. found dysregulated proteins in plasma from patients with fibrosis compared to healthy controls [23]. Dysregulation of the proteome was also found in tumorous compared to adjacent non-tumorous tissues when liver biopsies from HCC patients were compared [20]. When serum from CHB patients infected with genotype B or C and healthy controls were compared to each other, proteomic analysis revealed dysregulation of the expression of various proteins [22]. Proteome alterations involved in different biological processes included translation [20,23], oxidation reduction [20–23], intracellular transport [22,23], establishment of protein localization [22,23], protein transport [21–23], and translational elongation [20–22].

The objective of this study was to use mass spectrometry to determine the effect of G1862T on the proteome, by transfecting Huh7 cells with replication-competent clones of subgenotype A1, with or without G1862T. Having previously shown the accumulation of HBeAg in the ERGIC and the upregulation of the UPR [14], this in vitro system was used to determine the downstream effects of G1862T on host signalling pathways. Little is known about the difference in specific biological pathways induced by the accumulation of mutant HBeAg in the ER. Understanding the role of G1862T mutation may provide further insights on the pathogenesis of HCC in SSA patients infected with subgenotype A1.

2. Materials and Methods

1. *Plasmid Constructs*

1.28-mer replication-competent plasmids for wild-type subgenotype A1 (KM519453) and subgenotype A1 with the G1862T mutation (KM519452) were constructed and were able to replicate and express proteins in vitro [24,25]. Ethics approval to perform this study was obtained from the University of the Witwatersrand Research Ethics Committee (Medical), Johannesburg, South Africa (HREC: W-CBP-210416-01).

2. *Cell Line and Transfections*

Huh7 cells were transfected as described previously [24]. Twenty-four hours before transfection, 3 × 10^6 Huh7 cells were plated into each of the 10 cm culture dishes for mass spectrometry, whilst 2 × 10^5 Huh7 cells were plated in each of 12-well plates either with coverslips for indirect immunofluorescence or no coverslips for real-time polymerase chain reaction (RT-PCR) (viral load/RNA expression) or enzyme-linked immunosorbent assay ELISA and incubated overnight at 37 °C in a humidified incubator containing 5% (*w*/*v*) CO_2. For the transfection, 12,000 ng/10 cm-plate and 800 ng for each well of a 12-well plate were used. All experiments were carried out using 4 replicates per condition. To measure transfection efficiency 1-day post-transfection, an eGFP control was included in triplicate. This was measured using The FLoid™ Cell Imaging Station (Thermo Fisher, Waltham, MA, USA) by counting the number of cells successfully transfected.

3. *Mass Spectrometry Analysis*

Mass spectrometry was performed using the protocol described by Padarath et al. [26].

1. Cell Lysis and Protein Preparation

Five days post-transfection, cells were washed 3 times with PBS. Aliquots of 0.5 million cells were prepared and pelleted at 300× *g* for 10 min. Supernatants were removed and cell pellets were stored at −80 °C until further use. Cell pellets were thawed on ice and resuspended in 200 µL of lysis buffer (1% SDS, 50 mM Tris-HCl pH 8.0). Protein was extracted from cell pellets using a PIXUL multi-sample sonicator (Active Motif, Carlsbad, CA, USA). Sonication settings were: Pulse = 50; PRF = 1; Process Time = 30; Burst = 20. Thereafter, the lysates were incubated with 25 units of benzonase, and with $MgCl_2$ to a final concentration of 2 mM at 37 °C for 30 min. Next, the samples were centrifuged at 15,000× *g* to clear cell debris. Post BCA-based protein concentration determination, samples were incubated with 10 mM DTT (30 min at 37 °C) followed by addition of 40 mM IAA (30 min at RT).

2. Sample Clean-Up and Digestion

All experiments were performed with a KingFisher™ Flex (Thermo Fisher Scientific, Waltham, MA, USA) magnetic particle processing robot, as previously described [27]. The KingFisher™ Flex system was configured for automated HILIC-protein clean-up and on-bead trypsin digest. In brief, deep 96 well plates were loaded in each carousel position with each plate filled as follows: (1) 96 well tip heads (Thermo Fisher Scientific, Waltham, MA, USA); (2) 10 µL, 20 mg/mL hyper porous magnetic MagReSyn HILIC microspheres (ReSyn Biosciences, Pretoria, South Africa) in 20% EtOH and 180 µL Equilibration buffer (100 mM NH_4Ac, 15% MeCN pH 4.5); (3) Equilibration Buffer (500 µL); (4) Protein extract mixed 1:1 with Bind buffer (200 mM NH_4Ac, 30% MeCN pH 4.5), the final volume of 100 µL; (5) 500 µL 95% MeCN (wash 1); (6) 500 µL 95% MeCN (wash 2); (7) 200 µL 50 mM ammonium bicarbonate pH 8.0 and Promega sequencing grade Trypsin for an enzyme: protein ratio of 1:10; (8) Elution with 100 µl of 1% TFA. The Bindit programme was then run with the magnetic pins transferring the magnetic HILIC beads from position 2 to 8 and in the process binding proteins, washing off SDS and other contaminants and finally generating peptides ready for LC-MS analysis post the on-bead trypsin digest.

3. LC-MS Data Acquisition

Post-HILIC, peptide samples were vacuum dried and resuspended in 2% MeCN/0.2% FA. Analysis was performed on a Dionex Ultimate 3000 RSLC system coupled to an AB Sciex 5600 TripleTOF mass spectrometer (Sciex, Toronto, ON, Canada). Injected peptides were inline de-salted using an Acclaim PepMap C18 trap column (Thermo Fisher, Waltham, MA, USA) (75 µm × 2 cm; 2 min at 5 µL.min^{-1}using 2% ACN/0.2% FA).

Trapped peptides were gradient eluted and separated on a Waters Acquity CSH C18 NanoEase column (Waters™, Milford, MA, USA) (75 µm × 25 cm, 1.7 µm particle size) at a flow-rate of 0.3 µL.min ^{1} with a gradient of 6–40% B over 60 min (A: 0.1% FA; B: 80% ACN/0.1% FA). For Sequential window acquisition of all theoretical mass spectra (SWATH), precursor scans were acquired from 400 to 1100 *m*/*z* with 50 milliseconds accumulation time and fragment ions acquired from 200 to 1800 *m*/*z* for 48 variable-width precursor windows with 0.5 Da overlap between windows and 20 milliseconds accumulation time per window.

4. SWATH-MS Data Processing

SWATH data was processed using Spectronaut v17 software (Biognosys, Zurich, Switzerland). The default directDIA identification and quantification settings were used for data processing. Carbamidomethylation was added as a fixed modification, and N-terminal acetylation and methionine oxidation were added as variable modifications. Swiss-Prot Human sequences (www.uniprot.org, accessed on 12 December 2022) and common contaminating proteins were used as the search databases. A q-value ≤ 0.01 cut-off was applied at the precursor and protein levels. Quantification was performed at the MS1 and MS2 level. Label-free cross-run normalisation was employed using a global normalisation strategy. Candidate dysregulated proteins were filtered at a q-value ≤ 0.05, absolute fold change (FC) ≥ 1.5 and a minimum of two unique peptides identified.

5. Mass Spectrometry Biological Data Annotation

Pathway analysis, gene-set enrichment analysis, and STRING analysis were performed as described in Padarath et al. [26].

2.4. RNA Expression Levels

As described in Padarath et al. [26], total RNA was isolated from Huh7 cells-transfected with wildtype subgenotype A1, subgenotype A1 with G1862T mutation, and controls (untransfected and vector controls) using the RNAeasy mini extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. An aliquot of 500 ng RNA was reverse transcribed to generate cDNA using the SuperScript™ VI cDNA First-Strand Synthesis System synthesis kit (Invitrogen, Boston, MA, USA), as per the manufacturer's instructions. A total of 5 µL of cDNA (1:10 dilution) was amplified using SYBR®Green Realtime PCR Master Mix (Thermofisher, Waltham, MA, USA) and performed on a MyCycler Thermocycler (Bio-Rad Laboratories, Hercules, CA, USA). Thermal cycling conditions were as follows: initial denaturation at 95 °C for 2 min followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 1 min, and a final elongation at 72 °C for 10 min. Three relative expression levels of the target genes were calculated using the ΔCt method with GAPDH as the control housekeeping gene. Primers for the qPCR are listed in Table 1 below.

Table 1. The Primers qPCR for three genes.

Gene	Direction	Sequence
PRIM2	F	5′-CTTCAGCCTCTGCTCAATCACC-3′
	R	5′-GTAACTGACGCATGCAAGGTGG-3′
RPA	F	5′-GAGCACCTATCAGCAATCCAGG-3′
	R	5′-CCTTCAGGTCTTGGACAAGCCT-3′
GAPDH	F	5′-GAAGGTGAAGGTCGGAGT-3′
	R	5′-GAAGATGGTGATGGGATTTC-3′

5. Viral Loads

Viral loads were performed using the protocol described by Bhoola et al. [24]. On day 5 post-transfection, total DNA was extracted from transfected Huh7 cells harvested using the NucleoSpin® Tissue kit (Macherey-Nagel, Nordrhein-Westfalen, Düren, Germany). An aliquot of 5 mL of transfected medium collected on day 3 or 5 was centrifuged at 22,000× *g* for 5 min at 4 °C, after which a 100 µL aliquot was adjusted with 10 µL 10× Incubation Buffer (Roche Diagnostics, Basel, Switzerland) and treated with 1 µL 10 U/µL DNase I (Roche Diagnostics) and 10 µL 100 mg/mL RNase A (Fermentas Molecular Biology Tools, Waltham, MA, USA) and incubated at 37 °C for 20 min. Subsequently, the reaction was terminated by adding 5 µL 0.2 M EDTA, pH 8.0 and incubation at 75 °C for 10 min. Thereafter, extracellular total DNA and intracellular total DNA were extracted using the NucleoSpin® Tissue. PCR primers, HBV DNA F (5′-CGTGTGTCTTGGCCAAAATTCG-3′) and HBV DNA R (5′-CATCCAGCGATAACCAGGACAA-3′), with a FAM/NFQ, labelled TaqMan® MGB probe (5′-FAM-TCACTCACCAACCTCC-NFQ-3′) were used to quantify HBV DNA in the ABI 7500 Real-Time PCR system (Applied Biosystems by Life Technologies, Waltham, MA, USA) using the PCR thermal cycling conditions described previously [28]. A serial dilution of plasmid DNA containing a single genome of subgenotype A1, ranging from 1×10^4 to 1×10^8 copies/mL, was used as a template to generate the standard curve. The 2nd WHO International Standard, Hepatitis B Virus DNA (National Institute for Biological Standards and Controls (NIBSC), Hertfordshire, UK), which has a final concentration of 1×10^6 IU/mL was used as the positive control and to calibrate the standard curve.

