MICRORNA PROFILE IN PATIENTS WITH CHRONIC HEPATITIS B VIRUS (CHBV) AND HUMAN IMMUNODEFICIENCY VIRUS (HIV) CO-INFECTION IN A HIGH PREVALENCE SETTING

By

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PREFACE

All the experiments described in this thesis were carried out in the Department of Virology, Inkosi Albert Luthuli Central Hospital Academic Complex, School of Laboratory Medicine and Medical Science, Faculty of Health Sciences, University of KwaZulu-Natal, Durban, South Africa under the supervision of Prof R Parboosing and Dr. N Msomi. The research was financially supported by National Health Laboratory Services (NHLS) Research Trust, National Research Foundation (NRF) and Poliomyelitis Research Foundation (PRF).

This research work has never been submitted to any other tertiary institution, where the work of others has been used, it is acknowledged in the text. All of the results reported are due to investigations by the candidate.

Lulama Mthethwa Signed [redacted]..

As the candidate's supervisor and co-supervisor, we agree with the submission of this thesis.

Prof Raveen Parboosing Signed:[redacted]

Dr. Nokukhaya Msomi Signed:[redacted]

DECLARATION

I Lulama Mthethwa declare that:

The research reported in this thesis, except where otherwise indicated or acknowledged, contains the original work by the author and has not been submitted at any other University.

The use of other people's work has been duly acknowledged in the text.

All the experiments described in this thesis were carried out in the Department of Virology, Inkosi Albert Luthuli Central Hospital Academic Complex, School of Laboratory Medicine and Medical Science, Faculty of Health Sciences, University of KwaZulu-Natal, Durban, South Africa under the supervision of Prof R Parboosing and Dr. N Msomi.

SIGNED [redacted] **DATE** 06/04/2023

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I would like to dedicate this work to my late mother, though she may not be here but her spirit continues to live in my heart.

TABLE OF CONTENTS

ABBREVIATIONS

%	Percent
<	Less than
>	Greater than
ΔΔCt	delta-delta threshold cycle
ΔCt	delta threshold cycle
≤	less or equal to
°C	degrees Celsius
µl	microlitre
∞	infinite
AASLD	American Association for the Study of Liver Diseases
ADV	Adefovir
AFP	Alpha-fetoprotein
AGO2	Argonaute 2
ALT	Alanine aminotransferase
ANOVA	Analysis of Variance
Anti-HBe/HBeAb	Hepatitis B e antibody
APRI	Aspartate aminotransferase to Platelet Ratio Index
AST	Aspartate aminotransferase
BREC	Biomedical Research Ethics Committee
cccDNA	covalently closed circular Deoxyribonucleic Acid
CD4+/CD8+	Cluster of Differentiation 4+/8+
cDNA	complementary Deoxyribonucleic Acid
CHB	Chronic Hepatitis B
CHBV	Chronic Hepatitis B Virus
Ct	threshold cycle
DGCR8	DiGeorge syndrome chromosomal region 8
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside triphosphate
DR	direct repeats

Ds-rcDNA	double-stranded relaxed circular Deoxyribonucleic Acid
dTTP	Deoxythymidine triphosphate
EASL	European Association for the Study of the Liver
Enh1/2	Enhancers 1/2
ETV	Entecavir
FAM	Fluorescein amidites
HBcAg	Hepatitis B core Antigen
HBeAg	Hepatitis B e Antigen
HBsAg	Hepatitis B surface Antigen
HBV	Hepatitis B virus
HBx	Hepatitis B x
HCC	Hepatocellular carcinoma
HCV	Hepatitis C Virus
HD	Hepatic decompensation
HIV	Human immunodeficiency virus
IALCH	Inkosi Albert Luthuli Central Hospital
IFN	Interferon
IQR	Interquartile range
IU/ml	International Units per milliliter
Kb	kilobase
LFTs	Liver function tests
MicroRNA/miRNA	Micro-ribonucleic acid
mRNA	Messenger Ribonucleic Acid
NAs	Nucleos(t)ide Analogs
ng/mL	nanogram per millilitre
NHLS	National Health Laboratory Services
nm	nanometre
NRF	National Research Foundation
nt	Nucleotides
NTCP	sodium-taurocholate cotransporting polypeptide
ORF	open reading frames
P53	Tumour protein
PCR	Polymerase Chain Reaction
Peg-IFN	Pegylated interferon

pgRNA	pre-genomic Ribonucleic Acids
pH	power of hydrogen
Pre-microRNA	Precursor micro- Ribonucleic Acids
PRF	Poliomyelitis Research Foundation
Pri-microRNA	Primary micro- Ribonucleic Acids
PTA	Prothrombin activity
r	Spearman's rank correlation coefficient
rcDNA	relaxed circular Deoxyribonucleic Acid
RISC	Ribonucleic Acid-induced silencing complex
RNA	Ribonucleic Acid
RT	Reverse transcription
RT-PCR	Real-Time Polymerase Chain Reaction
snRNA	small nuclear Ribonucleic Acids
TAF	Tenofovir alafenamide fumarate
TDF	Tenofovir disoproxil fumarate
TE	Tris-EDTA
TRBP	Transactivation-response RNA-binding protein
UKZN	University of KwaZulu-Natal
ULN	upper limit of normal
UNG	Uracil N-Glycosylase
UTR	Untranslated region
vs	versus
WHO	World Health Organisation

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Introduction: HBV and HIV/HBV co-infection are significant public health issues, despite the availability of an effective HBV vaccine for nearly three decades. HBV and HIV both modulate microRNA (miRNA) expression to support viral replication. It has been estimated that 2.7 million people are co-infected with HBV and HIV worldwide. South Africa is an endemic setting for HBV and HIV infections. MiRNAs are small single-stranded non-coding RNAs that bind to the complementary strand of messenger RNA and suppress translation at the post-transcriptional level. The role played by miRNAs in HBV replication and pathogenesis is being increasingly recognized. This retrospective study aimed to describe the pattern of miRNA expression in patients coinfected with chronic HBV and HIV with varying disease severity, as indicated by HBeAg status, HBV viral load, ALT levels, and HIV viral load.

Methods: Plasma miRNAs, specific to HBV, were measured by reverse-transcription quantitative polymerase chain reaction (qRT-PCR) in HBV and HIV negative healthy controls (n = 23) and patients coinfected with chronic HBV-HIV (n = 50). Samples from HBV-HIV coinfected patients were obtained from a previous study and controls were randomly selected from samples submitted for routine hepatitis and HIV serology at the Department of Virology, Inkosi Albert Luthuli Central Hospital. The studied miRNAs included hsa-miR-15b-5p, hsa-miR-20a-5p, hsa-miR-29a-3p, hsa-miR-122-5p, hsa-miR-125b-5p, hsa-miR-181b-5p, hsa-miR-192-5p, hsa-miR-193b-3p, hsa-miR-194-5p, and U6 snRNA (endogenous control). MiRNA expression levels were measured and compared between patients with high vs low HBV viral load, HBeAg positive vs HBeAg negative, high vs low ALT levels, and high vs low HIV viral load. Additionally, HBV viral load, ALT levels, and HIV viral load were correlated with miRNA expression levels.

Results: The healthy control group consisted of 69.9% females while the chronic HBV-HIV coninfected group had 42% of females. Significantly higher expression levels of our HBV-specific miRNAs were observed in chronic HBV-HIV coinfected samples compared to healthy controls. Samples with high HBV viral load had significantly higher expression levels of hsa-miR-122-5p (p = 0.0001), hsa-miR-192-5p (p = 0.0003), and hsa-miR-193b-3p (p = 0.0002) compared to samples with low HBV viral load. In HBeAg-negatives samples, significantly higher levels of hsa-miR-15b-5p (p = 0.0054) and hsa-miR-181b-5p (p = 0.03) were observed

compared to HBeAg-positive samples. No significant differences were observed in low vs high ALT levels and low vs high HIV viral load samples. A significant moderate positive correlation was observed between HBV viral load and the expression levels of hsa-miR-122-5p, hsa-miR-192-5p, and hsa-miR-193-3p.

Conclusion: Our study provides evidence for the potential use of hsa-miR-15b-5p, hsa-miR-122-5p, hsa-miR-181b-5p, hsa-miR-192-5p and has-miR-193b-3p as additional or confirmatory tests, together with currently available prognostic and diagnostic markers in chronic HBV disease progression.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

1.1.1 Background

Even though an effective vaccine has been available for nearly three decades, hepatitis B remains a significant public health burden (Alter, 2012, Amponsah-Dacosta, 2021). Hepatitis B virus (HBV) infection causes acute and chronic hepatitis and complications such as hepatocellular carcinoma (HCC) and liver cirrhosis (Liaw and Chu, 2009, Wu and Yeh, 2021). According to the World Health Organisation (WHO) estimates, in 2019, 296 million individuals were infected with chronic hepatitis B with new infections of about 1.5 million, and 820 000 individuals died due to HBV infection-related causes (WHO, 2021). There is a high prevalence of both HBV and HIV in South Africa (Mphahlele et al., 2006, Msomi et al., 2020).

The role of micro-ribonucleic acids (microRNAs/miRNAs) in HBV pathogenesis and prognosis has been investigated (Jin et al., 2019). MiRNAs are single-stranded, small non-coding RNAs that inhibit or degrade messenger ribonucleic acids (mRNAs) during post-transcriptional gene expression by binding to the 3'-untranslated region (3'-UTR) of the target mRNA (Bartel, 2004, Bartel, 2009, Yoshida et al., 2021). They were first discovered in *Caenorhabditis elegans (C. elegans)* but have now been identified in all multi-cellular eukaryotes and some viruses (Lee et al., 1993, Axtell et al., 2011, Qureshi et al., 2014). Several studies have been conducted to investigate the role of miRNAs in cellular response regulation such as proliferation, protein synthesis, differentiation, energy production, and apoptosis (Taganov et al., 2007, O'Brien et al., 2018).

Through direct interactions with viruses or viral components, cellular miRNAs can negatively or positively affect viral replication. Several studies have investigated the role of miRNAs in HBV. In one study, in patients chronically infected with HBV, Hepatitis B e Antigen (HBeAg) negative patients had lower plasma levels of several miRNAs compared to HBeAg positive patients, and the levels of these miRNAs were associated with lower HBV deoxyribonucleic acid (DNA) levels (van der Ree et al., 2017).

A study conducted on children identified 16 miRNAs that were elevated in HBeAg-positive children compared to those who were HBeAg-negative (Winther et al., 2013). Another study on

adults found a significant positive correlation between serum miRNA-210 level and alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), and total bilirubin, while there was a significant negative correlation between serum miRNA-210 and prothrombin activity (PTA) levels (Song et al., 2014).

1.1.2 Study Rationale

Despite the availability of an effective vaccine, HBV remains a global public health burden. Patients with chronic HBV (CHBV) infection are at risk of cirrhosis and HCC. The ALT and AST levels are commonly measured to assess liver injury, however, their specificity and sensitivity to virus-induced liver damage are inadequate. Hence, assessing the severity of HBV-induced liver damage remains a significant clinical challenge. It is important to explore other potential biomarkers, such as miRNAs, to better monitor the progression of chronic HBV. MiRNAs may enhance our understanding of the interactions between the host and virus and thereby contribute to data relevant to the management of the disease.

1.1.3 Research question

Does the expression of miRNAs correlate with markers of disease severity in patients coinfected with chronic HBV and HIV infection?

1.1.4 Aim

Describe the pattern of miRNA expression in patients coinfected with chronic HBV and HIV with varying disease severity, as indicated by HBeAg status, HBV viral load, ALT, and HIV viral load.

1.1.5 Objectives

- Description of miRNA expression pattern in patients with high vs low HBV viral load.
- Description of miRNA expression pattern in HBeAg positive vs. HBeAg negative patients.
- Description of miRNA expression pattern in normal vs. abnormal ALT levels.
- Describe how the expression pattern of miRNA differs in patients with high vs low HIV viral load.
- Description of miRNA expression levels correlation with HBV viral load, ALT levels, and HIV viral load.

1.2. Literature Review

1.2.1 HBV Infection

1.2.1.1 Global and local HBV epidemiology

Eradication of HBV infection is still a global health challenge despite the availability of an effective prophylactic vaccine (Alter, 2012, Amponsah-Dacosta, 2021). Even though little is known about HBV prevalence and distribution in some populations and regions, it is estimated to be the 7th major cause of morbidity and mortality globally (Stanaway et al., 2016, Sheena et al., 2022). HBV affects 296 million people globally and about 81 million of these are in sub-Saharan Africa, where 990 000 new infections occurred in 2019 and 80 000 individuals died from HBV infection-related complications, as depicted in Figure 1.1 (WHO, 2017, WHO, 2021). In 2019, there were 1.1 million deaths globally due to viral hepatitis, of which 96% were due to HBV and Hepatitis C Virus (HCV), which is greater than HIV mortality (WHO, 2021). Most of the viral hepatitis deaths were due to liver cirrhosis and HCC. The HBV epidemic affects mostly the WHO African Region and the WHO Western Pacific Region (WHO, 2017, WHO, 2021). There are an estimated 3.5 million people infected with HBV in South Africa (Schweitzer et al., 2015, Samsunder et al., 2019).

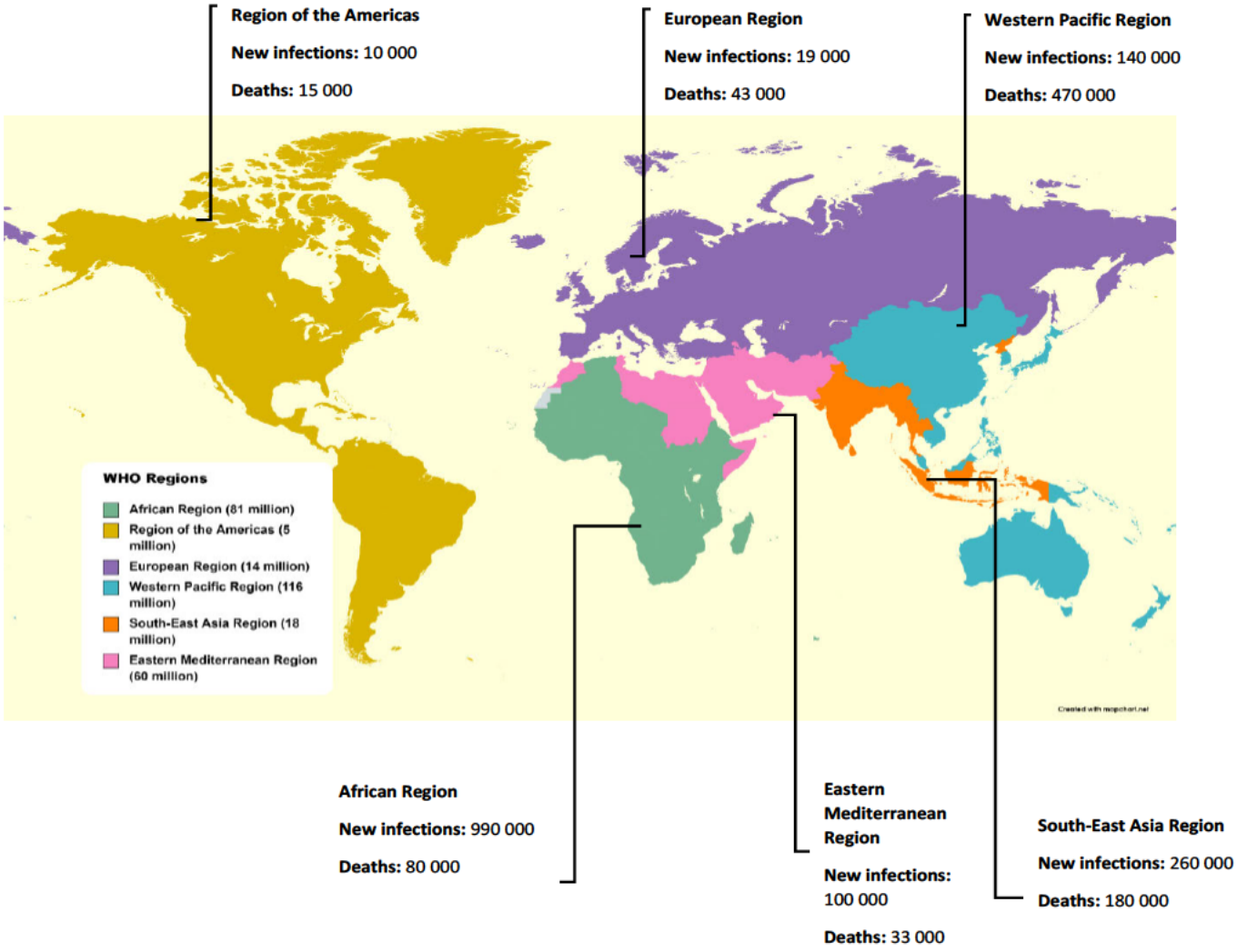


Figure 1.1: Hepatitis B prevalence with new infections and mortality per region, 2019. The WHO Western Pacific region has the highest number of infections followed by the WHO African region. The Western Pacific region is also leading in the number of deaths while the African region comes third. The map was drawn using MapChart (https://www.mapchart.net/) based on data from (https://apps.who.int/iris/handle/10665/342813).

1.2.1.2 HBV transmission

HBV is transmitted by three major modes which are sexual contact, horizontal transmission (through sharing infected objects), parenteral (subcutaneous, intravenous, intramuscular, and intrasternal injections) and vertical (mother to child). The predominant mode of transmission is perinatal (WHO, 2011, WHO, 2021). In highly endemic areas, such as sub-Saharan Africa (where HBsAg prevalence exceeds 8%) transmission occurs both horizontally and vertically (Edmunds et al., 1996, SA NDOH, 2019). The transmission risk for mothers who are HBV-carriers and HBeAg positive in Africa is 40% while the transmission risk for mothers who are HBV-carriers and HBeAg negative is 5%; indicating that infants born to mothers who are HBV-carriers and HBeAg positive

are at higher risk of acquiring infection (Keane et al., 2016, WHO, 2017). This highlights the significance of primary prevention in infancy through immunization to reduce perinatal transmission. In the first 24 hours a single dose of the hepatitis B vaccine must be administered to infants followed by three additional doses by the age of one (WHO, 2015, Pattyn et al., 2021, Yonghao et al., 2022). But this is not yet universally implemented as South Africa and other countries do not yet give the birth dose of the HBV vaccine (de Villiers et al., 2021).

1.2.1.3 HBV and HIV co-infection

HBV and HIV have similar modes of transmission and patterns of endemicity but HBV is about 100 times more infectious than HIV (Tarantola et al., 2006, WHO, 2011, Kafeero et al., 2021). In 2020, among 37.7 million individuals living with HIV, approximately 2.7 million individuals were chronically infected with HBV. Of the 2.7 million cases of co-infection, 71% of patients reside in sub-Saharan Africa (Platt et al., 2016, WHO, 2017, WHO, 2021). In people living with HIV and co-infected with HBV, liver diseases are a primary cause of mortality and morbidity (WHO, 2017, WHO, 2021). Management of hepatitis B in HIV co-infected patients is intricate due to the dual activity of various nucleoside analogs and the emergence of resistant HBV or HIV strains (Thio, 2009, Kourtis et al., 2012, Ramesh et al., 2021). The natural history of HBV infection is modified in the presence of HIV infection, with a higher titer of HBV in the blood, increased reactivation, liver fibrosis, and HCC (Puoti et al., 2006, Kew, 2012, Chen et al., 2013, Sarmati and Malagnino, 2019).

1.2.1.4 HBV genomic structure

The structure of HBV comprises a nucleocapsid surrounding a partially double-stranded relaxed circular DNA genome (ds-rcDNA) of about 3.2 kilobases (kb) pairs. Figure 1.2 displays a typical genomic organization of HBV which is made up of four promoters, four overlapping open reading frames (ORF), and two enhancers for the regulation of viral DNA transcription. The ORFs P, Pre-C/C, X, and Pre-S1/Pre-S2/S encode viral DNA polymerase, pre-core, and core proteins, Hepatitis B x (HBx) protein, and surface proteins, respectively. The direct repeats (DR1 and DR2) are essential for strand-specific DNA synthesis and the complete viral transcript expression is regulated by the enhancers (enh1 and enh2) (Al-Sadeq et al., 2019).

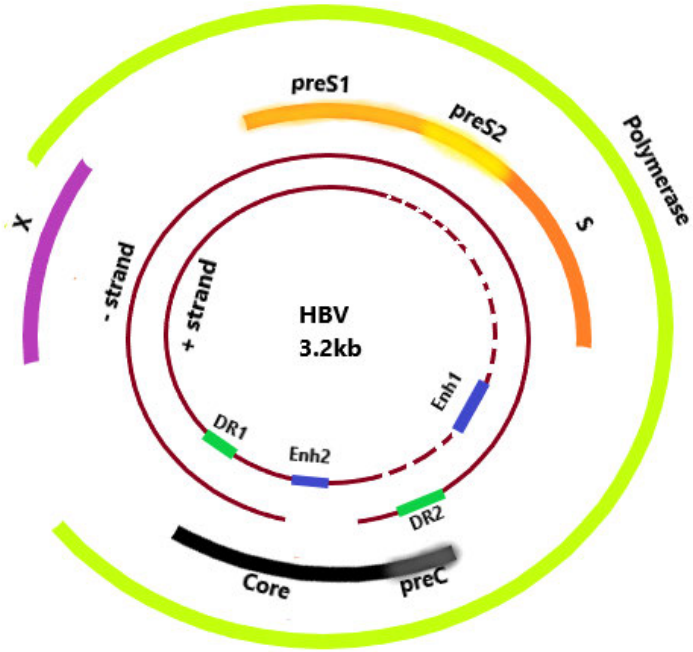


Figure 1.2: Genomic organization of HBV. HBV is composed of approximately 3020-3320 base pairs, which consist of open reading frames (ORF S/Pre-S1/S2, P, X, and C/Pre-C), two enhancers (Enh1 and Enh2), direct repeats (DR1 and DR2), four promoters embedded in the ORFs, an incomplete positive strand and a negative strand which is complementary to the viral mRNA. (Diagram drawn using Paint 3D [Microsoft Corporation, London, England], Adapted with modifications from Al-Sadeq DW [2019, May 11]. Hepatitis B Virus Molecular Epidemiology, Host-Virus Interaction, Coinfection, and Laboratory Diagnosis in the MENA Region: An Update. Pathogens. Retrieved October 2020, [Al-Sadeq et al., 2019])

1.2.1.5 HBV replication

The virus utilizes sodium-taurocholate cotransporting polypeptide (NTCP) as a functional surface receptor to enter the hepatocyte via endocytosis. Then the viral nucleocapsid is transported to the nucleus (Cooper and Shaul, 2006, Huang et al., 2012, Herrscher et al., 2020). The relaxed circular DNA (rcDNA) is released into the nucleus (Rabe et al., 2003, Cai et al., 2020). The cellular RNA polymerase II repairs the rcDNA to form covalently closed circular DNA (cccDNA) which is a template for HBV transcription (Fan et al., 2022).

The cccDNA is stable thereby making it difficult to remove during HBV infection, therefore it plays a pivotal role in viral persistence (Zoulim, 2005, Martinez et al., 2019). The cccDNA is transcribed into five types of mRNA transcripts: a 3.5-kb pre-genomic RNA (pgRNA), a 3.5-kb pre-core RNA, a 0.7-kb HBx RNA, and a 2.1-kb and 2.4-kb HBV surface antigen (HBsAg) RNA (Figure 1.3) (Chapus et al., 2021). The pre-core RNA translates into HBeAg, and pgRNA is translated to reverse

polymerase and Hepatitis B core Antigen (HBcAg). The pgRNA is a template for HBV DNA production via reverse transcription. The mature virions exit the cell through the endoplasmic reticulum and Golgi apparatus after encapsidation and reverse transcription. Alternatively, they are recycled back to the nucleus for a new round of replication but the details of the exiting/recycling process have not been completely revealed (Kim et al., 2016, Prange, 2022).

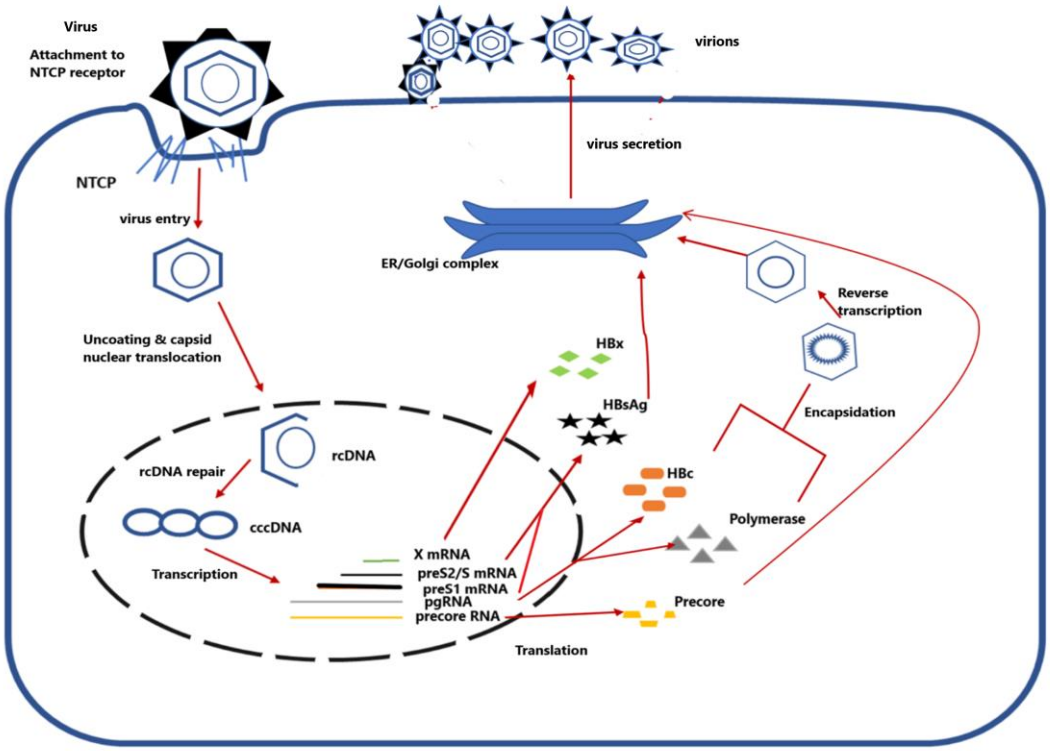


Figure 1.3: Schematic diagram of the Hepatitis B virus replication cycle. The virus enters the cell via endocytosis and its genome is transported into the nucleus to be converted to cccDNA. It is then transcribed, translated, and reverse-transcribed to produce mature virions to be released into circulation. (Diagram drawn using Microsoft PowerPoint, Adapted with modifications from Kim DH [2016, August 21]. Roles of hepatocyte nuclear factors in hepatitis B virus infection. World Journal of Gastroenterology. Retrieved October 2020, from [Kim et al., 2016])

1.2.1.6 HBV pathology

1.2.1.6.1 Natural history and clinical features

HBV causes acute and chronic infection; 90-95% of adults with HBV infections have acute hepatitis, where the infection is resolved (undetectable viral DNA and the presence of antibodies against the surface antigen) (Liang, 2009, WHO, 2017). HBV-infected individuals may present with jaundice, nausea, vomiting, and abdominal pain but many cases of HBV infection are asymptomatic (SA NDOH, 2019). Acute infection resolves within six months (WHO, 2011). An early replicative phase of HBV occurs during acute infection. Patients are most infectious during this phase and are

at a higher risk of developing progressive fibrosis (Iannacone and Guidotti, 2022). Chronic infection is characterized by the persistence of detectable HBsAg for at least six months after the initial infection. More than 90% of children infected with HBV become chronically infected, compared to 5%-10% of adults (Lai et al., 2003, Jing et al., 2020). Other determinants of chronicity include age, sex, comorbidities (e.g. diabetes), coinfections (e.g. HBV-HIV), substance abuse (alcohol and tobacco), HBV genotypes (A, B, C, D, and F) and the presence of specific pre-core/ core mutations (Fattovich et al., 2008, Trépo et al., 2014, Al-Qahtani et al., 2018). About 20-30% of chronically infected adults will develop cirrhosis or liver cancer (WHO, 2017).

Chronic HBV infection can be clinically divided into phases based on virus-host interaction as shown in Figure 1.4 (McMahon, 2005, Tan et al., 2015). The immunotolerant phase occurs in typically young patients and is characterized by the lack of symptoms, the presence of HBeAg, and high serum levels of HBV DNA with normal ALT and no liver inflammation (Livingston et al., 2007, Liaw and Chu, 2009, Perrillo et al., 2022). More than 90% of patients with chronic HBV infection after the age of 20 years slowly enter an immune clearance phase, defined by continually elevated ALT levels over the upper limit of normal (ULN) as well as the presence of HBeAg and decreasing HBV DNA levels. These events might in due course lead to the seroconversion of HBeAg to anti-HBe (spontaneous or under treatment pressure). Seroconversion of HBeAg is persistent, with only 2% to 4% of patients becoming HBeAg-positive again (HBeAg reversion), and remission occurs in >95% of patients entering an inactive phase during which HBsAg loss may occur at the rate of 2% per year (Pisano et al., 2021, de Almeida Pondé, 2021). HBeAg-negative chronic HBV is caused by replicative HBV mutants that fail to produce HBeAg (precore mutants) or downregulated precore/core messenger RNA transcripts (basic core promoter mutants) (Carman et al., 1989b, Wei et al., 2022). HBeAg-negative carriers are a heterogeneous group and most of them have low viral DNA levels, relatively normal levels of ALT and a fair prognosis. HBeAg-negative chronic HBV emerges during the course of a typical HBV infection with the wild-type virus, and is selected during the immune clearance phase (HBeAg seroconversion) (Keeffe et al., 2004). HBeAg-negative chronic HBV can develop either soon after HBeAg seroconversion or decades later.