6. HBeAg Expression

Expression of HBeAg in the culture media (supernatants) was analysed with an ELISA kit (ELISA kit: ETI-EBK PLUS (HBeAg), (DiaSorin, Saluggia, Italy) according to the manufacturer's protocol. The concentrations were determined in OD/µL for the transfection of each construct and compared to the control provided in the kit.

7. Immunofluorescence

Protocols were adapted from Deroubaix et al. [18]. Cells cultured in 12-well plates onto coverslips were washed 3 times with PBS and fixed with 3.7% formaldehyde in 1× PBS for 10 min at room temperature. The fixed cells were washed 3 times again with 1× PBS and permeabilized with 0.1% Triton-PBS for 7 min and washed 3 times with PBS. Cells were incubated for 1 h at room temperature with 1% bovine serum albumin (BSA, Fraction V, Roche Diagnostics GmbH by Roche Applied Science, Basel, Switzerland) (diluted in PBS 1×). Next, the cells were incubated with the primary antibodies (transfected cells: rabbit anti-HBV core protein DAKO [29], Agilent Technologies (Santa Clara, CA, USA), 1/1000, mouse anti-RPA, Invitrogen, 1/200) for 1 h at 37 °C. Then, the cells were washed 8 times with PBS 1× and incubated with the secondary antibody (Alexa Fluor 488/labelled anti-mouse (1:1000, Invitrogen) and Alexa Fluor 546 labelled anti-rabbit (1:1000, Invitrogen) for 1 h at 37 °C. Cells were washed 8 times with PBS 1×. DNA was stained with 4′,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, 1 mg/mL, 1/2000, Sigma-Aldrich, Saint Louis, MO, USA) for 10 min at room temperature in the dark. Coverslips were mounted using Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich), sealed using clear nail polish after 3 h and analysed by fluorescent microscopy the following day.

8. Microscopy and Image Analysis

Microscopy was performed using a Zeiss Laser Scanning Confocal Microscope 780 (Carl Zeiss AG, Oberkochen, Germany), equipped with a 63× objective (oil immersion alpha Plan-Apochromat 63×/1.40 Oil CorrM27 (Zeiss)) and Zen Blue software 2.1. Quantification of fluorescence was performed using ImageJ software (Version 1.53j,

Fiji/Image J, https://imagej.net/Fiji/Downloads, accessed on 29 June 2023), as described by Deroubaix et al. [18]. Briefly, the mean of replication protein A (RPA) fluorescence in the nucleus of 20 transfected cells was determined per condition and compared to the untransfected control. Laser intensity (488 nm laser for Alexa Fluor 488, 546 nm for Alexa 546 and 405 nm laser for DAPI), was kept constant to obtain comparable results between different slides of an experiment. Using immunofluorescence, a range of 58–68%/field of cells transfected with either WT or G1862T were stained positively for viral antigens (HBcAg and HBeAg) using anti-HBc (Dako, Santa Barbara, CA, USA).

2.9. Data Statistical Analysis

Statistical tests, i.e., *t*-test, one-way ANOVA and two-way ANOVA, were calculated using GraphPad Prism version 10.2.2.

3. Results

1. Comparison of Viral Load and HBeAg Expression

Cells transfected with G1862T mutant, showed a significant decrease in extracellular levels of HBeAg when compared to cells transfected with wildtype A1 (Figure S1A). Five days post-transfection, there was no significant difference between the intracellular and extracellular viral loads of cells transfected with either of the two constructs (Figure S1B).

2. Comparison of the Proteome of Huh7 Cells Transfected with Wildtype and Mutant Subgenotype A1 Compared to the Vector Control

The proteome of cells transfected with one of the following: wildtype, G1862T mutant or vector control, was analysed. At 1% false discovery rate (FDR), 3906 proteins and 36,107 peptides were identified, five days post-transfection. As an internal validation, principal component analysis (PCA) was used to differentiate the proteome of untransfected cells relative to cells transfected with one of the following: vector control, wildtype (WT) and G1862T mutant (Figure S2A). Principal component analysis (PCA) differentiated the untransfected control, vector control, wildtype and G1862T mutant sample groups.

Volcano plots were generated to visualize differentially expressed proteins (DEPs) in transfected and untransfected cells (Figure 1A). A minimum fold change ≥1.5 and maximum FDR adjusted *p*-value (q-value) ≤0.05 was used to filter proteins that were significantly different between the cells transfected with either WT or G1862T against the vector control. Relative to the untransfected cells, the vector control showed 90 DEPs, 16 downregulated and 74 upregulated. To compensate for the effect of the vector on the proteome of Huh7 cells and of transfection, all comparisons of the proteome of transfection with HBV plasmid constructs were carried out against cells transfected with vector alone (vector control). Thus, relative to the vector control, cells transfected with WT had 247 DEPs (196 upregulated and 78 downregulated), whereas cells transfected with G1862T had 123 DEPs (74 upregulated and 49 downregulated) (Figure 1B).

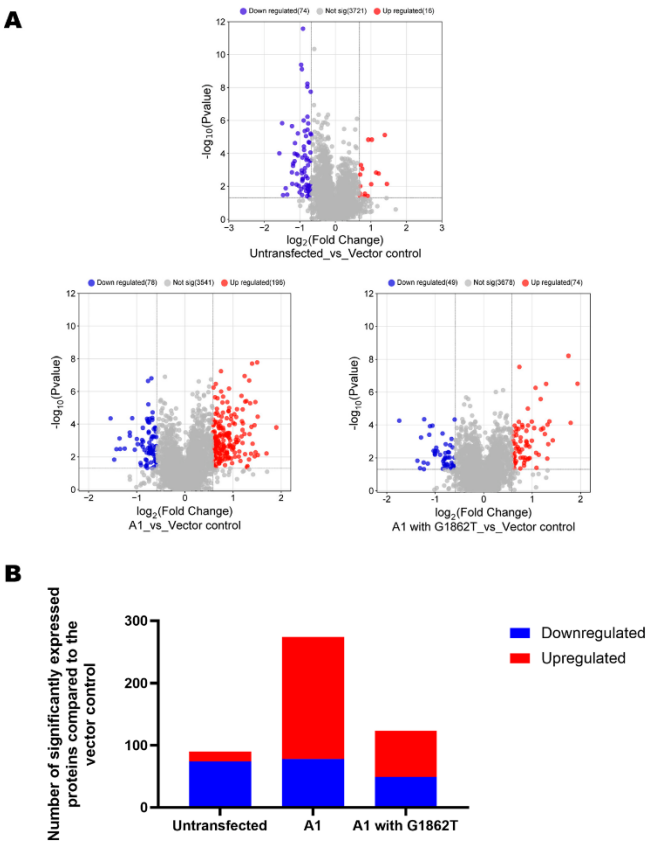
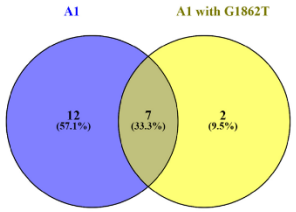


Figure 1. DEPs detection. (**A**) Volcano plots of the differentially expressed proteins, in Huh7 cells, transfected with replication-competent A1 with or without G1862T mutation and untransfected control against the vector-only control. Downregulated versus upregulated proteins are highlighted in blue and red, respectively. (**B**) A bar graph showing the number of significantly upregulated (red) and downregulated (blue) proteins in subgenotype A1, with and without the G1862T mutation and the untransfected compared to the vector control.

For clarification, DEPs were classified according to their corresponding pathways using the Panther bioinformatics tool, accessed 23 March 2023. Pathways were ranked according to the number of DEPs, and pathways with fewer than two DEPs were excluded from the analysis (Figure 2). In comparison to the vector control, 12 pathways were unique to WT-transfected cells, 2 were unique to G1862T-transfected cells and 7 were shared between cells transfected with either WT or G1862T (Figure 2A). In cells transfected with WT, 12 pathways were differentially expressed. The expression of the proteins in the 10 pathways were either up or downregulated, whereas in the coenzyme A biosynthesis and PI3 kinase pathways the proteins were upregulated (Figure 2B). The seven shared pathways were integrin signalling, inflammation-mediated signalling, cytoskeletal regulation by Rho GTPases, rat sarcoma (Ras) signalling, fibroblast growth factor (FGF) signalling, epidermal growth factor (EGF) signalling and the p38 MAPK signalling pathways (Figure 2B). The dysregulation of five of these pathways was the same for WT and G1862T. On the other hand, cytoskeletal regulation by Rho GTPases and the Ras

pathway were upregulated in cells transfected with WT and dysregulated in G1862T-transfected cells. Two signalling pathways, oxidative stress and cell cycle signalling, were differentially expressed only in cells transfected with G1862T, with the former and latter pathways, dysregulated and upregulated, respectively (Figure 2B). Except for the p38 MAPK signalling pathway, WT-transfected cells showed higher DEPs than G1862T-transfected cells in all remaining six shared pathways (Figure 2C).