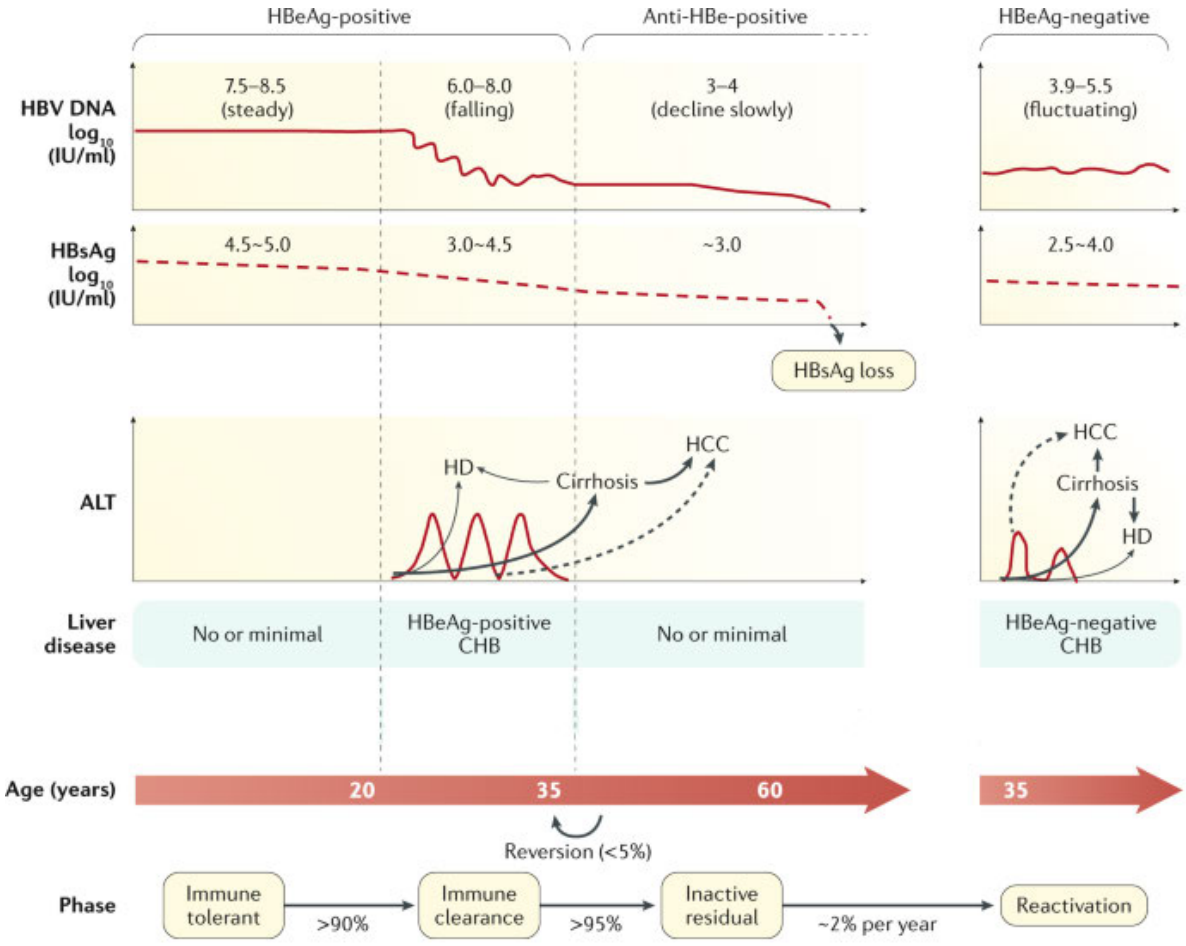


Figure 1.4: Chronic hepatitis B phases of infection. An HBeAg-positive immune tolerant state will transition to an immune-clearing phase, and finally to an inactive HBeAg-negative state after HBeAg seroconversion characterized by HBeAg loss with the appearance of anti-HBeAg antibodies. HD, Hepatic decompensation; ALT, Alanine transaminase; CHB, chronic Hepatitis B; HCC, hepatocellular carcinoma. (Reproduced from Liaw YF [2019, September 02] with permission from Nature Reviews Gastroenterology & Hepatology. Clinical utility of HBV surface antigen quantification in HBV e antigen-negative chronic HBV infection. Nature Reviews Gastroenterology & Hepatology. Retrieved October 2020, [Liaw, 2019]).

1.2.1.6.2 Pathogenesis and immune response

Hepatitis B infection's pathogenesis and clinical manifestations are due to the interaction of the virus and the host immune system. During HBV infection, activated CD4+ and CD8+ lymphocytes recognize several HBV-derived peptides on the surface of hepatocytes (Iannacone and Guidotti, 2022). The innate immune system serves as the first line of defense whereby interferon molecules are produced and secreted from infected cells, to activate anti-viral gene transcription and an antiviral state in general (Huang et al., 2019). Chronic HBV infection is denoted by a notably delayed early innate immune response with weak elicitation of antiviral interferons due to specific immunosuppression by viral proteins (Ferrari, 2015, Suslov et al., 2018). The host immune response

is activated by viral proteins as the viral load increases to induce HBV-specific T-cell response in the presence of a secondary inflammatory response and further increases interferon, free radicals, tumor necrosis factor, and hepatic injury (Mak et al., 2018). In infants, where the immune response is immature, exposure to HBV allows the virus to persist which manifests as chronic infection (Al-Sadeq et al., 2019, WHO, 2021).

1.2.1.6.3 Complications and prognosis

Chronic HBV infection causes approximately 50% of HCC worldwide (Kim et al., 2011, Gebremeskel et al., 2020). High HBV DNA levels (>2000 IU/ml), elevated ALT levels, and prolonged HBeAg-positivity are associated with the development of cirrhosis and HCC in individuals chronically infected with HBV. Persistent high level of HBV DNA has been suggested to be the most reliable and robust predictor of the development of cirrhosis and HCC (Han et al., 2011, Terrault et al., 2016, Yao et al., 2022). Seroconversion to Hepatitis B e antibody (anti-HBe) and undetectable HBV DNA is an improved clinical outcome for patients, characterized by favorable survival with fewer or no complications, slower disease progression, and reduced rate of progression to cirrhosis and HCC development (Kim et al., 2011, Liem et al., 2019, de Almeida Pondé, 2021).

1.2.1.7 HBV current treatment and limitations

HBsAg clearance and suppression of HBV replication is considered the treatment endpoint and main aim of antiviral therapies (EASL, 2017). Currently, there are two types of approved antivirals for the treatment of chronic hepatitis B; pegylated interferon (Peg-IFN) and nucleos(t)ide analogs (NAs) (EASL, 2012). NAs drugs include tenofovir alafenamide fumarate (TAF), tenofovir disoproxil fumarate (TDF), lamivudine (LAM), adefovir (ADV), entecavir (ETV), telbivudine (TBV) and emtricitabine. Peg-IFN is a subcutaneous injection administered once weekly to adults with HBeAg-positive and HBeAg-negative compensated liver disease (EASL, 2017). About 30% of HBeAg-positive patients respond to Peg-IFN with sustained HBeAg seroconversion, and HBsAg loss was attained in 3-7% of patients (Sonneveld et al., 2013, Sarin et al., 2016, Hu et al., 2022). Furthermore, Peg-IFN achieved HBsAg clearance in approximately 12% of patients with HBeAg-negative disease, five years after Peg-IFN treatment, and sustained virological responses (HBV DNA 2000 IU/ml) in up to 40% of patients (Lampertico et al., 2013, van Campenhout et al., 2019).

Nucleos(t)ide analogs function by hindering the reverse transcription of pgRNA into HBV DNA and therefore have no direct impact on cccDNA. Hence, NAs have a finite effect on HBsAg loss,

which explains the viral recurrence after treatment withdrawal. Nevertheless, due to their ease of administration and favorable tolerability profile, NAs are often endorsed as first-line treatment (Chevaliez et al., 2013, Lin et al., 2013, Gill et al., 2015, WHO, 2015, Ning et al., 2019). Most patients in sub-Saharan Africa are treated with lamivudine and have experienced drug resistance (Kouanfack et al., 2012, Msomi et al., 2022). Replacing lamivudine with TDF in low and middle-income countries is limited by the high cost (Kouanfack et al., 2012, Hamers et al., 2013, Aoudjane et al., 2014, WHO, 2015, Lukhwareni et al., 2020). Currently, the clearance of HBsAg is based primarily on sequential or combined treatment with NA and Peg-IFN (Terrault et al., 2018).

A thorough evaluation of the HBV viral load, the HBV genotype, the HBeAg/HBeAb status, the stage of liver disease, and the presence or absence of HIV coinfection should be conducted when deciding to treat chronically infected patients (Ive et al., 2013, Terrault et al., 2018). ETV, TDF, and TAF are the most recommended monotherapy regimens according to the 2017 European Association for the Study of the Liver (EASL) guideline, and the extension of Peg-IFN therapy beyond week 48 may be beneficial in certain patients with HBeAg-negative CHB (EASL, 2017). For adults with immune-active CHB, Peg-IFN, ETV, or TDF are the preferred initial treatments recommended by the 2018 American Association for the Study of Liver Diseases (AASLD) guidelines. As part of monitoring for potential transition to immune-active or immune-inactive CHB, they also suggest that ALT levels should be checked at least every six months in adults with immune-tolerant CHB (Terrault et al., 2018). The National Guidelines for the Management of Viral Hepatitis in South Africa recommends lamivudine and TDF for both individuals mono-infected with HBV and those with HBV/HIV co-infection in all levels of care. For tertiary-level care, TAF, entecavir, and Pegylated Interferon are recommended (Spearman et al., 2013, Spearman et al., 2017, SA NDOH, 2019).

1.2.1.8 HBV diagnostic approaches and their limitations

Diagnostic markers currently available are categorized as viral-specific markers and liver-disease markers. The current diagnostic approaches for HBV markers include immunoassays to detect viral antigens and antibodies, and polymerase chain reaction (PCR)-based amplification assay for measuring viral DNA. HBsAg is the first serological marker to appear indicating active infection, its persistence for more than six months characterizes chronic infection (Coffin et al., 2019). Individuals positive for HBsAg do not necessarily have liver disease and a majority of inactive HBV carriers have a normal liver (Bonino et al., 2010, Pisano et al., 2021). Immunity to HBV infection after vaccination is characterized by the presence of anti-HBs only. Quantitative HBsAg levels

measurement has been proposed as an approach to distinguish inactive HBsAg carriers from individuals with active disease (Brunetto et al., 2010, WHO, 2015, Kim et al., 2020). HBeAg indicates viral replication and a higher risk of transmission but does not provide any diagnostic indication of liver disease (de Almeida Pondé, 2021). ALT levels normalize and HBV replication declines following spontaneous seroconversion, conferring a good prognosis and not requiring treatment (WHO, 2015, de Almeida Pondé, 2021). However, infection with pre-core mutants may be accompanied by active HBV replication with negative HBeAg and positive anti-HBe (WHO, 2015, Coffin et al., 2019).

Real-time polymerase chain reaction (RT-PCR) analyses of serum HBV DNA correlate with disease progression, enabling the distinction between active HBeAg-negative disease and chronic infection, as well as monitoring and treatment needs. (Hadziyannis and Laras, 2018, Laras et al., 2022). The current recommendation is to monitor HBV DNA levels over a few months or longer. However, there is no consensus regarding how low levels of DNA are indicative of "inactive" disease, or how high levels should be to initiate treatment (WHO, 2015, Terrault et al., 2018). A rise in HBV DNA concentration may indicate the emergence of resistant variants, which can also be monitored as a response to treatment (EASL, 2017, Terrault et al., 2018). In resource-limited settings, the availability of HBV DNA testing remains very poor (WHO, 2015, WHO, 2020).

In assessing liver severity, clinical features such as cirrhosis and evidence of decompensation are evaluated as well as liver enzyme levels. Fluctuations of aminotransferase levels may occur with time, and the disease stage is not indicated by single measurements of AST and ALT. AST concentrations are normally lower than ALT concentrations, but with cirrhosis, the AST/ALT ratio may be reversed (Kim et al., 2007, Güzelbulut et al., 2012, WHO, 2015). Decompensated cirrhosis is characterized by decreased serum albumin levels, increased bilirubin levels, and prolonged prothrombin times (Gümüşay et al., 2013, Castro-Narro et al., 2022). In resource-limited settings, an AST to Platelet Ratio Index (APRI) is a recommended measurement method for the evaluation of liver fibrosis due to its cost-effectiveness (WHO, 2015, Yue et al., 2019).

1.2.2 MiRNAs in HBV gene expression and replication

1.2.2.1 Basis of MiRNAs

MiRNAs are small single-stranded non-coding RNAs ~19-25 nucleotides (nt) in length They bind to the complementary strand of messenger RNA and suppress translation (and hence protein

production) at the post-transcriptional level (Filipowicz et al., 2005, Bartel, 2009). More than 2000 different miRNAs have been identified in the human genome, which collectively regulates the expression of over 30% of human genes (Plotnikova et al., 2019). Genes that encode miRNAs may occur within the untranslated regions of mRNA (Poliseno et al., 2010, Ramalingam et al., 2014, Plotnikova et al., 2019). MiRNAs have been shown to regulate diverse biological functions such as apoptosis, tumorigenesis, development, cellular differentiation, and targeting foreign pathogens (Farazi et al., 2011, Pedroza-Torres et al., 2019).

1.2.2.2 MiRNAs biogenesis and functions

The biogenesis of miRNAs occurs in both the nucleus and cytoplasm and involves several crucial steps. MiRNAs are transcribed from genes in the nucleus by RNA polymerase II into primary miRNAs (pri-miRNA), which have a stem-loop structure of ~80 nt (Lee et al., 2004, Quick-Cleveland et al., 2014, O'Brien et al., 2018). The processing of pri-miRNA starts with the double-stranded stem being recognized by the DiGeorge syndrome chromosomal region 8 (DGCR8) protein. The DGCR8 protein then associates with the enzyme DROSHA to form a complex to cleave the pri-miRNA to produce the precursor miRNA (pre-miRNA) of ~60-70 nt (Denli et al., 2004, Wu et al., 2012, Zhao et al., 2019). The pre-miRNA is then transported by a transporter protein exportin-5 into the cytoplasm. Once in the cytoplasm, the pre-miRNA is recognized by an RNase III enzyme called Dicer. The dicer together with the transactivation-response RNA-binding protein (TRBP) cleaves the hairpin loop structure to generate a short double-stranded miRNA molecule (Yi et al., 2003, Lund et al., 2004, O'Brien et al., 2018).

The miRNA molecule is ~22 nt in length comprising a mature strand (seed strand) and a complementary star strand. The Argonaute 2 protein (AGO2) interacts with the Dicer and binds the miRNA molecule, and the complementary star strand is released and degraded (Gregory et al., 2005, Treiber et al., 2019). Several proteins including AGO2, TRBP, and Dicer form the RNA-induced silencing complex (RISC) which facilitates the binding of the mature miRNA to the RISC (Cheloufi et al., 2010, Dai et al., 2019). The mature strand guides the RISC complex to the target messenger RNA to accomplish translational repression and mRNA degradation (He and Hannon, 2004, Dai et al., 2019). The mature miRNA binds to the 3'-UTR of the mRNA. When there is complete complementarity between the miRNA and the mRNA, mRNA is degraded by RISC. However, in the case of a partial base-pairing complementarity, which is usually 2-8 nt, mRNA translation into protein is inhibited therefore the gene is effectively silenced (Guo et al., 2010, Djuranovic et al., 2012, Fukaya et al., 2014, Dai et al., 2019, Treiber et al., 2019).

1.2.3 Cellular miRNA's association with HBV infection/ Role of miRNAs in HBV infection.

1.2.3.1 Direct interaction with HBV transcripts

Several miRNAs reportedly regulate HBV replication by interacting directly with HBV transcripts, thereby influencing the HBV lifecycle (Kitab et al., 2015, Tsukuda and Watashi, 2020, Ligat et al., 2021). The targets of miRNAs in HBV transcripts that affect HBV replication have been screened using two different approaches in previous studies. One approach utilizes bioinformatics analysis to screen miRNAs that can regulate HBV gene expression and then pairs the selected miRNAs with HBV sequences to determine if the binding sites for these miRNAs exist (Mishra et al., 2020, Sarfaraz et al., 2022, Loukachov et al., 2022a).

In an attempt to determine the influence of host-encoded miRNAs on HBV replication and gene expression, Zhang et al. (2010) used the loss of function approach; anti-sense oligonucleotides of 328 identified human miRNAs were transfected into HepG2 2.2.15 cells and then measured the expression level of HBsAg (Zhang et al., 2010). Among these miRNAs, miR-199a-3p and miR-210 were observed to proficiently decrease HBsAg expression and HBV replication without an effect on the HepG2 2.2.15 cell proliferation. They further used bioinformatics analysis to predict the viral targets of miR-199a-3p and miR-210 the algorithm analysis showed that miR-199a-3p and miR-210 have binding sites on the HBV genome, the HBsAg-encoded region, and the pre-S1 region, respectively. This suggests that these miRNAs may hinder the production of HBsAg and HBV virion by post-transcriptional gene silencing (Zhang et al., 2010). The miR-125a-5p was also demonstrated to affect the expression of HBsAg by binding to HBsAg mRNA, thereby inhibiting its translation (Potenza et al., 2011).

A highly liver-specific miRNA, miR-122 accounts for ~70% of miRNA content in the liver. In a study by Chen et al. (2011) an inhibitory effect of miR-122 on HBV replication and gene expression was demonstrated and in silico tools were used to predict the target for miR-122 within the pregenomic RNA and the overlapping region of the HBV pre-C mRNA. It was found that miR-122 binds to a highly conserved segment of HBV pgRNA resulting in a decrease in HBV core-associated DNA level (Chen et al., 2011b). These findings demonstrate the ability of cellular miRNAs to alter HBV gene expression by directly targeting HBV transcripts.

1.2.3.2 Interaction with the regulators of HBV infection

HBV transcripts are controlled by two enhancers and four promoters and various transcriptional factors interact with these elements to regulate HBV transcription (Quasdorff and Protzer, 2010, Turton et al., 2020). Several miRNAs target HBV-associated genes including transcription regulators. Epigenetic mechanisms such as histone modifications, DNA methylation, and acetylation tightly regulate the transcription of HBV cccDNA (Liu et al., 2011, Dandri, 2020). The p53 protein acts as a suppressor for HBV replication by binding to HBV enhancer elements. This inhibitory effect is blocked by miR-122, which inhibits p53 from interacting with cyclin G1 thereby blocking its specific binding to the HBV enhancer elements (Ori et al., 1998, Wang et al., 2012, Lim et al., 2022).

1.2.3.3 MiRNAs and HBV-related Hepatocellular carcinoma

Chronic HBV infection is a major risk factor for hepatocellular carcinoma. MiRNAs in HCC can function as tumor suppressor genes or oncogenes depending on the cellular function of their targets (Negrini et al., 2007, Di Leva et al., 2014, Sarvepalli et al., 2022, Khan et al., 2022). There is accumulating evidence suggesting that HBV changes the host gene expression by downregulating or upregulating selected miRNAs thereby promoting the development of HCC (Wu et al., 2011, Sartorius et al., 2019, Sarvepalli et al., 2022). Several recent studies focusing on miRNA profiling in HBV-related HCC have identified deregulated miRNAs that are significant for the development of HCC (Pratedrat et al., 2020, Ghosh et al., 2020, Huang et al., 2021, Zhang et al., 2022). Gao et al. (2011) studied the expression levels of seven cancer-related miRNAs in HCC patients known to be deregulated in human cancers. The downregulation of miR-145 and miR-199a and the up-regulation of miR-244 were frequently observed. Furthermore, these changes persisted throughout HCC development (Gao et al., 2011).

1.2.3.4 Correlation of miRNA levels with virological and clinical parameters

Several studies have investigated the correlation between miRNAs and clinical features of chronic HBV infection. For example, a negative correlation was found between miR-29 and liver necroinflammation and fibrosis in patients with chronic HBV infection (Huang et al., 2014). Van der Ree et al. (2017) conducted a study to identify miRNAs that are associated with the HBeAg status and response to treatment in patients with chronic HBV infection. Expression levels of miR-122-5p, miR-125b-5p, miR-192-5p, miR-193b-3p, and miR-194-5p were observed to be lower in HBeAg-negative patients compared to HBeAg-positive patients and their levels were correlated with low levels of HBV DNA and HBsAg (van der Ree et al., 2017).

Another study found a correlation between serum levels of miR-181b and HBeAg-seropositivity and HBV DNA levels (Yu et al., 2015a). A study that was conducted to investigate the correlation between miR-125b, viral replication, and liver necroinflammation in adults with chronic HBV infection, found that miR-125b correlated with an enhanced viral replication capacity *in vitro*. They also observed that serum miRNA-125b level alone, or in combination with miRNA-124, may be able to differentiate the severity of liver necroinflammation in Chinese CHB patients with normal or mildly elevated ALT levels (Li et al., 2016).

1.2.3.5 Cellular miRNAs as a therapeutic target

Cellular miRNA's can be considered therapeutic targets because their potential to affect biological function lies in their ability to suppress gene expression when levels are elevated in a disease state or to upregulate when levels are reduced. Hence, both loss and gain of function techniques have been used to fully explore their therapeutic potential (Peek and Behlke, 2007, Krützfeldt, 2016, Andrew et al., 2018). Establishing the relationship between the virus genome and the miRNAs in the blood circulation of hepatic patients can be used to develop methods to be used in eradicating cccDNA, which is a major challenge in the treatment of HBV patients (Yu et al., 2015b, Loukachov et al., 2022b).

1.2.4 MiRNAs as a biomarker in the management of HBV

1.2.4.1 HBV disease progression

MiRNAs have the potential to be used as non-invasive biomarkers for HBV due to being stable in serum or plasma (Friedman et al., 2009, Chen et al., 2013, Wang et al., 2019). Several studies investigating the role of miRNAs as potential biomarkers have been conducted. Huang et al. (2014) investigated the role of serum miR-29 as a biomarker in predicting chronic HBV disease progression. It was found that miR-29 levels were negatively correlated with necroinflammation grades and liver fibrotic stages in patients with chronic HBV infection (Huang et al., 2014). In another study, miRNA-125b was positively correlated with HBV DNA level in the serum and an increase in the HBV replication capacity was observed after transfection with miRNA-125b mimics. It was concluded that miRNA-125b has the potential to distinguish grades of liver necroinflammation (Li et al., 2016), more studies are needed to validate or argue this statement. A study by Yousefpouran et al. (2020) found a positive correlation between the expression levels of miR-122 and the ALT levels. In a study investigating the association of miRNA expression levels

with HBeAg status in patients infected with chronic HBV, higher miRNA expression levels of miR-122-5p, miR-125b-5p, miR-192-5p, miR-193b-3p, and miR-194-5p were observed in HBeAg-positive patients compared to HBeAg-negative patients, and the correlation between the levels of these miRNAs and HBsAg levels, and HBV DNA was established (van der Ree et al., 2017). Another study showed higher expression levels of miR-181b in HBeAg- positive patients compared to HBeAg-negative patients (Yu et al., 2015a).

1.2.4.2 MiRNAs in HBV treatment response

Nucleos(t)ide analogs and pegylated-interferon (peg-IFN) are the current treatment options for chronic HBV. Treatment with nucleos(t)ide analogs can effectively inhibit viral replication but rarely leads to a functional cure while in patients treated with the peg-IFN, a functional cure is achieved in only 3-7% of the patients (Cornberg et al., 2020, Zeng et al., 2020, Yang et al., 2022). It is therefore significant that chronic HBV patients who will benefit from the peg-IFN treatment are identified before commencing therapy. In a study aimed at finding novel markers associated with the prognosis of interferon (IFN) therapy, Zhang et al. (2012) investigated the potential of pre-treatment plasma miRNA profile in predicting early virological response to IFN treatment. They found 11 miRNA profiles that might improve the overall sustained response to IFN treatment and provide an accurate method in assisting with medication decisions (Zhang et al., 2012). In another study, 9 miRNA profiles were demonstrated to be able to differentiate between patients that were responding to treatment and the patients that were not responding to treatment (Tan et al., 2015).

1.2.4.3 MiRNAs as biomarkers for hepatocellular carcinoma

There is a need for more sensitive and specific non-invasive biomarkers for early diagnosis of HCC as the sensitivity of alpha-fetoprotein (AFP) is poor at relatively low cut-offs (e.g, 20 ng/mL, sensitivity 64.3%), and is even more limited in smaller tumors (25% sensitivity at tumor size <3 cm) (EASL-EORTC, 2012, Marrero et al., 2018). MiRNAs have been shown to have potential as biomarkers in the screening and early detection of HBV-HCC as well as in predicting HCC prognosis (Xu et al., 2020). Li et al. (2010) identified five miRNA signatures (miR-23a, miR-23b, miR-375, and miR-342-3p) that could differentiate HBV-positive HCC individuals from the healthy control group. In another study, serum miRNAs (miR-10b, miR-106b, and miR-181a) were shown to differentiate between HCC patients and healthy controls (Jiang et al., 2015). In another study, a seven serum miRNA classifier (miR-505, miR-192, miR-145, miR-143, miR-133a, miR-29a, and miR-29c) was shown to have a significantly higher sensitivity than AFP in distinguishing HCC

patients from healthy controls, chronic HBV patients, inactive HBsAg carriers, and HBV cirrhosis patients (Lin et al., 2015).

1.2.4.4 MiRNAs as biomarkers for HBV-HIV coinfection or HIV infection

HIV may exploit cellular miRNAs to regulate its replication by directly targeting host mRNAs that negatively affect HIV replication (Huang et al., 2018, Sadri Nahand et al., 2020). Additionally, miRNAs are associated with a possible predisposition to HIV infection in macrophages and monocytes (Wang et al., 2009, Huang et al., 2018, Balasubramaniam et al., 2018). In one study, the expression levels of miRNAs (miR-29a, miR-122, and miR-181b) differed between patient groupings (HIV-1, HBV, HCV, HBV-HIV, HCV-HIV infection) and healthy controls (Yousefpouran et al., 2020). In another study, HIV viral load and the degree of immunosuppression were negatively correlated with miR-29a levels in young people with chronic HIV-1 infection (Rosca et al., 2016). In a recent study, miR-122 has significantly upregulated in HBV-HIV coinfected patients compared to HBV mono-infected patients, HIV mono-infected patients, and healthy controls. A significant negative correlation was also found between miR-122 expression levels and HIV viral load in HIV mono-infected patients (Moochani et al., 2022).

1.2.5 Conclusions

MiRNAs have been reported to have the potential of being utilized in clinical settings as biomarkers for disease progression and decision-making in the treatment of HBV patients. But there is a paucity of miRNA studies in South Africa and the African continent therefore local studies of miRNAs are necessary, given geographical differences in genotypes of HBV. This research project aims to fill that gap and contribute to a better understanding of the role of miRNAs in chronic HBV patients in a South African high prevalence setting. This study will report and add to the body of knowledge on miRNA profiles on HBV genotype A1.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Study design

This retrospective study is aimed at describing the pattern of miRNA expression in patients infected with chronic HBV at different stages of disease severity, as indicated by plasma HBeAg, HBV viral load, and liver function tests. The miRNA profile was compared in samples stratified according to serological markers, viral load, and ALT. The 10 candidate miRNAs included hsa-miR-15b-5p, hsa-miR-20a-5p, hsa-miR-29a-3p, hsa-miR-122-5p, hsa-miR-125b-5p, hsa-miR-181b-5p, hsa-miR-192-5p, hsa-miR-193b-3p, hsa-miR-194-5p, and U6 snRNA which had been identified as being potentially specific for Hepatitis B viral infection (http://miRTarBase.cuhk.edu.cn/).

2.1.2 Study samples

The study cohort consisted of 50 chronic HBV-infected samples stored at the Department of Virology, Inkosi Albert Luthuli Central Hospital (IALCH), Durban, from a previous study "Hepatitis B virus variants in HBV mono-infected and HIV/HBV co-infected samples in a high dual infection setting" (BREC REF No.: BE 324/16) (Appendix A). All samples were hepatitis B surface antigen (HBsAg)-positive for at least 6 months. HBV viral load, ALT, HBeAg, and HIV viral load results were available for these samples in the database from the previous study and were downloaded anonymously together with demographic (age/gender) and other relevant clinical data (e.g., antiretroviral treatment, co-infections, co-morbidities, history of liver disease). This study cohort included 29 males (18-52 years) and 21 females (23-61 years). The study cohort was grouped according to HBeAg status (positive or negative), and ALT levels (≤ 35 or > 35); normal ALT levels for males are ≤35 U/L and ≤25 U/L for females therefore ALT ≤ 35 U/L was taken as an inclusive cut-off value for low levels, HBV viral load (≤ 1000 or > 1000); patients with HBV viral load of 1000 IU/ml identify as inactive carriers which are accompanied by normal or low ALT levels, and HIV viral load (≤ 1000 or > 1000). The study groups were also stratified according to age and gender, the cut-off for age was determined according to the measure of central tendency used which was the median per study group. Twenty-three subjects, negative for the total HBV core antibody, HBsAg and HIV were used as the healthy control group. The control cohort included 7 males (18-47 years) and 16 females (13-62 years).

2.1.3 Ethical Considerations

This study was approved by UKZN Biomedical Research Ethics Committee (BREC 00002418/2021) (Appendix B). The study samples were from a previous study conducted at the Department of Virology, UKZN, and ethics were also approved by Biomedical Research Ethics Committee (BREC REF No.: BE 324/16). Bio-demographic and serology results data were recorded, and any personal identifiers were removed from the database to maintain patient confidentiality.

2.2 Methods

2.2.1 Sample preparation

Plasma samples stored at –80 °C were thawed and mixed using a vortex mixer, in preparation for RNA extraction.

2.2.2 RNA extraction and quantification

RNA was extracted from 500 µl plasma using the NucliSens easyMAG system (Biomeriux, Marcy I'Etoile, France) according to the manufacturer's protocol. Briefly, samples were vortexed and 500 µl of the sample was added to 1 ml of lysis buffer for offboard lysis. The mixture was vortexed for 10 seconds and incubated at room temperature for 20 minutes. The mixture was transferred into the disposable NucliSens easyMAG consumables for the instrument and 50 µl of silica was added and mixed by pipetting up and down using a BioHit multichannel pipette set at P3. The NucliSens easyMAG disposables were loaded into the instrument and the barcodes were scanned to start the program. RNA was eluted with 25 µl of NucliSens buffer 3. The concentration of RNA was measured at an absorbance of 260 nm in a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, United States) using nuclease-free water as a blank. RNA samples were stored at -80 °C.

2.2.3 Reverse transcription (RT) or complementary DNA (cDNA) synthesis

A panel of ten HBV-associated miRNAs (hsa-miR-15b-5p, hsa-miR-20a-5p, hsa-miR-29a-3p, hsa-miR-122-5p, hsa-miR-125b-5p, hsa-miR-181b-5p, hsa-miR-192-5p, hsa-miR-193b-3p, hsa-miR-194-5p, U6 snRNA) were chosen based on their reported relevance to HBV infection (Appendix C Table 1). The TaqMan MicroRNA reverse transcription kit (Applied Biosystems, Vilnius, Lithuania) and custom RT primer pool catalogue number 4427975 (Applied Biosystems,

Pleasanton, United States) were used to reverse transcribe the total RNA to cDNA according to the manufacturer's protocol. The custom primer pool was prepared by combining 10 µl of each 5X RT stem-loop specific miRNA primer in 1.5 ml microcentrifuge, and 1X Tris-EDTA (TE) buffer was added to bring the final volume to 1000 µl. The RT reactions were performed in 8-strip tubes with 4 µl of RNA sample, 8 µl RT primer pool (containing 0.05X of each stem-loop miRNA-specific RT primer), 2 µl of 10X RT buffer, 0.4 µl of 2 mM of dNTPs with dTTP, 4 µl of 10 U/µl MultiScribe Reverse Transcriptase, 0.25 µl of 0.25 U/µl RNase inhibitor and 1.35 µl nuclease-free water. The stem-loop miRNA-specific RT primers catalogue number 4427975 were designed by Thermo Fisher Scientific, Pleasanton, United States. The reaction tubes were sealed, inverted to mix, and centrifuged for 10 seconds then incubated on ice for 5 minutes. The reaction tubes were placed on the ProFlex 96-well PCR System (Applied Biosystems, Foster City, United States) thermal cycler, and the mixture was incubated for 30 minutes at 16 °C and 30 minutes at 42 °C, followed by 5 minutes of incubation at 85 °C for reverse transcriptase enzyme inactivation and infinity (∞) hold at 4 °C. The RT products were used immediately for the cDNA preamplification step or stored at -20°C for up to one week.