A



B

Pathways differentially expressed in A1	Pathways shared between A1 and A1 with G1862T	Pathways differentially expressed in A1 with G1862T
1. VEGF signalling pathway [↓↑]	1. Integrin signalling pathway [↓↑]	1. Oxidative stress response [↓↑]
2. DNA replication [↓↑]	2. Inflammation mediated signalling pathway [↓↑]	2. Cell cycle [↑]
3. CCKR signalling [↓↑]	3. Cytoskeletal regulation by Rho GTPase [↑ in WT and ↓↑ in G1862T]	
4. Apoptosis signalling pathway [↓↑]	4. Ras Pathway [↑ in WT and ↓↑ in G1862T]	
5. Angiogenesis [↓↑]	5. FGF signalling pathway [↓↑]	
6. Coenzyme A biosynthesis [↑]	6. EGF receptor signalling pathway [↓↑]	
7. Ubiquitin proteasome pathway [↓↑]	7. p38 MAPK pathway[↓↑]	
8. Heterotrimeric G-protein signalling pathway[↓↑]		
9. T cell activation [↓↑]		
10. PI3 kinase pathway [↑]		
11. PDGF signalling pathway [↓↑]		
12. B cell activation [↓↑]		

Legend	
[↓↑]	Proteins in this pathway are both up and downregulated
[↑]	Proteins in this pathway are upregulated

C

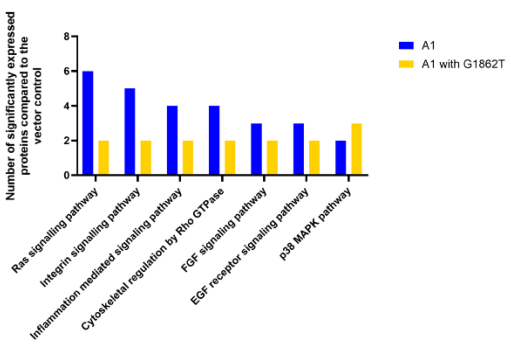


Figure 2. Proteomic analysis. (**A**) Venn diagram indicating the pathways associated with differentially expressed pathways between A1 with (yellow) and without (blue) the G1862T. Proteins were normalized against both the untransfected and vector control. (**B**) Proteomic analysis revealed significantly differently expressed proteins ($p < 0.05$) between the A1 with and without the G1862T. These differentially expressed proteins were further classified into pathways displayed in the table. Blue pathways are exclusively found in A1, grey pathways are shared between A1 with and without G1862T, and yellow pathways are exclusively found in A1 with G1862T. (**C**) Comparison table of the number of significantly differentially expressed proteins, amongst the common pathways between A1 with and without G1862T.

3.3. Proteomic Analysis of Huh7 Cells Transfected with Wildtype Compared to the Mutant

Next, the proteome of Huh7 cells transfected with G1862T was compared to WT-transfected cells. Compared to the WT, cells transfected with G1862T had 92 DEPs (24 upregulated and 68 downregulated) (Figure 3A,B). ShinyGO Gene Ontology enrichment analysis [30] was performed on the DEPs. In total, 15 pathways were dysregulated in G1862T-transfected cells compared to those transfected with WT. The 15 dysregulated pathways were involved in the following processes: MAPK signalling, DNA synthesis and methylation, and extracellular matrix organisation (Figure 3C).

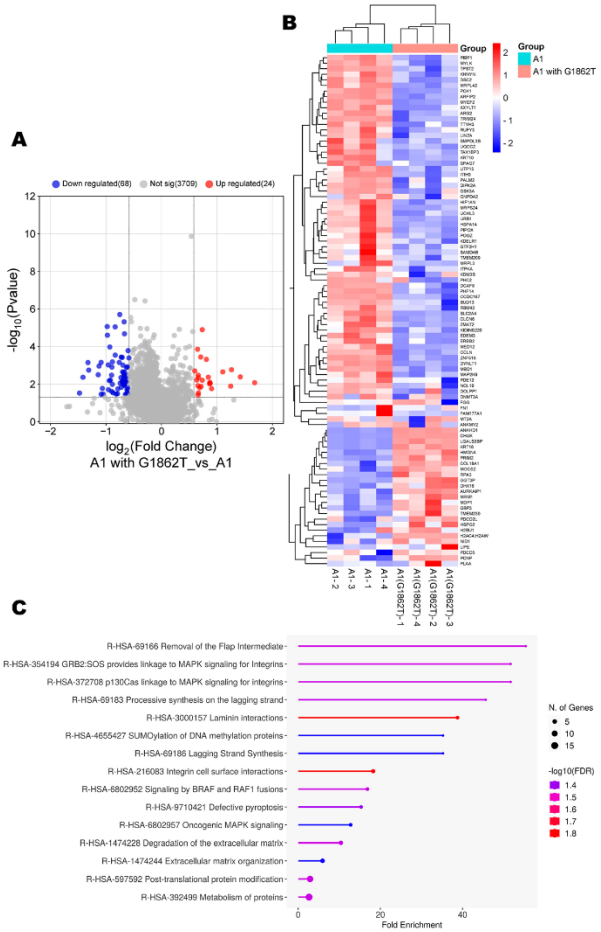


Figure 3. A1 with G1862T vs. A1 analysis. (**A**) Volcano plots of the differentially expressed proteins in Huh7 cells, transfected with replication-competent A1 with or without G1862T mutation. The negative x-axis represents downregulated (blue) in the A1 mutant, and the positive axis represents upregulated (red) proteins in the A1 mutant. (**B**) The heatmap showing the average log2 expression of the 92 significantly expressed proteins in G1862T compared to the WT. (**C**) A dot plot generated using the DEPs from image B in ShinyGO analysis, with Reactome pathway enrichment and fold enrichment, based on the number of genes present in each pathway. The FDR cut-off was set at 0.05, and the number of pathways was set to 10.

The proteome of G1862T-transfected cells was analysed by using Gene Set Enrichment Analysis (GSEA) [31]. The significantly enriched (upregulated) or depleted (downregulated) genes were classified according to their Reactome pathway. The top five GSEA-upregulated pathways were: RNA polymerase II transcription pre-initiation and promoter opening, transcriptional regulation by RUX1, base excision repair and apoptotic cleavage of cellular proteins pathways, and detoxification of reactive oxygen species (Figure S3A). The top five GSEA-downregulated pathways were: rRNA modification in the nucleus and cytosol, mitochondrial tRNA aminoacylation, translocation of SLC2A4-GLUT4 to the plasma membrane, RAF activation and phosphatidylinositol (PI) metabolism (Figure S4A). STRING analysis showed some interconnection between several upregulated and downregulated proteins (Figures S3B and S4B).

3.4. Increased Transcription of DNA Primase Subunit 2 (PRIM2) and RPA in G1862T-Transfected Cells Compared to WT-Transfected Cells

Using several proteomic bioinformatic tools, enrichment was observed in proteins relating to DNA synthesis. Amongst the 28 upregulated proteins, 2 proteins of interest were identified: DNA primase subunit 2 (PRIM2) and RPA for their involvement in DNA synthesis and their oncogenic association [32]. Both proteins were significantly upregulated (>2.0-fold change increase) in cells transfected with G1862T compared to WT (Figure 3B).

Increased transcription of both PRIM2 and RPA genes was shown using RT-qPCR. PRIM2 and RPA mRNA levels were significantly higher in G1862T-transfected cells compared to WT-transfected cells (Figure 4).

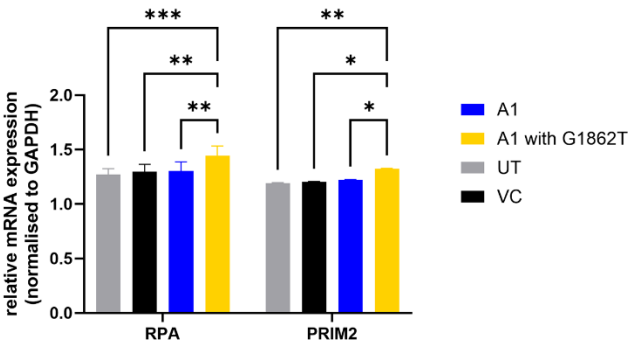


Figure 4. mRNA expression levels of DNA primase subunit 2 (PRIM2) and Replication Protein A (RPA) genes five days post-transfection. Results are expressed as a ratio between the mRNA expression of the DNA synthesis-associated proteins and GAPDH. Statistical analysis was performed using a one-way ANOVA statistical test (* = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001).

3.5. Immunofluorescence Reveals Increased Expression of RPA in G1862T-Transfected Cells Compared to WT-Transfected Cells

For further confirmation, immunofluorescence using the RPA-specific antibody was performed. Confocal microscopy showed that the mean of fluorescent intensity was significantly higher in the nuclei of G1862T-transfected cells compared to the WT-transfected cells and the untransfected control (Figure 5A,B). It should be noted the anti-HBc (Dako) was used to detect HBV proteins (HBcAg and HBeAg), and therefore, this antibody does not differentiate between core and precore proteins.

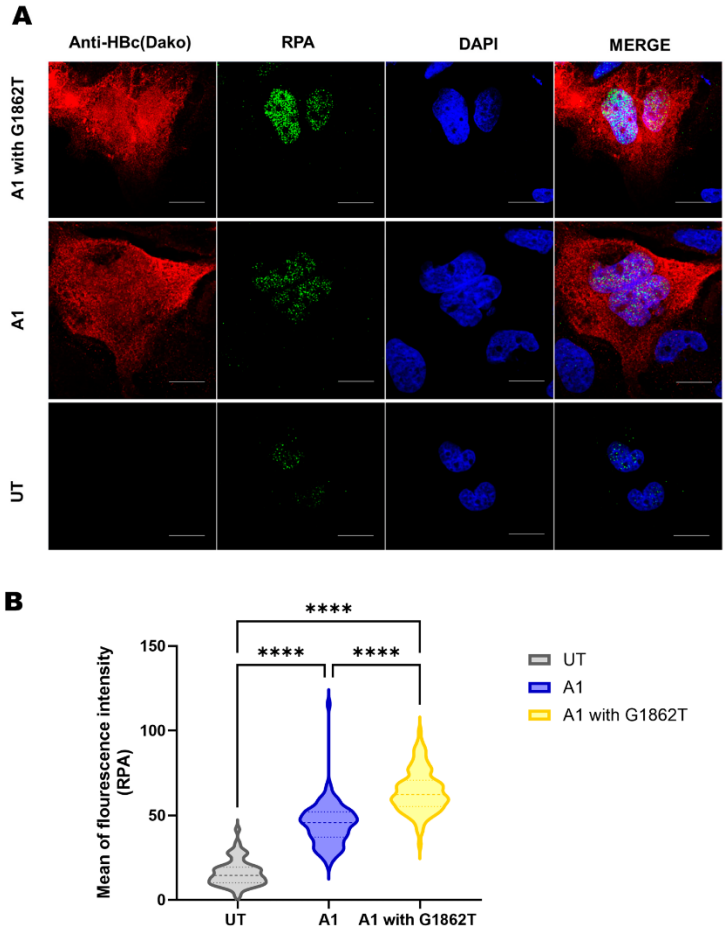


Figure 5. RPA expression using confocal microscopy. (**A**) Microscopy of transfected Huh7 cells with or without G1862T and untransfected cells after five days, viewed with a confocal microscope. Cells were immunostained with a polyclonal rabbit anti-HBc antibody (DAKO) and monoclonal mouse anti-RPA antibody. The nuclei were visualised by DAPI staining. Scale bars = 15 µm. (**B**) A volcano plot indicating the mean of fluorescence intensity for the RPA expression in cells transfected with and without G1862T compared to the untransfected (UT) control. Significant differences were analysed using a one-way ANOVA statistical test and compared to the expression in subgenotype A1 (**** = p-value < 0.0001).

4. Discussion

In agreement with previous studies performed by our team and others [16–18,33], HBeAg expression was reduced in cells transfected with G1862T precore mutation (Figure S1A). Viral loads did not differ between cells transfected with WT compared to those transfected with G1862T mutant (Figure S1B). Thus, any difference in protein expression cannot be attributed to differences in viral load.

Using a DIA SWATH-MS-based quantitative proteomic approach, a comparative analysis of the whole proteome of Huh7 cells transfected with either WT or G1862T was performed to determine the effect of G1862T on host signalling pathways. Compared to the vector control, G1862T-transfected cells showed a decrease in the number of significantly expressed proteins compared to WT-transfected cells (Figure 1B). The introduction of G1862T leads to the accumulation of the precursor of HBeAg p25 in the ERGIC [16–18,33]. This accumulation causes ER stress and the dysregulation of the UPR cascade [17] and can interfere with proteostasis, which regulates a balanced, functional proteome [19]. In fact, the accumulation of HBsAg in the ER has been shown to affect the expression of host proteins [34]. Moreover, the cell's transcriptional and translational programmes are also altered in order to overcome the ER stress and resolve protein-folding defects [35–38]. These consequences may account for the reduced number of significantly expressed proteins observed in cells transfected with the G1862T.

Proteomic analysis revealed that there were several common pathways shared between G1862T- and WT-transfected cells (Figure 2B). The p38 MAPK pathway was the only shared pathway where G1862T-transfected cells had more differentially expressed proteins than WT-transfected cells (Figure 2C). ShinyGo analysis further supported this finding with the enrichment in oncogenic MAPK signalling when G1862T was compared to WT (Figure 3C). p38 MAPK signalling has been shown to play an important role in immune escape as well as HBV replication, the promotion of tumour cell survival and drug resistance [39–41]. Previous studies have shown that HBeAg suppresses p38 MAPK phosphorylation [42]. This downregulation of MAPK would allow HBV to evade immune surveillance and thus persist. Therefore, viral mutations such as G1862T, which reduce HBeAg expression, may lead to the removal of the tolerogenic effect of HBeAg and the activation of the immune response [4,6,12].

Furthermore, proteomic analysis revealed that proteins involved in the cell cycle and oxidative stress pathways were exclusively found in cells transfected with the G1862T compared to WT (Figure 2B). This result was further supported by the GSEA analysis, which showed the base excision repair and detoxification of reactive oxygen species pathways were part of the top five enriched pathways when G1862T was compared to WT (Figure S3A). Oxidative stress is increased in HBV infections [43] and can be further exacerbated by the ER stress caused by G1862T [17] through the production of reactive oxygen species from the UPR [44]. Furthermore, increased oxidative stress in HBV infection was also demonstrated to increase DNA damage [45,46]. Therefore, there seems to be an interplay between these two exclusive pathways, which could contribute to the hepatocarcinogenic potential of HBV with G1862T. In addition, it has been shown that p22 and p25, which accumulate in the cytoplasm of G1862T-transfected cells, can translocate to the nucleus [47]. In the nucleus, these HBeAg precursors may interact with the host genome leading to genomic instability and mutations, a known hallmark of cancer [48].

Defective and inappropriate repair of DNA damage can result in genomic instability and promote tumorigenesis [49]. Therefore, the DNA synthesis and repair pathways were further explored. DNA primase subunit 2 (PRIM2) is a subunit of the DNA primase complex and a component of the DNA polymerase alpha complex, which plays an essential role in the initiation of DNA synthesis during the S-phase of the cell cycle [50–52]. On the other hand, RPA binds and stabilizes single-stranded DNA intermediates that form during DNA replication or upon DNA stress. Therefore, both proteins have an essential role in DNA replication and the cellular response to DNA damage [53]. Both these proteins have displayed oncogenic potential in various cancers [54,55] and were thus chosen as the proteins of interest. PRIM2 and RPA were upregulated in G1862T- compared to WT-transfected cells. DIA SWATH-based proteomics has become a well-established and unbiased technique for global proteome analysis [56]. For proteomics analysis in the current study, candidate dysregulated proteins were filtered at a q-value ≤ 0.05, absolute fold change (FC) ≥ 1.5 and a minimum of two identified unique peptides, which are acceptable metrics to limit the number of false observations. In addition, the interpretation

of the proteomics data was mainly carried out in the context of functional and pathway enrichment, limiting the impact of any potential false observations. The increased expression of the two proteins of interest, PRIM2 and RPA, were validated by RT-PCR by measuring the respective RNA expression levels for both and confocal microscopy for RPA.

In this study, transfection of Huh7 cells with HBV subgenotype A1 plasmid constructs, with or without G1862T, was used to determine the effect of this mutation on the cellular proteome. Huh7 cells are a well-differentiated, immortalized cell line derived from an HCC patient. The proteome of Huh7 cells differs from primary human hepatocytes (PHHs) or liver, and it has been suggested that caution must be exercised especially when performing drug metabolism and toxicity experiments [57]. Such experiments were not performed in the present study. Although, PHHs may be considered an ideal system, they also have several disadvantages including limited availability and lifespan, loss of hepatocyte function and susceptibility to HBV infection within days of isolation and unpredictable variability between donors [58]. Despite its limitation as an in vitro system, the Huh7 cell line provides a widely-used model that allows us to follow HBV infection and dissect complex biological processes [58–60] in a reproducible manner, which has been demonstrated to match findings in liver tissue and PHHs [58]. Therefore, the findings of the present study should be interpreted taking these caveats into account. Dysregulation of p38 MAPK, oxidative stress, and DNA synthesis and repair were shown in Huh7 cells transfected with G1862T compared to the wildtype. Further studies will be required using tissue and/or sera of patients infected with G1862T HBV to confirm these observations in vivo and relate them to the high hepatocarcinogenic potential of subgenotype A1 at an earlier age in SSA.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cimb46070419/s1, Figure S1: HBeAg expression and viral load comparison, Figure S2: Internal validation of mass spectrometry data, Figure S3: Upregulated proteins in G1862T-transfected cells compared to WT-transfected cells, and Figure S4: Downregulated proteins in G1862T-transfected cells compared to WT-transfected cells.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors upon request.

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Conflicts of Interest: S.S. has a commercial interest in ReSyn Biosciences which supplied the magnetic microspheres used during mass spectrometry sample preparation. S.S. is also employed by Evosep Biosystems. The remaining authors declare that the research was conducted in the

absence of any commercial or financial relationships that could be construed as a potential conflict of interest

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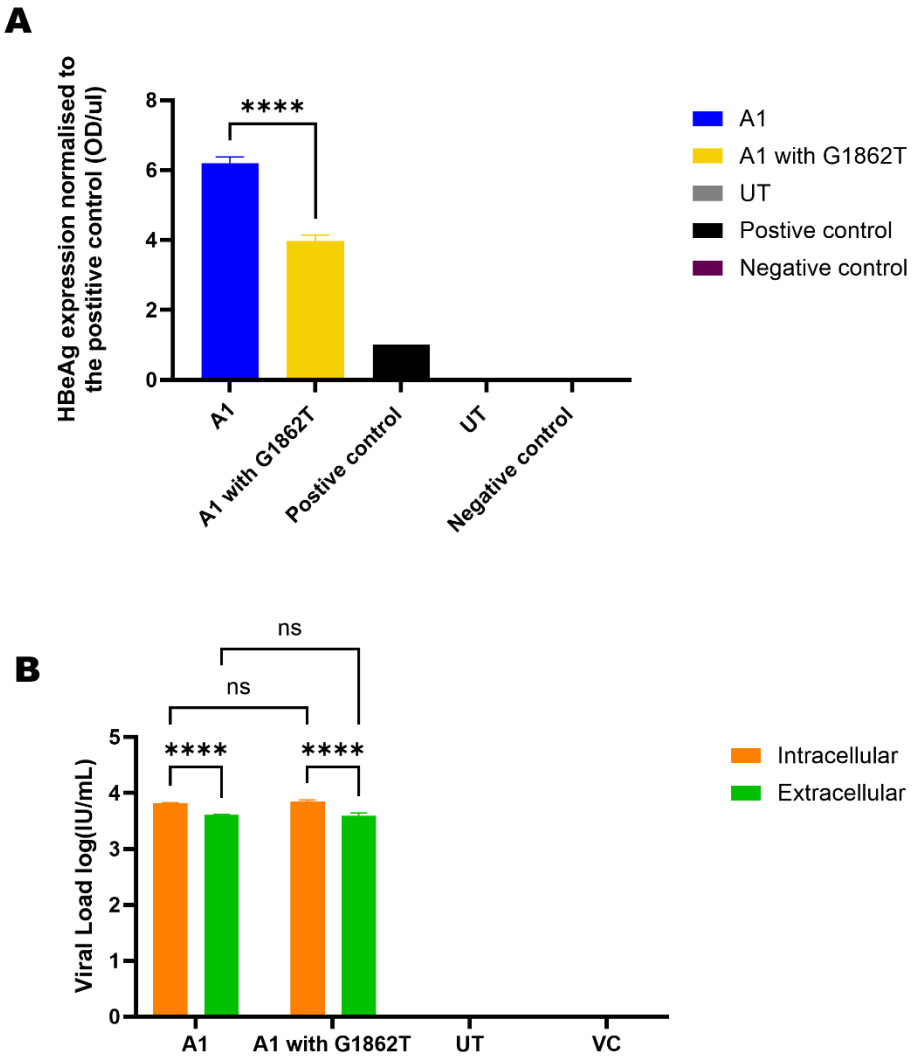
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Supplementary figure 1