2.2.4 cDNA Preamplification

To ensure that there would be sufficient cDNA product to amplify, a preamplification step was added. The custom preamplification primer pool was prepared by combining 10 µl of each 20X TaqMan MicroRNA assay (Applied Biosystems, Pleasanton, United States) in a 1.5 ml microcentrifuge. 1X TE buffer was added to bring the final volume up to 1000 µl. The 25 µl preamplification reactions were performed in 8-strip tubes with 2.5 µl of RT product, 12.50 µl of 2X TaqMan PreAmp master mix (Applied Biosystems, Vilnius, Lithuania), 3.75 µl of preamplification primer pool and 6.25 µl nuclease-free water. The reaction tubes were sealed, inverted to mix, and briefly centrifuged to collect contents to the bottom. The reaction tubes were placed on the ProFlex 96-well PCR System (Applied Biosystems, Foster City, United State) thermal cycler under the following cycling parameters, a cycle of enzyme activation at 95 °C for 10 minutes, an annealing cycle at 55 °C for 2 minutes, an extension cycle at 72 °C for 2 minutes, followed by 12 cycles of denaturation at 95 °C for 15 seconds and annealing/extension at 60 °C for 4 minutes, a cycle of enzyme inactivation step at 99.9 °C for 10 minutes and ∞ hold at 4 °C. The reaction tubes were removed from the thermal cycler, briefly centrifuged and 175 µl of 0.1X TE (pH 8.0) was added to each reaction tube to dilute the preamplification reaction product. The reaction tubes were

sealed, inverted to mix, and briefly centrifuged. The products were stored at -20°C for use in real-time quantitative PCR for up to one week.

2.2.5 Real-time quantitative PCR

The preamplification products were subjected to miRNA expression assay using real-time quantitative PCR. A PCR reaction master mix was prepared for each miRNA assay containing 1 µl of 20X TaqMan MicroRNA Assay (Applied Biosystems, Pleasanton, United States)**,** 10 µl of 2X TaqMan Universal Master mix II, No AmpErase UNG (Applied Biosystems, Vilnius, Lithuania) and 8.8 µl of nuclease-free water. The 20X TaqMan MicroRNA Assay contained specific reverse and forward primers and a TaqMan probe dye-labeled (FAM) catalogue number 4427975 (Applied Biosystems, Pleasanton, United States). The mixture was vortexed and centrifuged briefly to mix and collect contents properly. The 19 µl of the PCR reaction master mix was transferred to each well of the optical 96-well reaction plate, and 1 µl of the preamplified product was added. The plate was sealed with MicroAmp Optical Adhesive Film, vortexed briefly and centrifuged for 30 seconds to collect the contents to the bottom of the wells. The plate was placed on a QuantStudio 7 Flex Real-time PCR system (Applied Biosystem, Foster City, United States) for amplification. The cycling parameters used included a cycle of enzyme activation at 95 °C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, and annealing/extension at 60 °C for 1 minute. The U6 snRNA TaqMan MicroRNA assay (Applied Biosystems, Pleasanton, United States) was used as an endogenous control for normalization in miRNA expression analysis.

2.2.6 Data analyses and statistical analyses

The threshold cycle (Ct) value was defined as the number of cycles required for the fluorescent signal to cross the threshold in quantitative PCR. Each target was quantified in duplicates per sample. The amplification plots of miRNAs were analyzed using Design and Analysis software version 2.6 (Applied Biosystems, Foster City, United States) for analysis of the Ct values. The cut-off Ct value for miRNAs expression was 35, Ct values ≥35 were regarded as undetectable and were substituted with a Ct value of 35 for further analysis (Chen et al., 2011a). The expression of miRNA relative to small RNA U6 was reported as delta Ct (ΔCt), which was calculated by subtracting the average Ct of U6 RNA from the average Ct of the target miRNA. To interpret the results, the relative change in expression was assessed using a comparative Ct method. Relative miRNA expression levels were presented as equal to $2^{-\Delta\Delta Ct}$ and delta-delta Ct (ΔΔCt) was calculated by subtracting ΔCt average for the control group from ΔCt of the target miRNA (Livak and Schmittgen, 2001). The

study cohort was grouped and analysed according to age (≤37 years or >37 years for CHBV samples and ≤31 years or >31 years for healthy controls), gender (male or female), HBeAg status (positive or negative), ALT levels (≤ 35 U/L or > 35 U/L), HBV viral load (≤ 3 $\log_{10}$ IU/ml or > 3 $\log_{10}$ IU/ml); and HIV viral load (≤ 3 $\log_{10}$ IU/ml or > 3 $\log_{10}$ IU/ml). Expression levels of miRNAs in different groups were compared using an unpaired Mann-Whitney U test, and a Spearman's correlation coefficient (r) analysis was used to evaluate the association of clinical parameters with miRNAs. Possible confounding variables were tested using linear regression model. The false discovery rate was corrected by using the Benjamini–Hochberg FDR method (Benjamini and Hochberg, 1995). All statistical analyses were performed using GraphPad prism version 7.0 (GraphPad Software, San Diego, United States) and IBM SPSS Statistics version 28.0 (IBM SPSS, New York, United States). A two-tailed *p* value of <0.05 was regarded as statistically significant.

CHAPTER 3: RESULTS

3.1 Results

3.1.1 Sample characteristics

The study analyzed samples from 50 patients with chronic HBV and 23 controls without serological evidence of HBV. Characteristics of female chronic HBV samples (42%), healthy female controls (69.6%), male chronic HBV samples (58%), and healthy male control samples (30.4%) are summarised in Table 3.1. A significant difference was observed between chronic HBV samples and healthy controls in terms of age and gender (Table 3.1). Chronic HBV samples had genotype A1 chronic hepatitis B (Msomi et al., 2022). Among the 10 miRNA panels chosen, the U6 snRNA miRNA was used as an endogenous control for the normalization of the nine targets in the panel, it was chosen as one of the recommended endogenous controls for miRNA profiling by the testing kits manufacturers as well as support by other studies (Liu et al., 2014, Li et al., 2015). Amplification plots were analyzed using the Design and Analysis software (Appendix D Figure 1). There was no significant difference in miRNA expressions between age groups and gender groups in chronic HBV and healthy control samples (Appendix E Table 1). The levels of expression for the miRNA targets were assessed in five subgroupings, chronic HBV vs healthy control samples, High vs low HBV viral load, HBeAg status (positive and negative), ALT levels, and high vs low HIV viral load.

Table 3.1: Characteristics of Chronic Hepatitis B samples and healthy controls

	CHBV samples (n=50)	**Healthy controls (n=23)**	**p-value**
Age (years), median (IQR)	36.5 (12.25)	31 (21)	0.04
Gender, n (%)			
Male	29 (58 %)	7 (30.4 %)	0.04
Female	21 (42 %)	16 (69.6 %)	
ALT (U/L), median (IQR)	35 (18)	Not Tested	
HBV DNA (log_{10} IU/ml), median (IQR)	2.19 (2.71)		
HBeAg Status, n (%)			
Positive	37 (74 %)		
Negative	13 (26 %)		
HIV viral load (log_{10} IU/ml), median (IQR)	2.42 (3.38)		
CD4+ count (cells/mm^3), median (IQR)	171 (255)		

HBV active ART, n (%)	
TDF+LAM	48 (96)
TDF only	1 (2)
LAM only	1 (2)

ALT; Alanine aminotransferase, CHBV; chronic Hepatitis B virus, ART; antiretroviral treatment, TDF; tenofovir disoproxil fumarate, LAM; lamivudine

3.1.2 Expression levels of miRNA panel in chronic HBV samples vs healthy controls

Compared to healthy control groups, patients with chronic HBV infection showed significantly higher expression levels of all miRNAs. The greatest difference in miRNA expression was found with hsa-miR-122-5p (median [IQR]; 3.42 [1.64]) compared to healthy controls (median [IQR]; -0.05 [0.53]; $p<0.0001$), followed by hsa-miR-29a-3p levels (median [IQR]; 2.51 [1.06]; $p<0,0001$), hsa-miR-192-5p (median [IQR]; 2.44 [1.64]; $p<0,0001$) and hsa-miR-125b-5p (median [IQR]; 2.41 [0.88]; $p<0,0001$). Hsa-miR-193b-3p had the smallest difference in expression levels in chronic HBV (median [IQR]; 1.14 [1.46]) vs healthy controls (median [IQR]; 0.18 [0.72]; $p<0,0001$) (Figure 3.1: A-I and Appendix F Table 1).The linear regression analysis revealed that age and gender did not influence the miRNA expression level differences but the differences were due to HBsAg positivity (Appendic G Table 1).

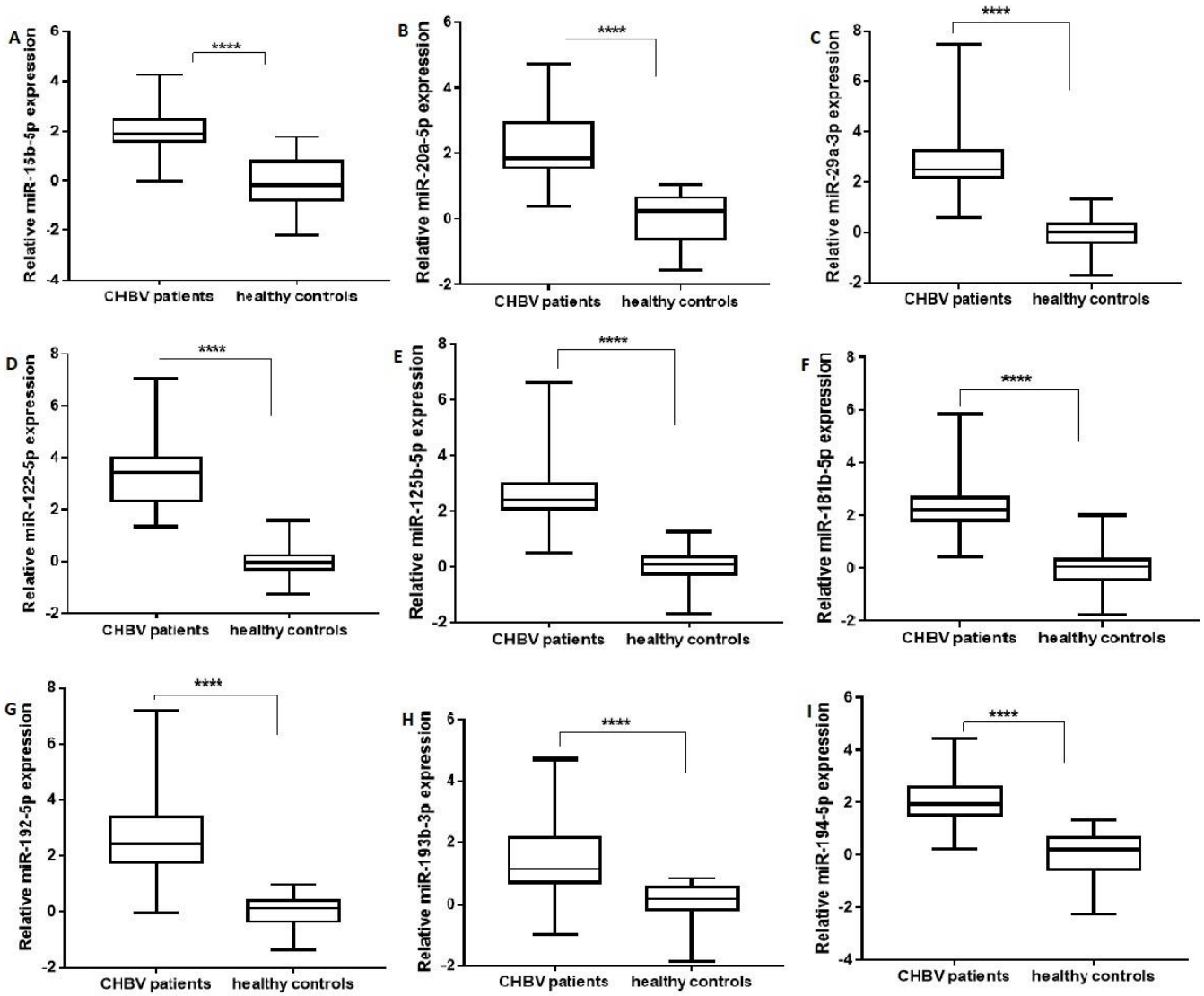


Figure 3.1: MiRNA panel expression levels in chronic HBV samples vs healthy controls. The box and whisker diagrams show significantly higher expression levels of miRNAs in chronic HBV samples in relation to healthy controls, (A) plasma hsa-miR-15b-5p, (B) plasma hsa-miR-20a-5p, (C) plasma hsa-miR-29a-3p, (D) plasma hsa-miR-122-5p, (E) plasma hsa-miR-125b-5p, (F) plasma hsa-miR-181b-5p, (G) plasma hsa-miR-192-5p, (H) plasma hsa-miR-193b-3p, (I) plasma hsa-miR-194-5p. Relative miRNA level is shown as 2 ^ – (ΔCt of target miRNA– arithmetic mean of ΔCt for the control group). Statistical comparisons were performed using an unpaired Mann-Whitney U test. Significant differences are shown by an (*) system (**** $P < 0.0001$).

3.1.3 Different miRNA expression signatures in chronic HBV samples with high vs low HBV DNA

Low HBV viral load (<3 log_{10}IU/ml) is whereby partial treatment response is generally considered and high HBV viral load (>3 log_{10}IU/ml) is whereby a virological breakthrough is defined (Zoulim and Locarnini, 2009, Orrell et al., 2011, Yu et al., 2015a, Terrault et al., 2018). Samples with high HBV viral load had significantly higher expression levels of hsa-miR-122-5p (median [IQR], 3.74 [1.18]; p = 0.0001), hsa-miR-192-5p (median [IQR], 3.07 [1.72]; p = 0.0003) and hsa-miR-193b-3p (median [IQR], 2.14 [1.97]; p = 0.0002) (Figure 3.2 D, G, H) compared to samples with low HBV viral load. The expression levels of hsa-miR-20a-5p, hsa-miR-29a-5p, and hsa-miR-194-5p were slightly higher in samples with high HBV viral load compared to samples with low HBV viral load, but the difference was not significant (Figure 3.2 B, C, I). There was no significant difference in expression levels of miRNAs, hsa-miR-15b-5p, hsa-miR-125b-5p, and hsa-miR-181b-5p between high HBV and low HBV viral load samples (Figure 3.2 A, E, F). In samples with high HBV viral load hsa-miR-15b-5p had the lowest expression level (median [IQR]; 1.97 [0.94]; p = 0.75) followed by hsa-miR-194-5p (median [IQR]; 2.01 [1.75]; p = 0.08), while hsa-miR-122-5p (median [IQR], 3.74 [1.18]; p = 0.0001) had the highest expression level (Appendix F Table 1).

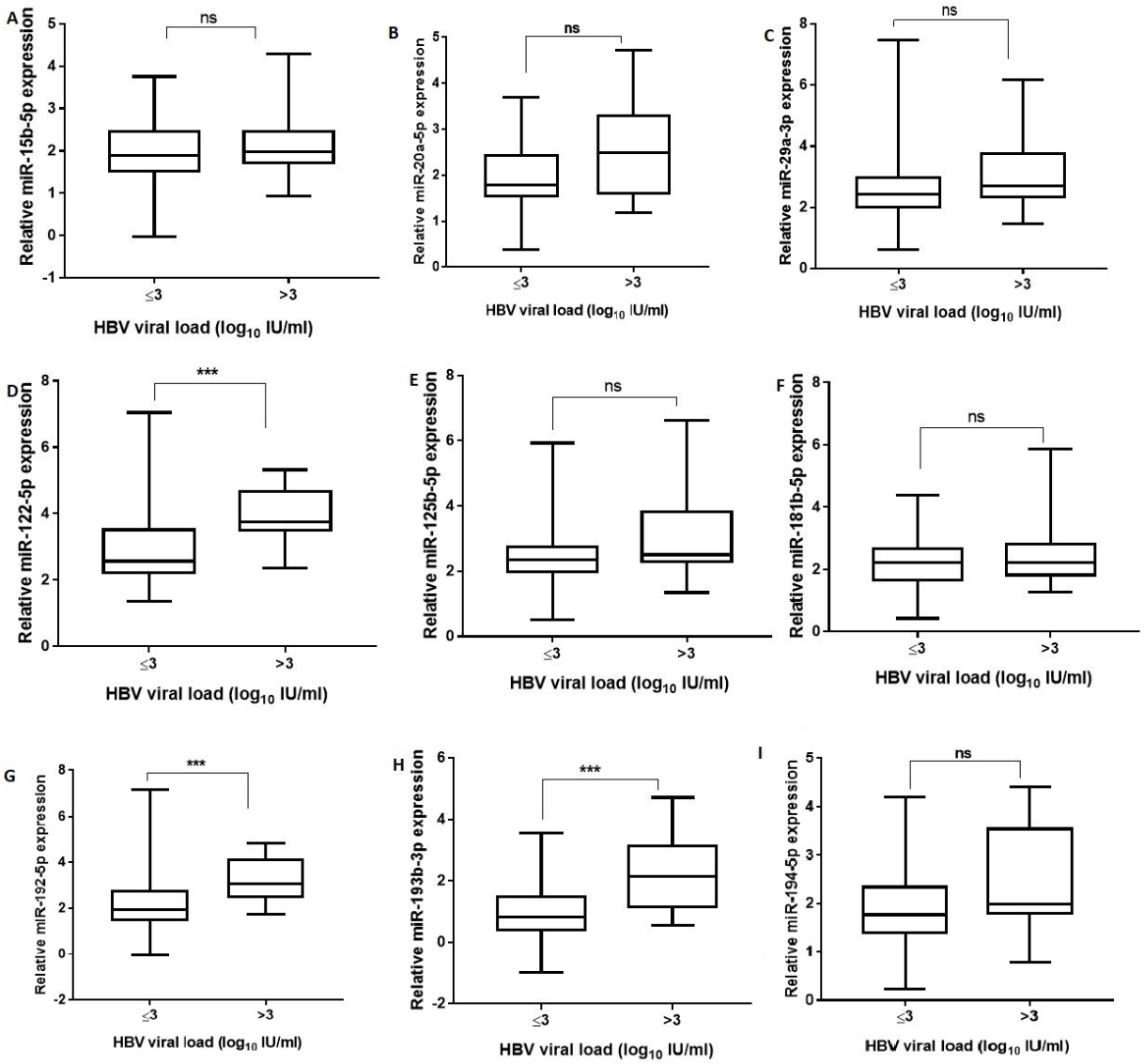


Figure 3.2: Different miRNA **expression levels in chronic HBV samples with high and low HBV viral load.** The box and whisker diagrams show, (**A**) no significant difference in the expression of hsa-miR-15b-5p. (**B**) hsa-miR-20a-5p was significantly expressed in samples with high HBV viral load, (**C**) no significant difference in expression of hsa-miR-29a-3p between samples with high vs low HBV viral load, (**D)** Samples with high HBV viral load showed significantly higher hsa-miR-122-5p expression levels compared to low HBV viral load samples. (**E**) Both groups showed similar hsa-miR-125b-5p expression levels, (**F**) hsa-miR-181b-5p expression levels were similar in both groups, (**G**) hsa-miR-192-5p expression levels were significantly higher in samples with high HBV viral load compared to low viral load. (**H**) A significantly higher hsa-miR-193b-3p expression level was observed in samples with high HBV viral load compared to low viral load, (**I**) no significant difference was observed for hsa-miR-194-5p expression level. Relative miRNA level is shown as 2 ^ – (ΔCt of target miRNA– arithmetic mean of ΔCt for the control group). Statistical comparisons were performed using an unpaired Mann-Whitney U test. Significant differences are shown by an (*) system (*** P<0.001, ns P>0.05).

3.1.4 Expression levels of miRNAs associated with HBeAg status

Plasma miRNA levels were compared between chronic HBV samples who are positive and negative for the HBeAg. HBeAg-negative samples had significantly higher levels of hsa-miR-15b-5p (median [IQR]; 2.45 [1]) compared to HBeAg-positive samples (median [IQR]; 1.76 [0.69]; p = 0.0054) (Figure 3.3 A). Significantly high expression levels of hsa-miR-181b-5p were observed in HBeAg negative samples (median [IQR]; 2.58 [1.06]) compared to HBeAg positive samples (median [IQR]; 2.22 [0.85]; p = 0.03) (Figure 3.3 F). Slightly higher expression levels were observed for hsa-miR-20a-5p, hsa-miR-29a-3p, hsa-miR-125b-5p, hsa-miR-192-5p, hsa-miR-193b-3p and hsa-miR-194-5p in HBeAg negative samples even though the differences were not significant compared to HBeAg positive samples (Figure 3.3 B, C, D, E, F, G, H, I). HBeAg positive samples had higher expression levels of hsa-miR-122-5p (median [IQR]; 3.43 [1.49]) compared to HBeAg negative samples (median [IQR]; 2.56 [2.22]; p = 0.88) but the difference was not significant (Appendix F Table 1).

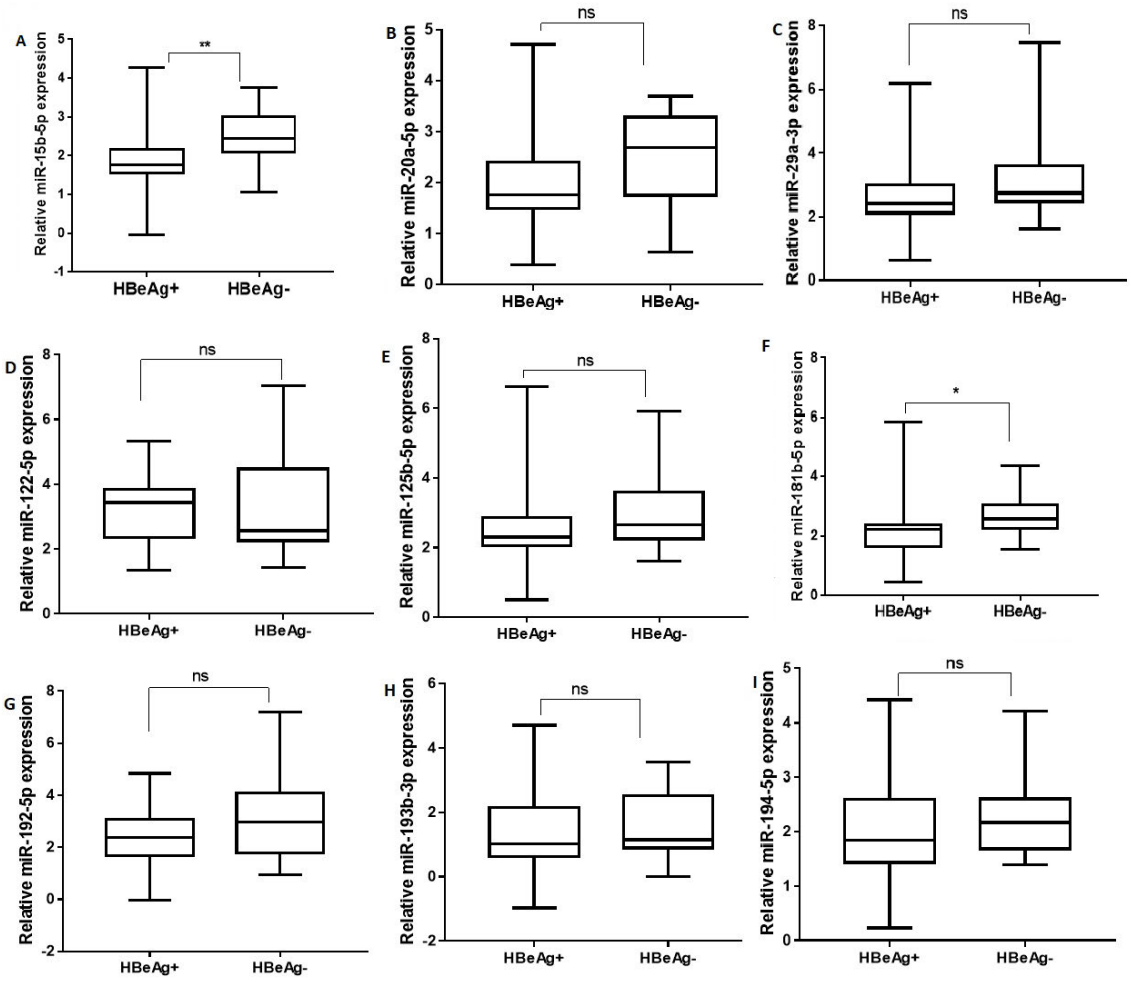


Figure 3.3: Plasma miRNA **levels in chronic HBV samples from HBeAg (+) and HBeAg (-) groups.** Box and whisker diagrams showing **(A**) HBeAg negative samples show significantly higher hsa-miR-15b-5p expression levels compared to HBeAg positive samples, (**B**) no significant difference in expression of hsa-miR-20a-5p was observed, (**C**) hsa-miR-29a-3p expression levels were not significantly different between HBeAg negative and positive samples, (**D**) Expression levels of hsa-miR-122-5p were not significantly different between HBeAg negative and positive samples, (**E**) HBeAg negative samples showed no significant difference hsa-miR-125b-5p expression levels compared HBeAg positive samples, (**F**) hsa-miR-181b-5p expression levels was significantly higher in HBeAg negative samples compared to HBeAg positive samples. (**G**) Expression levels of hsa-miR-192-5p were not significantly different between HBeAg negative and positive samples. (**H**) No significant difference in hsa-miR-193b-3p expression levels was observed, (**I**) no significant difference in hsa-miR-194-5p expression levels was observed in HBeAg negative and positive samples. Relative miRNA level is shown as 2 ^ – (ΔCt of target miRNA–

arithmetic mean of ΔCt for the control group). Statistical comparisons were performed using an unpaired Mann-Whitney U test. Significant differences are shown by an (*) system (** P<0.01, * P < 0.05, ns P>0.05).

*3.1.5 MiRNA **profiles in samples with varying ALT levels***

MiRNA expression profiles were compared between chronic HBV samples with lower and higher ALT levels, normal ALT levels for males are ≤35 U/L and ≤25 U/L for females (SA NDOH, 2019, Li et al., 2021). No significant differences were observed in the expression levels of all the studied miRNAs between ≤35 U/l vs >35 U/l ALT levels (Figure 3.4 A-I and Appendix F Table 1).

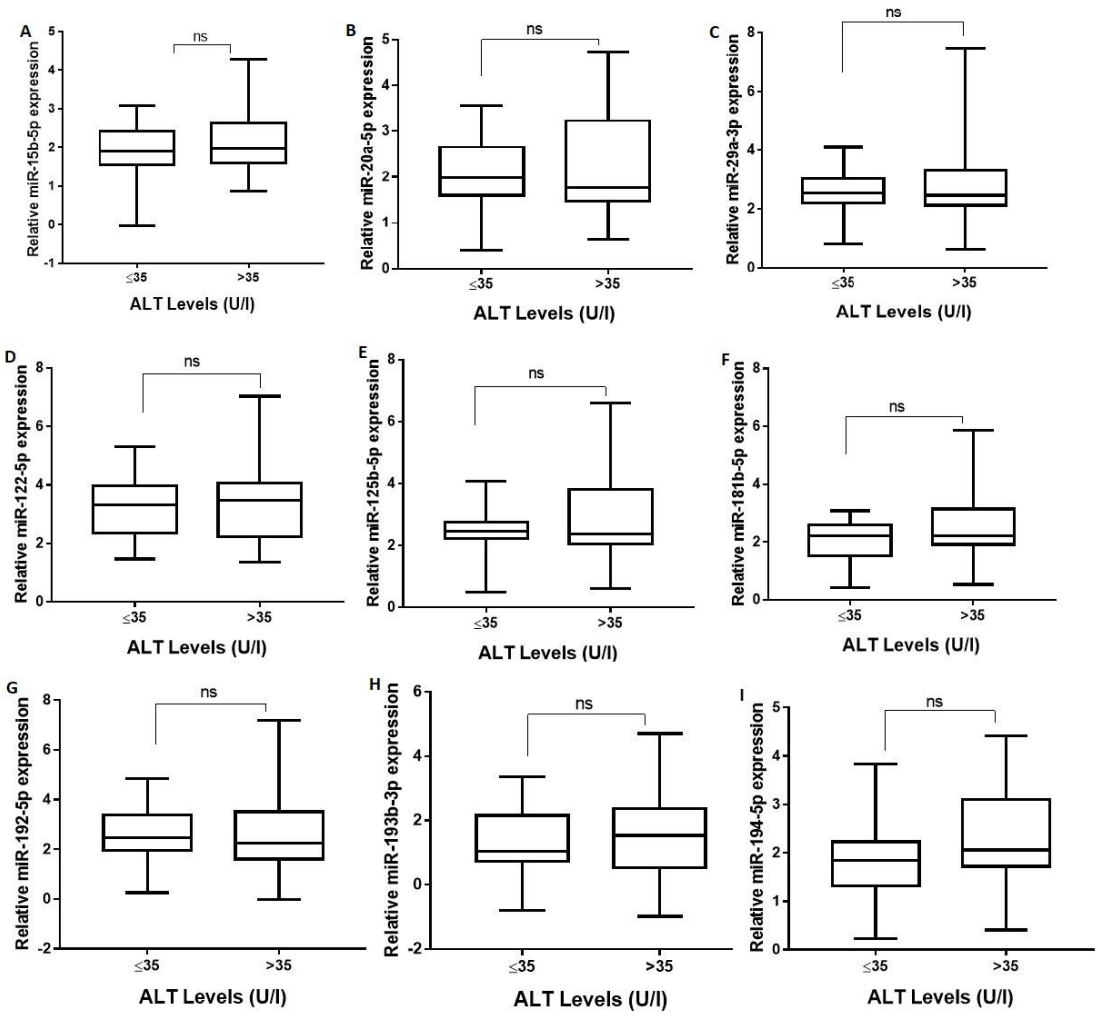


Figure 3.4: MiRNA **expression levels comparison in different groupings of ALT levels.** Box and whisker diagrams showing (**A**) no significant difference in hsa-miR-15b-5p expression levels, (**B**) no significant difference in hsa-miR-20a-5p expression levels were observed in both groups,

(**C**) hsa-miR-29a-3p expression levels were not significantly different between the compared groups, (**D**) hsa-miR-122-5p expression levels were not significantly different between the compared groups. (**E**) No significant difference was observed in hsa-miR-125b-5p expression levels between the compared ALT levels, (**F**) hsa-miR-181b-5p expression levels were not significantly different between the compared ALT levels, (**G**) hsa-miR-192-5p expression levels showed no significant difference, (**H**) no significant difference was observed in hsa-miR-193b-3p expression levels in both groups, (**I**) no significant difference in hsa-miR-194-5p expression levels was observed. Relative miRNA level is shown as 2 ^ – (ΔCt of target miRNA– arithmetic mean of ΔCt for the control group). Statistical comparisons were performed using an unpaired Mann-Whitney U test. Significant differences ($p < 0.05$) are shown by an (*).

3.1.6 Comparison of miRNA expression in samples with high versus low HIV viral load

Samples with high HIV viral load (>3 $\log_{10}$ IU/ml) and samples with low HIV viral load (≤3 $\log_{10}$ IU/ml) showed no significant differences in miRNA expression levels for all the studied miRNAs (Figure A-I and Appendix F Table 1).