S1 Figure. HBeAg expression and viral load comparison. (A) Extracellular expression of HBeAg in Huh7 cells transfected with the wildtype (blue) and mutant (Yellow) subgenotypes A1 constructs after 5 days. Data was normalized to the positive control provided in the ETI-EBK PLUS kit Sig-nificant differences were analysed using a one-way ANOVA statistical test and compared to the expression in A1. (B) The intracellular (orange) and extracellular (green)HBV DNA level was determined after 5 days post-transfection from Huh7 cells that were transfected with the wildtype and mutant A1 constructs. There was no significance between the intracellular and extracellular viral loads of the different (sub)genotypes, only significance difference ($p<0.05$) between the extracellular and intracellular viral loads per (sub)genotype, using a two-way ANOVA statistical test

Supplementary figure 2

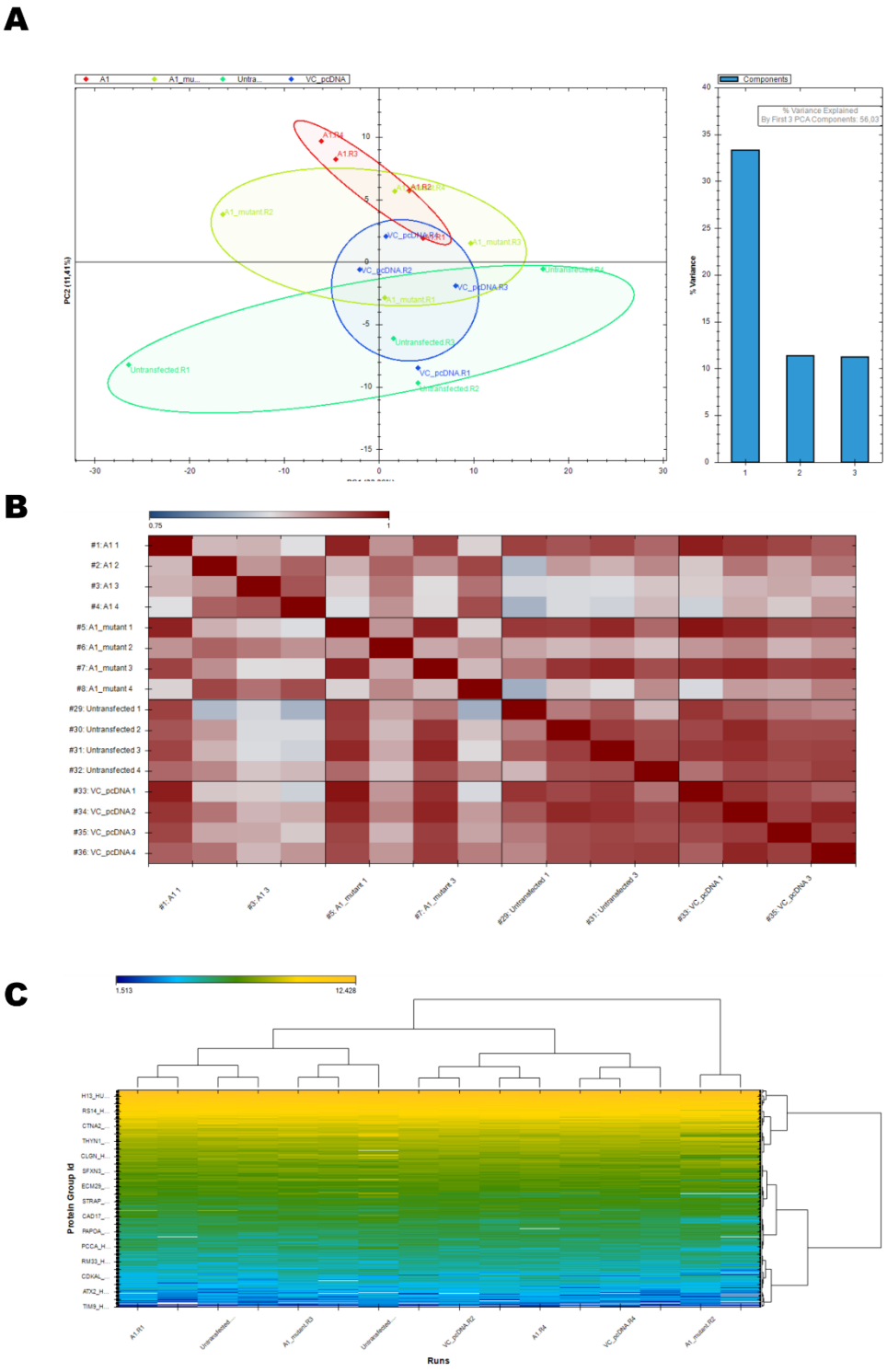


Figure S2. Internal validation of mass spectrometry data
(A)-Principal component analysis (PCA) showing subgenotype A1 with and without G1862T mutation, untransfected control, and vector control (pcDNA and pUC19) as different circles. Subgentype A1 is represented in red, subgenotype A1 with G1862T in light green, untransfected in light blue, and vector control (pcDNA) in dark blue. The PCA plot was generated using peptide abundance data of all peptides analysed per (sub)genotype with 4 replicates. (B)-Sample correlation analysis between subgenotypes A1 with and without G1862T, untransfected control and vector control. (C)-A comparative heatmap of all the replicates per sample and the different protein groups identified

Supplementary figure 3

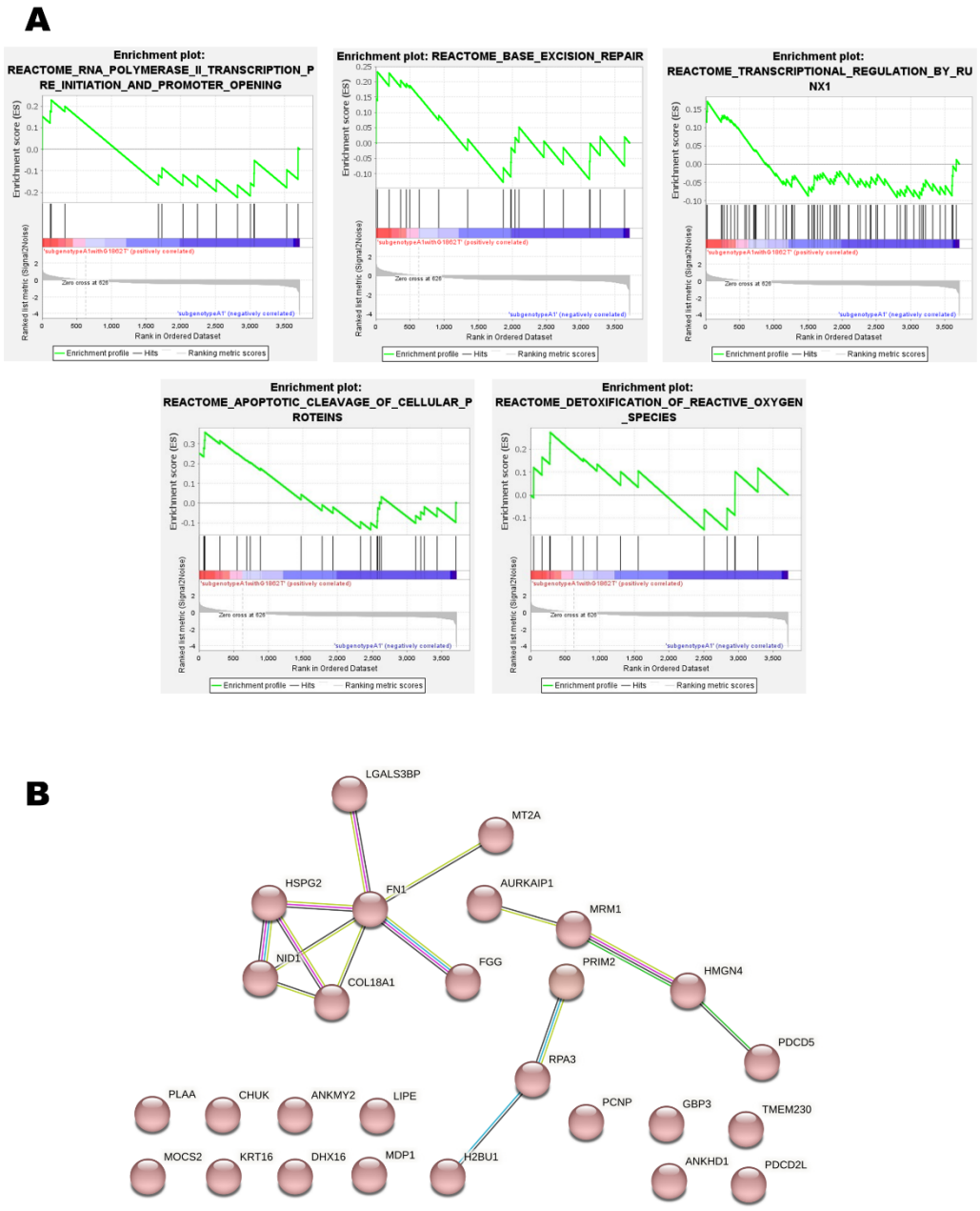


Figure S3 . Upregulated proteins in G1862T-transfected cells compared to WT-transfected cells.
(A)- GSEA enrichment plot between subgenotype A1 with G1862T and subgenotype A1, showing the top 5 upregulated Reactome pathways (B) Network analysis of upregulated proteins using string network (Fold cut-off set to 0.4).