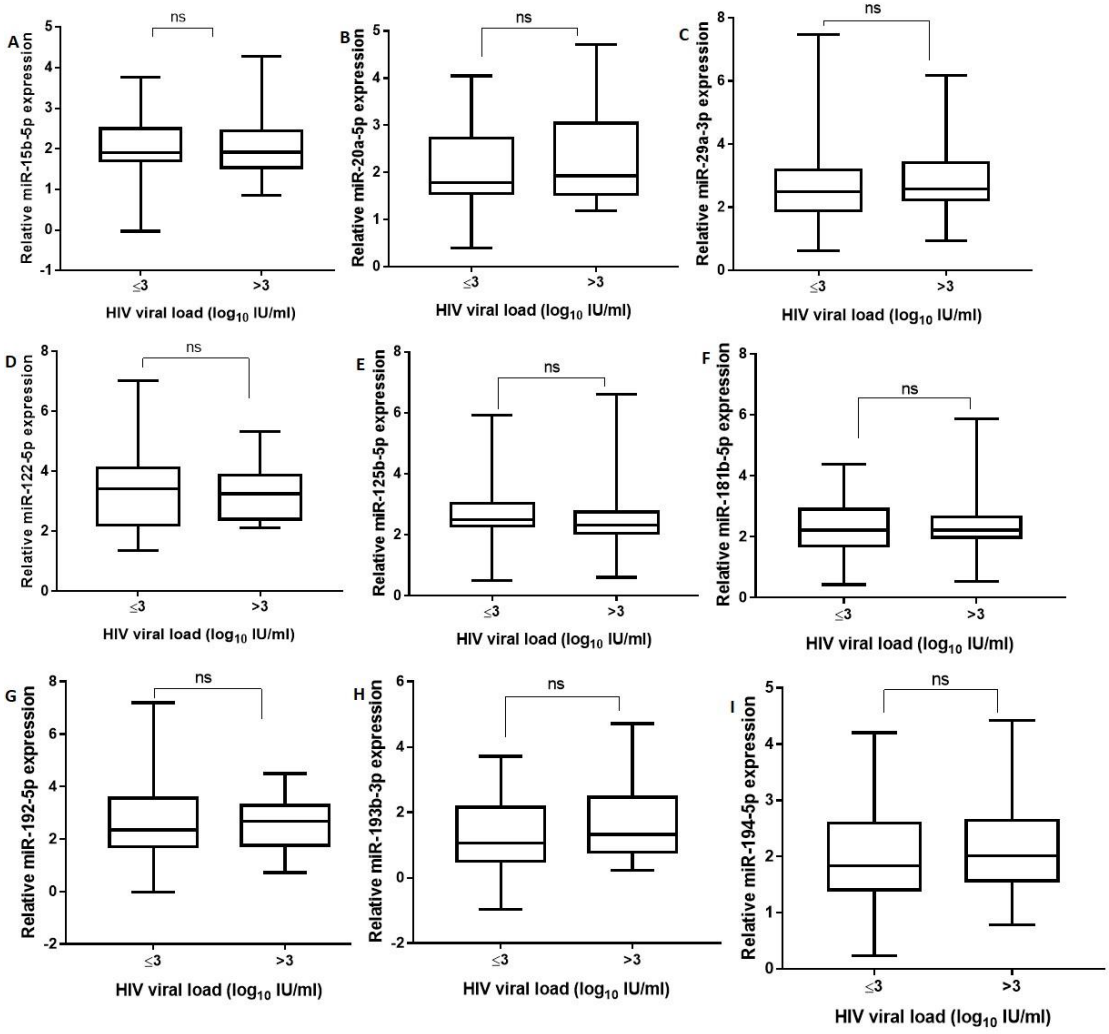
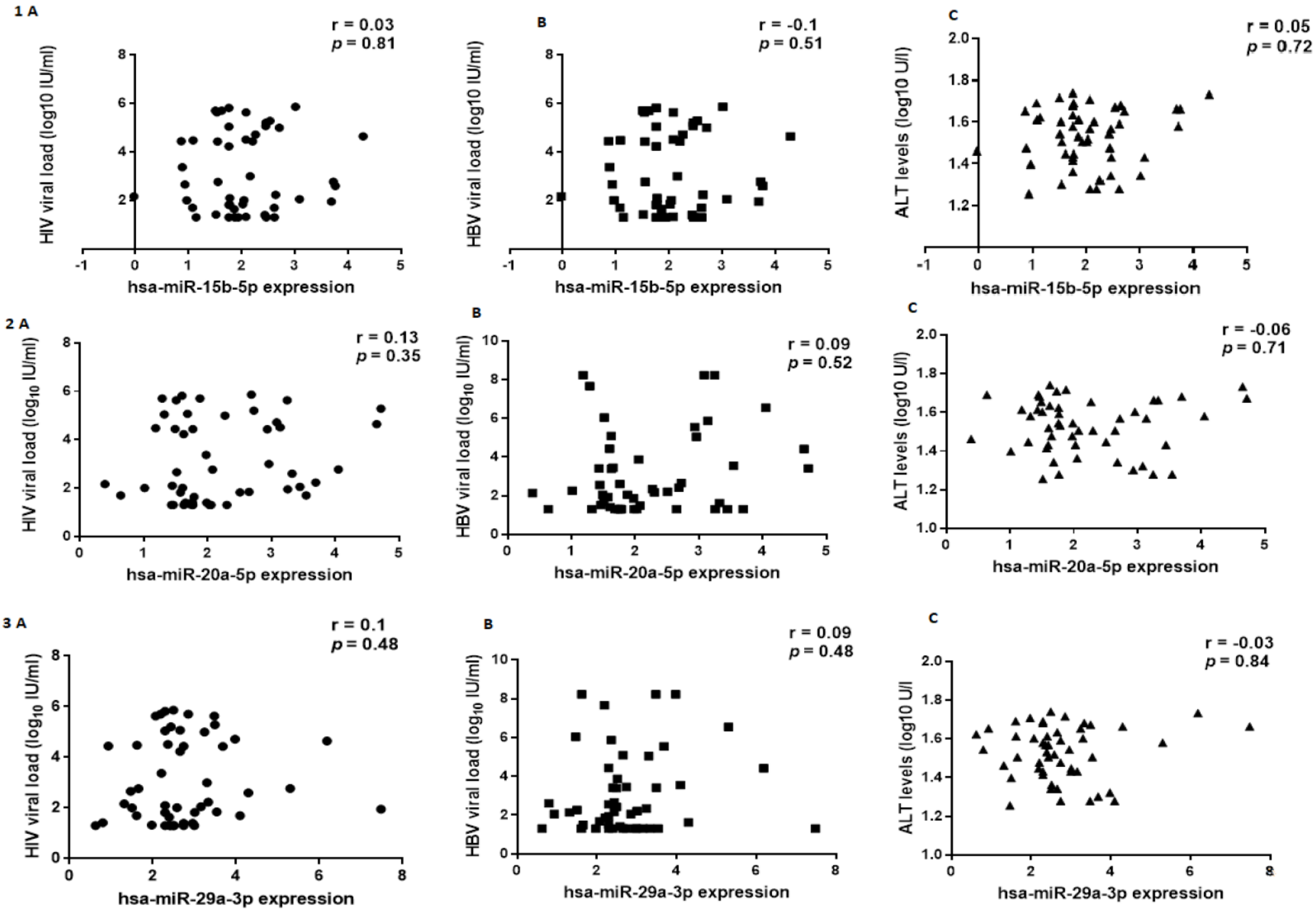


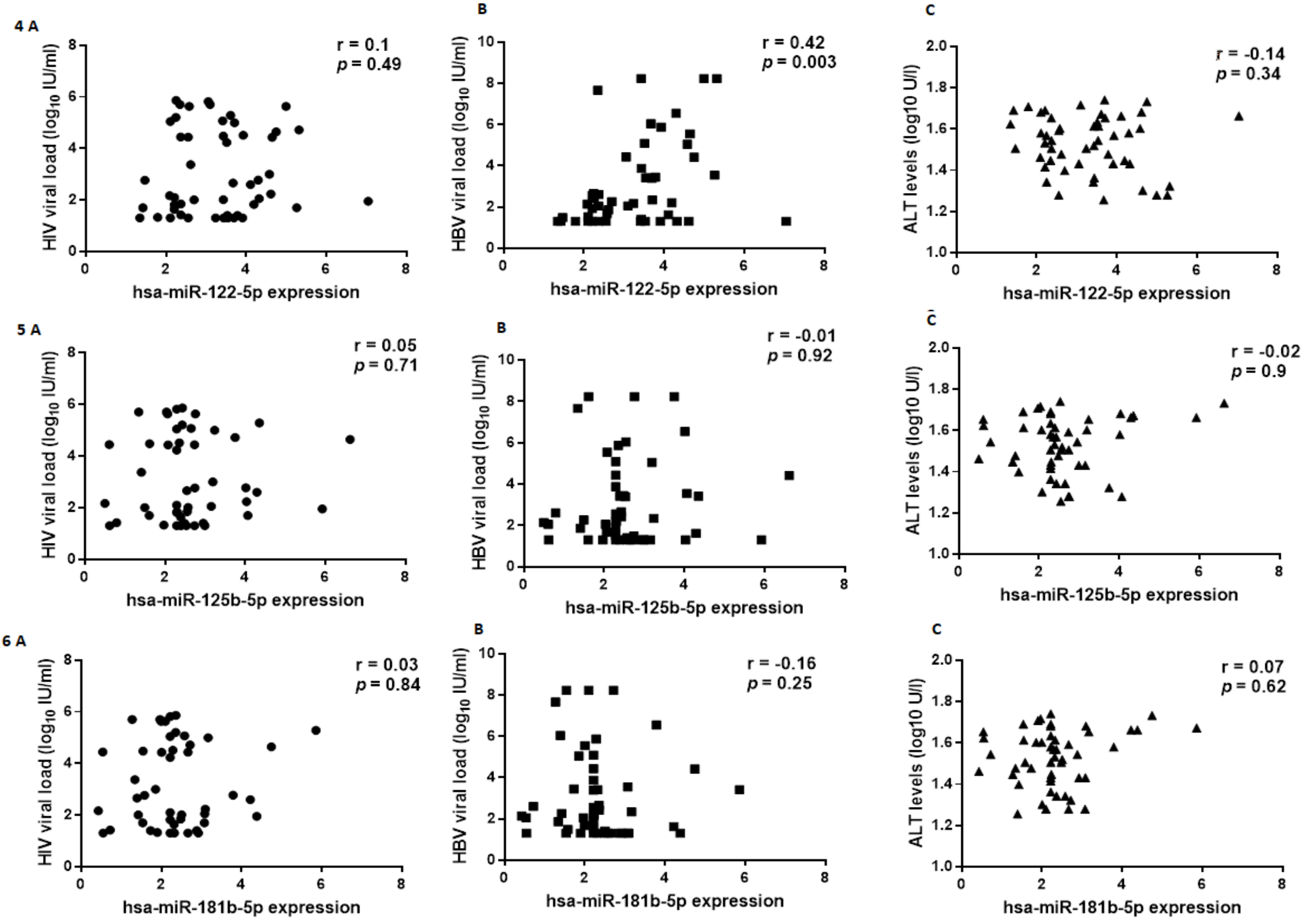
Figure 3.5: MiRNA **expression signature comparison between samples with high vs low HIV viral load.** Box and whisker diagrams showing, (**A**) No significant difference was observed in hsa-miR-15b-5p expression levels, (**B**) no significant difference was observed in hsa-miR-20a-5p expression levels in samples with high HIV viral load vs low HIV viral load. (**C**) No significant difference was observed in expression levels of hsa-miR-29a-3p between the compared groups. (**D**) No significant difference in hsa-miR-122-5p expression was observed between the compared groups. (**E**) No significant difference in hsa-miR-125b-5p expression levels was observed between the compared groups. (**F**) No significant difference in hsa-miR-181b-5p expression levels was observed between the compared groups. (**G**) insignificantly high hsa-miR-192-5p expression levels in samples with high HIV viral load, (**H**) No significant difference in hsa-miR-193b-3p expression levels were observed between the compared groups, (**I**) no significant difference in hsa-miR-194-5p expression levels was observed in samples with high vs low HIV viral load. Relative miRNA level is shown as 2 ^ – (ΔCt of target miRNA– arithmetic mean of ΔCt for the control group).

Statistical comparisons were performed using an unpaired Mann-Whitney U test. Significant differences ($p < 0.05$) are shown by an (*).

3.1.7 Correlation of miRNA expression levels with markers of disease severity

A significant moderate positive correlation was observed between HBV viral load and hsa-miR-122-5p, hsa-miR-192-5p, and hsa-miR-193-3p (Figure 3.6 4B, 7B & 8B). To further confirm the association between HBV viral load and the abovementioned miRNA levels and to avoid any potential confounding effects of other variables such as HIV viral load, ALT levels, and age, a Benjamini-Hochberg correction test was performed. The significant positive correlation between HBV viral load with hsa-miR-122-5p and hsa-miR-193-3p was confirmed while the positive correlation between hsa-miR-192-5p and HBV viral load was found to be not significant. A weak positive correlation was observed between HBV viral load and hsa-miR-194-5p, and a weak negative correlation was observed between HBV viral load with hsa-miR15b-5p and hsa-miR-181b-5p, but these associations were not significant (Figure 3.6 1B, 6B, 9B). No correlation was observed between HBV viral load and expression levels of hsa-miR-20a-5p, hsa-miR-29a-5p, and hsa-miR-125b-5p. A weak positive correlation trend was observed between miRNAs (hsa-miR-20a-5p, hsa-miR-29a-5p, hsa-miR-122-5p, hsa-miR-192-5p, hsa-miR-193b-3p, and hsa-miR-194-5p) and HIV viral load but the associations were not significant (Figure 3.6 2A, 3A, 4A, 7A, 8A, and 9A). No correlation was observed between HIV viral load and expression levels of hsa-miR15b-5p, hsa-miR-125b-5p, and hsa-miR-181b-5p. A weak negative correlation was shown between ALT levels and expression levels of hsa-miR-122-5p and hsa-miR-192-5p, but the associations were not significant (Figure 3.6 4C, 7C). No correlation was observed between ALT levels and the other studied miRNAs (Figure 1C-9C).