Supplementary figure 4

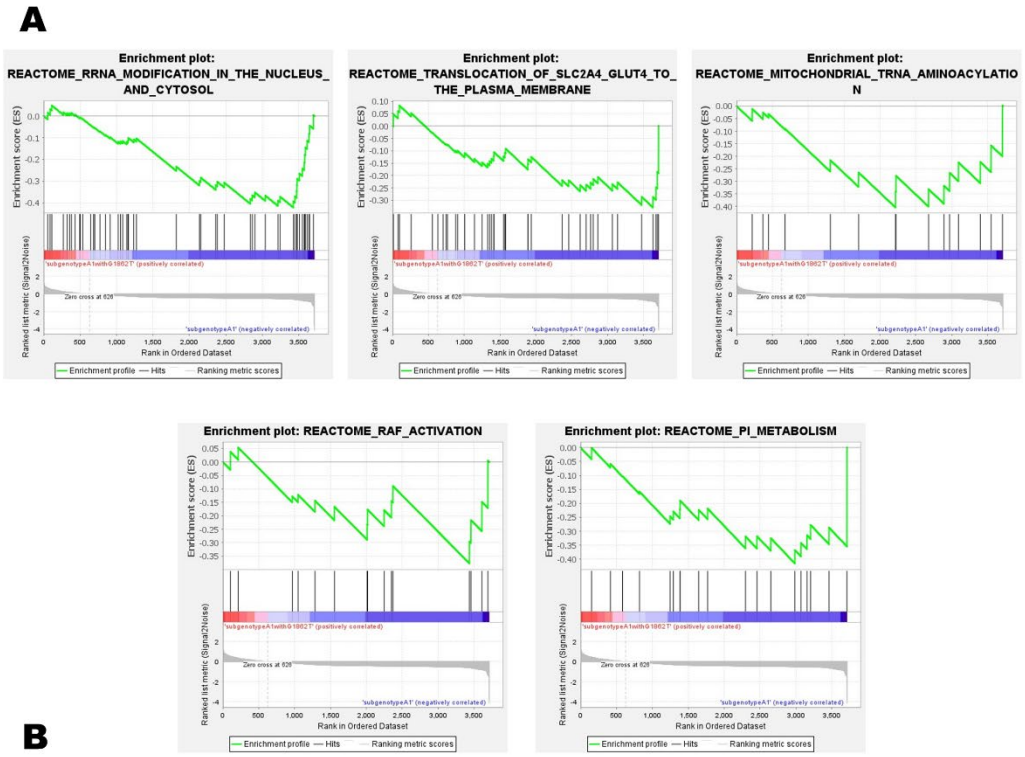


Figure S4. Downregulated proteins in G1862T-transfected cells compared to WT-transfected cells. (A) GSEA enrichment plot between subgenotype A1 with G1862T and subgenotype A1, showing the top 5 downregulated Reactome pathways (B) Network analysis of downregulated proteins using string network (Fold cut-off set to 0.4).

Chapter 5: Discussion and Conclusion

Viral infection is a major contributor to the global cancer burden. Seven known oncogenic viruses promote tumourigenesis through shared host cell targets and pathways (Krump and You 2018). Although the 7 oncogenic viruses differ in their genomes, being DNA or RNA, single or double-stranded, linear or circular, they share several features that can lead to human cancers. As reviewed in Chapter 2 there are at least 10 hallmarks of cancer (Krump and You 2018, Padarath, Deroubaix et al. 2023). Additionally, four emerging hallmarks have been described including: unlocking phenotypic plasticity, non-mutational epigenetic reprogramming, senescence cells and polymorphic microbiomes (Hanahan 2022)

HBV has a significant oncogenic potential, which is associated with its ability to establish chronic infection, persist by evading the immune response and ultimately leading to the clinical manifestation of cirrhosis and/or HCC (Jiang, Han et al. 2021). The course and outcomes of the infection depend on several virus-host interactions and can be (sub)genotype-dependent. In chapter 3, using mass spectrometry the proteome of Huh7 cells transfected with the different (sub)genotypes of HBV circulating in SSA, A1, D3 and E, and subgenotype A2, which prevails outside of Africa was analysed. Subgenotype A1, which has a higher hepatocarcinogenic potential, at an earlier age in sub-Saharan Africans (Kew, Kramvis et al. 2005), develops unique basic core promoter (BCP) and precore (PC) mutations, which affect HBeAg expression at a transcriptional, translational and post-translational level (Kramvis, Kew et al. 1998, Ahn, Kramvis et al. 2003, Kramvis 2007, Kramvis 2018). One such mutation is the G1862T precore mutation, found frequently in HBV isolated from HCC patients. This mutation affects HBeAg expression by interfering with the post-translational modification of the HBeAg precursor (Chen, Crowther et al. 2008, Bhoola and Kramvis 2017). The literature review in chapter 2 demonstrated that the HBeAg and its precursors can play roles in at least four hallmarks of cancer: avoiding immune destruction; tumour-promoting inflammation; resisting cell death; and sustained proliferative signalling (Figure 1; Chapter 2). Therefore, in Chapter 4, the proteome of Huh7 cells transfected with wild-type and mutant G1862T subgenotype A1, was also analysed by mass spectrometry to determine the effect of this mutation on cellular signalling pathways. Considering that subgenotype A1 regulates the expression of HBeAg using different mechanisms to other (sub)genotypes and also has a higher hepatocarcinogenic potential this study used proteome analyses to identify biological processes, which may be

impacted on by these unique features of subgenotype A1. From the present study, it became evident that the subgenotypes of HBV studied and the G1862T mutation may have a role in three of the 10 hallmarks of cancer (Padarath, Deroubaix et al. 2023) including immune evasion and persistence, sustaining proliferative signalling, and genome instability and mutation.

5.1 Immune evasion and persistence

Using a DIA SWATH–MS-based quantitative proteomic approach, a comparative analysis of the whole proteome of Huh7 cells transfected with the different SSA HBV (sub)genotypes relative to subgenotype A2 was performed. Although subgenotypes A1 and A2 belong to the same genotype, they demonstrate a higher divergence in the BCP/PC region between each other than between subgenotype A1 and (sub)genotypes D3 and E, respectively (Kramvis 2018). This could be the reason why the greatest difference in the proteome was between two subgenotypes of A, A1 and A2, and not between two different genotypes. Even though subgenotype A2 had the highest number of DEPs compared to subgenotype A1, the latter had a higher percentage of upregulated DEPs. Subgenotypes A1 and A2 have different clinical manifestations (Kramvis, Arakawa et al. 2008, Kramvis 2014, Kramvis 2016, Kramvis 2018). Subgenotype A1 develops different mutations in the BCP and pre-core region, whereas subgenotype A2 only develops mutations in the BCP, decreasing precore mRNA levels, which affect the expression level of HBeAg (Kramvis, Arakawa et al. 2008, Kramvis 2014, Kramvis 2016, Kramvis 2018), resulting in significantly higher HBeAg negativity in subgenotype A1-infected patients than subgenotype A2-infected patients (Kew, Kramvis et al. 2005, Kramvis and Kew 2007, Kramvis 2007). (Sub)genotypes D3 and E cannot be differentiated in the precore region, and showed similar DEPs. The difference in BCP/PC region can result in varying expression of HBeAg amongst the (sub)genotypes, resulting in different effects on host signalling pathways.

HBeAg has the dual role as a tolerogen and immunogen and its ability to suppress or activate the immune response reveals the complexity of the interactions between HBeAg and the host (Milich 2019). Host immunity plays a role in determining whether cancer develops after infection with an oncogenic virus, as demonstrated by the increased incidence of virus-induced cancers in immunocompromised individuals (Morales-Sánchez and Fuentes-Pananá 2014, Cesarman, Damania et al. 2019). Viruses, in turn, have developed a profusion of strategies to escape from the immune system. One of these mechanisms is viral proteins that can dysregulate

the host's immune response. From the review in chapter 2, it is evident that HBeAg and its precursors can lead to the downregulation of pro-inflammatory transcription factors (Xie 2017, Tsai and Ou 2021), and activate transcription factors that mediate the expression of key cytokines and chemokines to direct the immune system away from HBV-infected hepatocytes and ensure immune evasion (Mitra, Wang et al. 2019).

Various host signalling pathways regulating the immune system were affected by the different (sub)genotypes (Figure 2, Chapter 3). Genotype E, which is associated with the highest frequency of HBeAg positivity, was the only SSA genotype to favour dysregulation of toll like receptor signalling pathways (TLR). HBeAg downregulates the expression of the TLR family within Kupffer cells and blood monocytes, to promote immune evasion and persistence (Riordan, Skinner et al. 2006, Visvanathan, Skinner et al. 2007). The TLR family is part of the pattern recognition receptor (PRR) family and is responsible for recognising diverse molecules derived from pathogens and damaged host cells, also called pathogen-associated molecular patterns (PAMPs) (Kawasaki and Kawai 2014). Considering that genotype E does not differ from genotype D in the precore region, this effect may be attributed to differences in other regions of the genome. Genotype has a unique serological subtype (*ayw*4) compared to the (sub)genotypes and this would be interesting to investigate this further in the future.

This study identified the p38 mitogen-activated protein kinase (MAPK) signalling pathway as one of the top signalling pathways dysregulated between cells transfected with subgenotype A1 compared to the vector control. MAPK signalling has been shown to play an important role in immune escape, HBV replication, persistence, and the promotion of tumour cell survival (Jiang, Han et al. 2021). MAPK pathways regulate gene transcription that controls cell proliferation and the antiviral immune response (Kim and Choi 2015). They are involved in the life cycle and propagation of several oncogenic viruses, such as HCV, HPV and MCPyV, by promoting viral assembly, production and release (Krump and You 2018).

Furthermore, proteomic analysis revealed that when compared to the vector control, G1862T transfected cells had more significantly differentially expressed proteins in the p38 MAPK pathway than WT-transfected cells. Previous studies have shown HBeAg suppresses p38 MAPK phosphorylation (Visvanathan, Skinner et al. 2007). This downregulation of MAPK would allow HBV to evade immune surveillance and thus persist. Therefore, viral mutations such as G1862T, which reduce HBeAg expression, can lead to the removal of the tolerogenic

effect of HBeAg, and the activation of the immune response (Alexopoulou and Karayiannis 2014, Kramvis 2018, Milich 2019).

5.2 Sustaining proliferative signalling (PI3K/AKT/mTOR pathway)

The top dysregulated pathway in subgenotype A1 was PI3K/Akt/mTOR pathway (Table 2, Chapter 3). The PI3K/AKT/mTOR pathway has an important role in the regulation of cellular growth, cell cycle progression, proliferation, and survival. The PI3K/Akt/mTOR pathway is overexpressed in nearly 50% of HCCs and the dysregulated activation of this pathway affects a wide range of processes, including cell proliferation, metabolism, tumour cell differentiation, lipid metabolism, autophagy, and epithelial to mesenchymal transition (EMT) (Matter, Decaens et al. 2014, Porta, Paglino et al. 2014). Furthermore, previous studies have shown that the interaction of the mTOR signalling pathway and HBV life cycle is complex. The mTOR signalling pathway can regulate HBV replication and gene expression (Guo, Zhou et al. 2007, Lin, Deng et al. 2017, Wang, Lin et al. 2020), whereas, HBV infection can also activate PI3K/Akt/mTOR pathway. This occurs following the synthesis of a large quantity of viral proteins, which leads to the accumulation of HBsAg/HBeAg in the endoplasmic reticulum (ER) lumen that triggers ER stress (Bhoola and Kramvis 2016, Choi, Lee et al. 2019). A previous study, using the same plasmid construct used in the present study, showed that subgenotype A1 exhibits a greater amount of ER stress compared to subgenotypes A2 and D3 (Bhoola and Kramvis 2016), which could lead to the activation of the PI3K/Akt/mTOR pathway. Other oncogenic viruses, including HPV, EBV, HTLV-1, KSHV and MCPyV, have evolved mechanisms to engage this pathway to increase virus survival and carcinogenesis (Krump and You 2018).

Additionally, Ras homolog gene family, member C (RHOC), which is an upstream regulator of the MAPK and PI3K/Akt/mTOR, was identified as a protein of interest. As cells transfected with subgenotype A1 compared to the different HBV (sub)genotypes or vector control showed enrichment in multiple pathways where RHO GTPases activity was upregulated (Figure 5C&D; Chapter 3). RHO GTPases regulate cell growth, motility, survival, and metabolism, and are upregulated in HCC (Qu, Qu et al. 2009, Saini, Loi et al. 2013, Setia, Nehru et al. 2014, Stefani, Miricescu et al. 2021). Furthermore, RHOC expression was confirmed using an RHOC-specific ELISA (Figure 8A, Chapter 3) and the cellular localization of RHOC in Huh7 cells transfected with subgenotype A1 was compared to the other (sub)genotypes or

untransfected cells using immunofluorescence (Figure 9, Chapter 3). The increased focal adhesion staining of RHO proteins in subgenotype A1 transfected cells compared to cells transfected with other (sub)genotypes, may be related to higher hepatocarcinogenic potential of this subgenotype. More in-depth studies on the role of RHO proteins in HCC are necessary.

5.3 Genome instability and mutation.

Proteomic analysis revealed that proteins involved in the cell cycle and oxidative stress pathways were exclusively found in cells transfected with the G1862T mutation compared to wildtype (Figure 3B, Chapter 4). Oxidative stress is increased in HBV infections (Bolukbas, Bolukbas et al. 2005), and can be further exacerbated by the ER stress caused by G1862T mutation through the production of reactive oxygen species from the UPR (Görlach, Klappa et al. 2006, Bhoola and Kramvis 2017). Furthermore, increased oxidative stress in HBV infection was also demonstrated to increase DNA damage (Abdollahi, Ranjbar et al. 2004, Duygu, Karsen et al. 2012). Therefore, there seems to be an interplay between these two exclusive pathways, which could contribute to the hepatocarcinogenic potential of HBV with the G1862T mutation. In addition, it has been shown that p22 and p25, which accumulate in the cytoplasm of G1862T transfected cells, can translocate to the nucleus (Deroubaix and Kramvis 2021). In the nucleus, these HBeAg precursors can interact with the host genome leading to genomic instability and mutations.

The host DNA damage response (DDR) system is a complex network of signalling pathways that collectively monitor and repair DNA damage that results from DNA replication, cellular metabolism and exogenous insults, such as radiation and viral infection (Ciccia and Elledge 2010). Viruses often elicit host DDRs; however, they have evolved mechanisms to undermine these responses and manipulate them to their advantage (Lilley, Schwartz et al. 2007, Weitzman, Lilley et al. 2010).

Replication stress, nucleotide deficiency and the production of reactive oxygen species (ROS) during viral infection can also contribute to genomic instability and oncogenesis by other viruses (Srinivas, Tan et al. 2019). For example, during persistent HCV and EBV infection, chronically activated inflammatory cells release ROS, which can cause oxidative DNA damage and promote a pro-carcinogenic microenvironment that drives tumour development (Gruhne, Sompallae et al. 2009, Takeda, Takai et al. 2017).

Defects in efficiently and appropriately repairing DNA damage result in genomic instability and potential tumourigenesis (Huang and Zhou 2020). Therefore, the DNA synthesis and repair pathways were further explored. Two proteins involved in DNA synthesis: DNA primase subunit 2 (PRIM2) and Replication Protein A (RPA) were upregulated in G1862T compared to the WT (Figure 3B, Chapter 4). This was validated at the RNA level for both proteins (Figure 4, Chapter 4) and for RPA using immunofluorescence (Figure 5, Chapter 4). PRIM2 is a subunit of the DNA primase complex and a component of the DNA polymerase alpha complex, which plays an essential role in the initiation of DNA synthesis, during the S-phase of the cell cycle (Schneider, Smith et al. 1998, Weiner, Huang et al. 2007, Baranovskiy, Zhang et al. 2015). Whilst RPA binds and stabilizes single-stranded DNA intermediates that form during DNA replication or upon DNA stress, thereby playing an essential role both in DNA replication and the cellular response to DNA damage (Lin, Shivji et al. 1998). Both these proteins have displayed oncogenic potential in various cancers (Algethami, Toss et al. 2023, SUN, SHAO et al. 2024).

5.4 Limitations of this study

As discussed in Chapters 3 and 4, there were several limitations in this study. Even though the Huh7 *in vitro* system is not perfect, transfection of Huh7 cells with HBV plasmid constructs provides us with a widely-used model system that allows us to follow HBV infection and dissect complex biological processes (Wose Kinge, Bhoola et al. 2020). Moreover, as discussed above, using this system it was demonstrated differences between the (sub)genotypes and the effect of G1862T mutation on the proteome of Huh7 cells intimating that these differences may influence immune evasion and persistence of HBV, sustained proliferative signalling and genome instability of cells, three hallmarks of cancer.

Differences between constructs, the method of transfection and the cell line used, can make inter-experimental comparisons challenging (Gripon, Diot et al. 1988, Galle, Hagelstein et al. 1994, Wose Kinge, Bhoola et al. 2020). Therefore, a panel of replication-competent plasmids of (sub)genotypes A1, A2, D3 and E, with endogenous promoters, were constructed (Bhoola, Reumann et al. 2014, Bhoola and Kramvis 2016). These clones, although useful in *in vitro* studies cannot fully represent the diversity of the HBV (sub)genotype seen in patients because they were chosen for being the least divergent from the census sequence of the corresponding (sub)genotypes. In both Chapter 3 and Chapter 4, the effects of the SSA subgenotypes and the

G1862T mutation were compared to a vector control rather than to untransfected cells. The vector control was chosen as the comparator to correct for any effects of the transfection on the proteome, even though this effect was minimal relative to untransfected cells.

Even though this system does not recapitulate the pathogenic mechanisms, immune response and clinical manifestations seen *in vivo* and in patients, it does allow the elucidation of the complex interplay between HBV and its host, and in particular in liver cells. However, to further explore the mechanisms of the pathogenesis of subgenotype A1-associated HCC and the effect of the G1862T mutation, further *in vivo* studies using patient tissues and/or sera will be required.

5.5 Conclusion

This thesis represents the first comprehensive proteomic analysis of the differences among SSA genotypes, providing novel insights into the molecular underpinnings of their oncogenic potential. Using an *in vitro* system, the different (sub)genotypes and the G1862T mutant of HBV are shown to impact various signalling pathways involved in three hallmarks of cancer including: immune evasion and persistence, sustained proliferative signalling and genome instability and mutation. The differences of the (sub)genotypes on the proteome, which have been demonstrated in the present study may have an impact on the natural history of infection and clinical consequences of the various strains of HBV. In particular, subgenotype A1, which has the highest hepatocarcinogenic potential, was shown to favour the dysregulation of MAPK (immune evasion and persistence) and PI3K/Akt/mTOR (sustained proliferative signalling) pathways and when accompanied by the G1862T mutation showed dysregulation of DNA synthesis pathway leading to uncontrolled proliferation (genome instability and mutation). These findings not only illuminate the complex molecular mechanisms by which subgenotype A1, may contribute to HCC, but also emphasise the pivotal role of HBeAg in the process of hepatocarcinogenesis. This research opens potential avenues for biomarker development and targeted therapeutic interventions, especially in SSA, which has a diversity of HBV strains and a high incidence of HCC.

5.6 Future perspectives

As demonstrated by this study, which is novel in the sub-Saharan African context, proteomics can provide us with an important tool, which allows us to study virus-host interactions and the endpoint of gene expression, the proteome (Mann, Kumar et al. 2021). MS proteomics is characterised by its specificity and sensitivity, which allows for the unbiased detection and quantification of cellular proteins. With the unprecedented evolution of the "omics" (genomics, transcriptomics, and proteomics) (Gharib 2012) and the advent of artificial intelligence, major breakthroughs can be anticipated in the field. Accompanied by the advancement of various *in vitro* systems, including the development of three-dimensional (3D) tissue culture (organoids) and induced pluripotent stem cell (iPSC) technology (Yang, Hu et al. 2023), we can anticipate clarifications of the complex pathways leading to hepatocarcinogenesis following infection with HBV. This will allow for the discovery of anti-viral and anti-tumour modalities that can overcome this viral infection with its serious clinical consequences.

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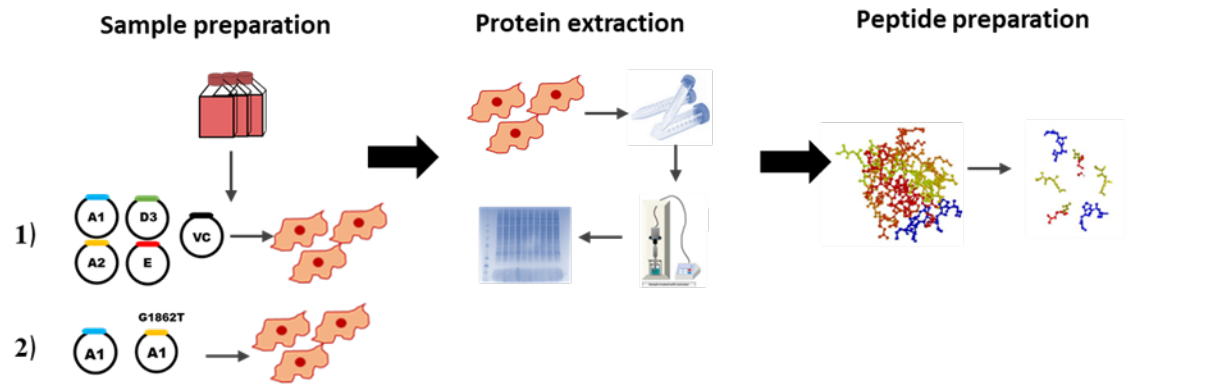
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Appendix A: An Annotated Mass Spectrometry workflow



- Huh7 cells were cultured at 37°C with 5% CO_2 in quadruplicate.
- Cells were then seeded in a 10cm^2, in order to obtain a minimum of 0.5 million cells in quadruplicate
- After 24 hours the cells were transfected with 1µg of plasmid DNA containing replication-competent clones

- 5 days post transfection proteins were extracted from the cell pellets using a PIXUL multi-sample sonicator
- The supernatants were then collected, and the concentrations were determined using a BCA assay.
- Proteins were run on a SDS-PAGE to check the quality of the proteins

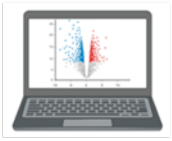
- Sample clean-up and digestion performed using a magnetic particle processing robot. KingFisher™ Flex system was configured for automated HILIC protein clean-up and on-bead Trypsin digestion
- Following HILIC, the peptide samples were vacuum dried.

LC-MS analysis



- Analysis was performed on a Dionex Ultimate 3000 RSLC system coupled to an AB Sciex 5600 TripleTOF mass spectrometer .
- For SWATH-MS, precursor scans were acquired from 400 to 1100 m/z with an accumulation time of 50 milliseconds and fragment ions acquired from 200 to 1800 m/z for 48 variable-width precursor windows, with a 0.5 Da overlap between the windows and an accumulation time of 20 milliseconds per window.

Bioinformatic analysis of MS data



- SWATH data was processed using Spectronaut v17 software Swiss-Prot Human sequences (downloaded on 12 December 2022 from www.uniprot.org) was used as a search database
- A FDR of 1% was applied
- Candidate dysregulated proteins were filtered at a q-value ≤ 0.05, absolute fold change (FC) ≥ 1.5, and a minimum of two unique peptides identified.

Data mining and interpretation



- Proteomic analysis was performed usings various bioinformatic tools e.g. Panter , Reactome , STRING etc
- DEPs were classified by their corresponding pathways using the panther database.
- Pathways were rank based of the number of DEPs and compared across the different (sub)genotypes and G1862T mutant .
- The Reactome databased was used in as part of an enrichment analysis to see which pathways were enriched or depleted
- STRING analysis was used to show if there was any interaction between selected proteins

*Abbreviations

- BCA- Bicinchoninic Acid
- SDS-PAGE-Sodium dodecyl sulfate polyacrylamide gel electrophoresis
- LC-MS-Liquid chromatography–mass spectrometry
- HILIC-Hydrophilic Interaction Liquid Chromatography
- SWATH-MS-Sequential Window Acquisition of All Theoretical Mass Spectra
- FDR- False discovery rate

Appendix B: Ethics Declaration

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

TO: Ms K Paradrath
School: Clinical Medicine
Department: Medicine
Division: Internal Medicine
Hepatitis Virus Diversity Research Unit
Medical School, University
E-mail: 806617@students.wits.ac.za

CC: Supervisor: Professor A Kramvis <Anna.Kramvis@wits.ac.za>
and <HREC-Medical.ResearchOffice@wits.ac.za>

FROM: Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 16/04/2021

REF: *R14/19*

PROTOCOL NO: W-CBP-210416-01 (This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)

PROJECT TITLE: *Comparison of the proteome of Huh-7 cells transfected with different (sub)genotypes of Hepatitus B virus prevailing in sub-Saharan Africa*

Please find attached the Ethics Waiver Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

MSWorks2000/Iain0007/ClearScan\Waiver.wps

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

16/04/2021

Ref: W-CBP-210416-01

TO WHOM IT MAY CONCERN

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)

Investigator: Ms K Paradrath
Student No. (if appropriate): 806617
Staff No. (if appropriate):

Supervisor: Professor A Kramvis

School: Clinical Medicine
Department: Medicine
Internal Medicine
Hepatitis Virus Diversity Research Unit
Medical School, University

Project title: *Comparison of the proteome of Huh-7 cells transfected with different (sub)genotypes of Hepatitus B virus prevailing in sub-Saharan Africa*

Reason: In vitro laboratory study
No human participants will be involved in the study

Dr CB Penny
Chairperson: Human Research Ethics Committee (Medical)

Research Office Secretariat:
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Appendix C: Permission from co-authors to use publication in thesis

Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, Kiyasha Padarath, student number 806617, declare that this Thesis/Dissertation/Research Report is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Thesis/Dissertation/Research Report .

Signature of Student ..... **Date**.....05/07/2024.....

Name of Primary Supervisor.....Professor Anna Kramvis .....

Signature of Primary Supervisor **Date**6 July 2024

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of his/her Thesis/Dissertation/Research Report. In cases where the student is not the 1st author of a published article, the primary supervisor must explain (under comments) why the student is entitled to use the paper for his/her degree purposes.

Article 1: Title: The Complex Role of HBeAg and Its Precursors in the Pathway to Hepatocellular Carcinoma
Journal name, year, volume and page numbers: Viruses 2023, 15(4), 857; https://doi.org/10.3390/v15040857 (Review)

Authors	Name	Signature	Date
1st author	Miss Kiyasha Padarath		05/07/2024
2nd author	Dr Aurelie Deroubaix		06/07/2024
3rd author	Prof Anna Kramvis		6 July 2024
4th author			
5th author			
6th author			

Comments by primary supervisor:

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Article 2: Title: Comparative proteomic analysis of Huh7 cells transfected with sub-Saharan African Hepatitis B Virus (sub)genotypes reveals potential oncogenic factors
Journal name, year, volume and page numbers: Viruses 2024, 16(7), 1052; https://doi.org/10.3390/v16071052 (Article)

Authors	Name	Signature	Date
1st author	Miss Kiyasha Padarath		05/07/2024
2nd author	Dr Aurelie Deroubaix		06/07/2024
3rd author	Dr Previn Naicker		05/07/2024
4th author	Dr Stoyan Stoychev		05/07/2024
5th author	Prof Anna Kramvis		6 July 2024

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6th author			

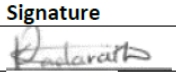
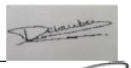
Comments by primary supervisor:

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Article 3: Title: Comparison of the Proteome of Huh7 Cells Transfected with Hepatitis B Virus Subgenotype A1, with or without G1862T

Journal name, year, volume and page numbers: Curr. Issues Mol. Biol. 2024, 46(7), 7032-7047; https://doi.org/10.3390/cimb46070419 (Article)

Authors	Name	Signature	Date
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4th author	Dr Stoyan Stoychev		05/07/2024
5th author	Prof Anna Kramvis		6 July 2024
6th author			

Comments by primary supervisor:

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Appendix D: Plagiarism declaration and check (turn it in report)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

FACULTY OF HEALTH SCIENCES

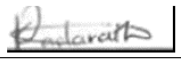
PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I Kiyasha Padarath (Student number:806617) am a student registered for the degree of Doctor of Philosophy(Ph.D.) in the academic year2024 .

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

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9th July 2024

Ms Kgomotso Tlala
Faculty Officer
Faculty of Health Sciences
Wits University

Dear Ms Tlala

Similarity index – PhD thesis- Ms Kiyasha Padarath

Dr Deroubaix and I, as supervisors, have checked the plagiarism report from the turn-it-in program, for the submission of the thesis submitted by Ms Kiyasha Padarath (Student number: 806617), PhD student in the Hepatitis Virus Diversity Research Unit doing a project entitled:

Comparison of the proteome of Huh-7 cells transfected with different (sub)genotypes of Hepatitis B Virus prevailing in sub-Saharan Africa

As far as we can tell the similarity index detected is as a result of common phrases used in the field, in the paper papers published by Ms Padarath and presented as part of the thesis.

Yours sincerely

A Kramvis

Professor Anna Kramvis
Professor Emerita
Director: Hepatitis Virus Diversity Research Unit

“What is not started, will never finish.”

-John Wolfgang von Goethe