1 A
r = 0.03
p = 0.81
HIV viral load (log10 IU/ml)
hsa-miR-15b-5p expression
B
r = -0.1
p = 0.51
HBV viral load (log10 IU/ml)
hsa-miR-15b-5p expression
C
r = 0.05
p = 0.72
ALT levels (log10 U/l)
hsa-miR-15b-5p expression
2 A
r = 0.13
p = 0.35
HIV viral load (log10 IU/ml)
hsa-miR-20a-5p expression
B
r = 0.09
p = 0.52
HBV viral load (log10 IU/ml)
hsa-miR-20a-5p expression
C
r = -0.06
p = 0.71
ALT levels (log10 U/l)
hsa-miR-20a-5p expression
3 A
r = 0.1
p = 0.48
HIV viral load (log10 IU/ml)
hsa-miR-29a-3p expression
B
r = 0.09
p = 0.48
HBV viral load (log10 IU/ml)
hsa-miR-29a-3p expression
C
r = -0.03
p = 0.84
ALT levels (log10 U/l
hsa-miR-29a-3p expression


4 A
HIV viral load (log10 IU/ml)
r = 0.1
p = 0.49
hsa-miR-122-5p expression
B
HBV viral load (log10 IU/ml)
r = 0.42
p = 0.003
hsa-miR-122-5p expression
C
ALT levels (log10 U/l)
r = -0.14
p = 0.34
hsa-miR-122-5p expression
5 A
HIV viral load (log10 IU/ml)
r = 0.05
p = 0.71
hsa-miR-125b-5p expression
B
HBV viral load (log10 IU/ml)
r = -0.01
p = 0.92
hsa-miR-125b-5p expression
C
ALT levels (log10 U/l)
r = -0.02
p = 0.9
hsa-miR-125b-5p expression
6 A
HIV viral load (log10 IU/ml)
r = 0.03
p = 0.84
hsa-miR-181b-5p expression
B
HBV viral load (log10 IU/ml)
r = -0.16
p = 0.25
hsa-miR-181b-5p expression
C
ALT levels (log10 U/l)
r = 0.07
p = 0.62
hsa-miR-181b-5p expression

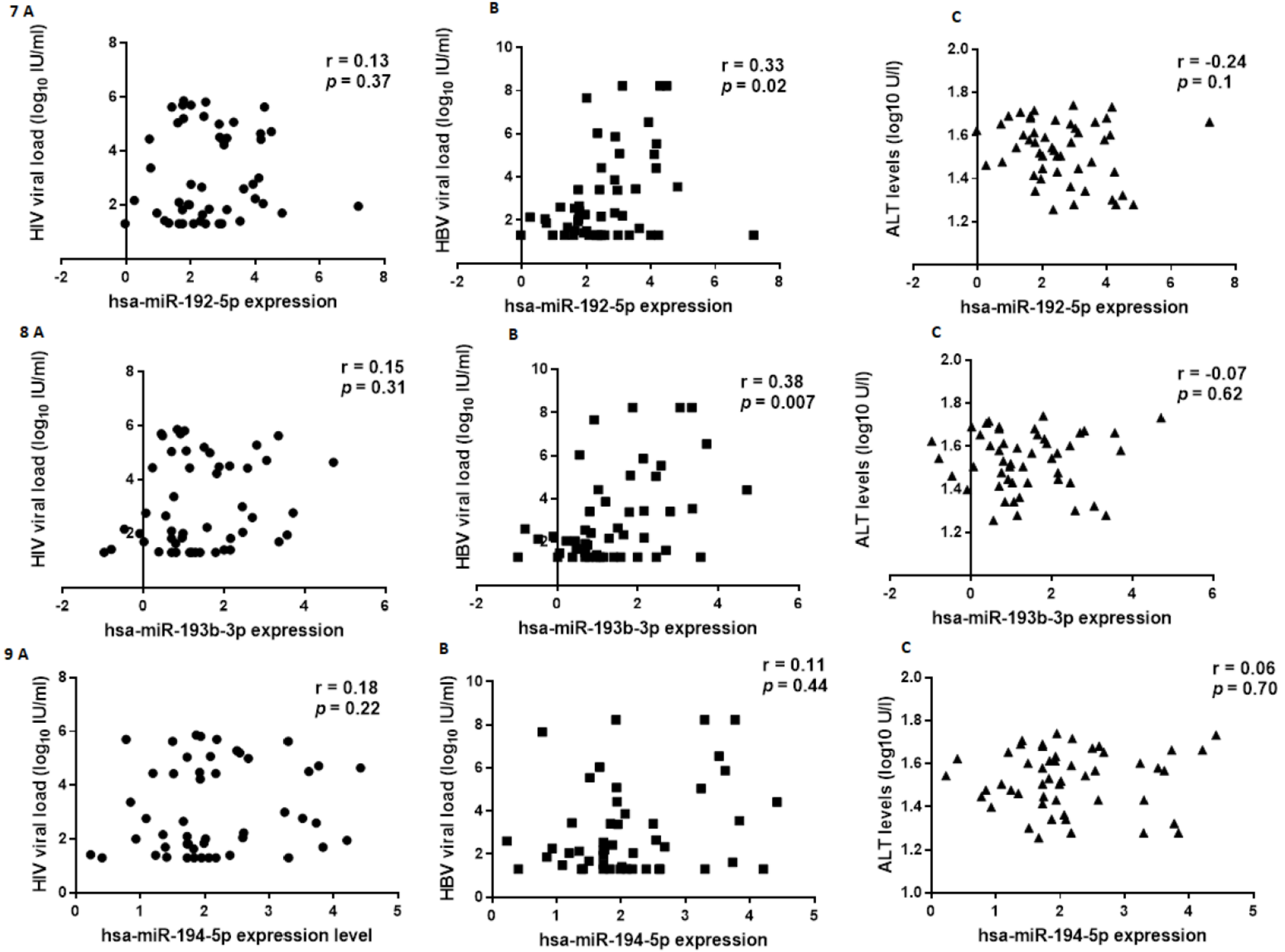

7 A
HIV viral load (log10 IU/ml)
r = 0.13
p = 0.37
hsa-miR-192-5p expression
B
HBV viral load (log10 IU/ml)
r = 0.33
p = 0.02
hsa-miR-192-5p expression
C
ALT levels (log10 U/l)
r = -0.24
p = 0.1
hsa-miR-192-5p expression
8 A
HIV viral load (log10 IU/ml)
r = 0.15
p = 0.31
hsa-miR-193b-3p expression
B
HBV viral load (log10 IU/ml)
r = 0.38
p = 0.007
hsa-miR-193b-3p expression
C
ALT levels (log10 U/l)
r = -0.07
p = 0.62
hsa-miR-193b-3p expression
9 A
HIV viral load (log10 IU/ml)
r = 0.18
p = 0.22
hsa-miR-194-5p expression level
B
HBV viral load (log10 IU/ml)
r = 0.11
p = 0.44
hsa-miR-194-5p expression
C
ALT levels (log10 U/l)
r = 0.06
p = 0.70
hsa-miR-194-5p expression

Figure 3.6: Correlations of microRNA panel with HIV viral load, HBV viral load and ALT levels. Dot plot diagrams showing association trends between HIV viral, HBV viral and ALT levels with (**1**: **A, B, C**) hsa-miR15b-5p expression levels, (**2: A, B, C**) hsa-miR-20a-5p expression levels, (**3: A, B, C**) hsa-miR-29a-5p expression levels, (**4: A, B, C**) has-miR-122-5p expression levels (**5: A, B, C**) hsa-miR-125b-5p expression levels, (**6: A, B, C**) hsa-miR181b-5p expression levels, (**7: A, B, C**) hsa-miR-192-5p expression levels, (**8: A, B, C**) hsa-miR-193-3p expression levels and (**9: A, B, C**) hsa-miR-194-5p expression levels. Correlation analysis was performed using a Spearman correlation coefficient. Significant relationships are shown by $P < 0.05$.

CHAPTER 4: DISCUSSION AND CONCLUSION

4.1 Discussion

Significantly higher expression levels of our HBV-specific microRNAmiRNAs were observed in chronic HBV-HIV coinfected samples compared to healthy controls. Samples with high HBV viral load had significantly higher expression levels of hsa-miR-122-5p ($p = 0.0001$), hsa-miR-192-5p ($p = 0.0003$), and hsa-miR-193b-3p ($p = 0.0002$) compared to samples with low HBV viral load. In HBeAg-negatives samples, significantly higher levels of hsa-miR-15b-5p ($p = 0.0054$) and hsa-miR-181b-5p ($p = 0.03$) were observed compared to HBeAg-positive samples. No significant differences were observed in low vs high ALT levels and low vs high HIV viral load samples. A significant moderate positive correlation was observed between HBV viral load and the expression levels of hsa-miR-122-5p, hsa-miR-192-5p, and hsa-miR-193-3p. It was noted that there is a difference in the age and gender between the controls and the chronic HBV infected individuals however when we performed the linear regression model analysis, it was shown that age and gender were not significant explanatory variables for miRNA expression levels and the differences in the miRNA expression levels were due to the HBsAg positivity. We ensured that good laboratory practice was exercised when conducting the testing to eliminate possibility of contamination, a multichannel pipette was used for loading the plates, appropriate personal protective equipment was always worn, and master mixes were prepared and aliquoted in the non-amplification area of the laboratory to avoid amplicon contamination. In our study, chronic HBV samples were compared with healthy samples and, all nine miRNA targets (hsa-miR-15b-5p, hsa-miR-20a-5p, hsa-miR-29a-3p, hsa-miR-122-5p, hsa-miR-125b-5p, hsa-miR-181b-5p, hsa-miR-192-5p, hsa-miR-193b-3p & hsa-miR-194-5p) revealed significantly higher levels in chronic HBV samples. The results from our study are congruent with the results from previous studies that investigated the expression pattern of the miRNAs in our panel in patients with chronic HBV (Winther et al., 2013, Huang et al., 2014, Yu et al., 2015a, van der Ree et al., 2017, Yousefpouran et al., 2020). Similar findings were shown in HCV-HIV coinfected patients. Hsa-miR-122-5p expression levels were significantly higher in HCV-HIV coinfected patients compared to healthy controls (Jansen et al., 2015). High expression levels of hsa-miR-20a-5p and hsa-miR-125b-5p were observed in chronic HCV patients compared with healthy controls, supporting the potential use of miRNAs in viral infections (Shrivastava et al., 2013, Shwetha et al., 2018). This suggests that the studied miRNAs may be useful in differentiating between patients infected with chronic hepatitis B

and healthy individuals. MiRNAs are stable and may be a useful biomarker for early detection, prognosis, and monitoring of HBV disease progression.

There is significant morbidity and mortality associated with chronic HBV infection. Chronic HBV infection is associated with a 15% to 40% risk of cirrhosis, liver failure, and/or HCC, as well as a 15% to 25% risk of death from HBV-associated liver diseases (SA NDOH, 2019). It is essential to predict disease progression in patients with chronic HBV infection so that antiviral therapy can be initiated at the right time. The current diagnostic markers for HBV infection can be used as indicators of specific infection phases, but they cannot predict the outcome of the infection (Coffin et al., 2019, Kramvis et al., 2022). Because of their non-invasive nature, serum or plasma miRNAs have attracted significant research attention as potential diagnostic and prognostic markers for chronic hepatitis B (Wang et al., 2019). Several studies suggest that the use of miRNA panels in serum or plasma could improve the specificity of HBV diagnostics (Bonino et al., 2010, Gümüşay et al., 2013, Jin et al., 2019, Peng et al., 2020, Tan et al., 2021). Our study aimed to investigate and describe the pattern of miRNA expression in samples of patients with chronic hepatitis B and different disease severity biomarkers (HBV viral load, HBeAg status, ALT levels, and HIV viral load) as well as miRNA expression's correlation with these biomarkers.

In our study, expression levels of miRNAs were compared in samples with high versus low HBV viral load. The samples with high HBV viral load (>3 $\log_{10}$ IU/ml) expressed significantly higher levels of miRNA (hsa-miR-122-5p, hsa-miR-192-5p & hsa-miR-193b-3p). Since HBV viral load levels are associated with disease progression, the levels of these miRNAs were also shown to be positively correlated with HBV viral, these results suggest the potential use of these miRNAs as prognostic markers for chronic hepatitis B disease progression. MiRNAs may contribute to defining the phase of chronic hepatitis B infection, and treatment indication and may even allow assessment of antiviral therapy effectiveness. Li et al. (2016) studied the expression levels of hsa-miR-125b-5p, and hsa-miR-122-5p in patients with chronic HBV, the patients with high HBV viral loads demonstrated high expression levels of these miRNAs compared to patients with low HBV viral loads. Additionally, elevated hsa-miR-125b-5p was also shown in patients at different phases of chronic hepatitis B infection and patients in the immune-tolerant phase. The patients in the immune-tolerant phase showed lower levels of hsa-miR-125b-5p compared to patients in the immune-reactive phase, which indicated that

miRNAs may not only be influenced by HBV replication but by other factors such as liver necroinflammation (Li et al., 2016). In contrast, our study did not show any difference in the expression levels of hsa-miR-125b-5p between samples with high vs low HBV viral load. This discrepancy between our study and a study by Li et al. (2016) may be due to the cohort selection criteria, our chronic HBV samples were infected with genotype A1 chronic HBV and they were also coinfected with HIV while the other study had samples monoinfected with chronic HBV and had HBV genotype B or C. Therefore, these results may be related to the variation between the study groups like the effect of different genotypes in the disease state. Our study did not show any difference in the expression levels of hsa-miR-181b-5p between samples with high vs low HBV viral load. In contrast to our findings, Yu et al. (2015) investigated whether the expression levels of hsa-miR-181b-5p were associated with HBV DNA levels and liver fibrosis progression, and significantly increased levels of hsa-miR-181b-5p were demonstrated in patients with high HBV DNA suggesting that liver exposure to HBV may induce hsa-miR-181b-5p. The difference between our study and the study by Yu et al. (2015) may be on account of the differences in the study cohort, their study cohort was monoinfected with chronic HBV while our study was coinfected with HBV-HIV. These results may be relevant in the potential use of hsa-miR-122-5p, hsa-miR-192-5p, and hsa-miR-193b-3p as prognostic markers for chronic HBV disease progression in our clinical setting.

In South Africa, the HBV genotype A is predominant with subtype A1 occurring in about 97% of rural Africans (Firnhaber et al., 2008, Boyles and Cohen, 2011, Kramvis, 2018). Patients with HBV genotype A infection are predisposed to chronicity, with low frequency of HBeAg-positivity, horizontal transmission of HBV, higher levels of liver damage, and an elevated risk of HCC (Kew et al., 2005). In this study expression levels of miRNAs were compared between HBeAg positive and HBeAg negative samples and the levels of hsa-miR-15b-5p and hsa-miR-181b-5p were found to be significantly higher in HBeAg negative samples. In contrast to our findings, a study by Yu et al. (2015) found significantly high expression levels of hsa-miR-181b-5p in HBeAg-positive patients. In our study, no significant differences were observed in the expression levels of hsa-miR-20a-5p, hsa-miR-29a-3p, hsa-miR-125b-5p, hsa-miR-192-5p, hsa-miR-193b-3p and hsa-miR-194-5p in the HBeAg status groups although these miRNAs were slightly higher in HBeAg negative samples. A previous study demonstrated significantly higher levels of hsa-miR-20a-5p, hsa-miR-122-5p, and hsa-miR-194-5p in HBeAg-negative patients compared to positive patients (Ji et al., 2011). However, several previous studies based on miRNA profiling showed high expression levels of miRNAs in HBeAg-positive patients

when compared to HBeAg-negative patients (Zhou et al., 2011, Zhang et al., 2012, Winther et al., 2013). Van der Ree et al. (2017), revealed significantly higher expression levels of hsa-miR-125b-5p, hsa-miR-192-5p & hsa-miR-194-5p in HBeAg-positive patients than in HBeAg-negative patients. This study by Van der Ree et al. (2017), had patients that had completed about 48 weeks of treatment which may explain the discordance with our findings, their results suggest the potential use of these miRNAs as prognostic markers as well as biomarkers in predicting antiviral treatment response. A study investigating miRNA expression profiles in children with chronic HBV, hsa-miR-122-5p, hsa-miR-125b-5p, hsa-miR-192-5p, hsa-miR-193b-3p & hsa-miR-194-5p expression levels were significantly higher in HBeAg-positive patients compared to HBeAg-negative patients (Winther et al., 2013). Ji et al. (2011), showed high expression levels of hsa-miR-20a-5p in HBeAg-positive samples. In contrast, our study showed higher levels of hsa-miR-15b-5p and hsa-miR-181b-5p in HBeAg-negative samples compared to HBeAg-positive samples. This discordance between the findings in the current study and the abovementioned previous studies can be explained by the difference in specific geographical HBV genotypes and our cohort has sub-genotype A1 chronic HBV as established on the previous study conducted on these samples (Msomi et al., 2022), most of the previous studies were done in settings predominated with genotype B, C & E chronic HBV. Different HBV genotype carriers are prone to different clinical outcomes or severity of the infection (Kramvis, 2018). There is growing evidence suggesting that HBV genotypes may influence HBV endemicity, HBeAg seroconversion rates, mutational patterns in the core and pre-core promoter regions, clinical outcomes, and response to treatment, nonetheless, it has not yet been established what biological characteristics underlie these differences (Liu and Kao, 2013, Elizalde et al., 2021). A study investigating HBV infection persistence found high persistence of HBV infection in patients with sub-genotype A1 when compared to non-A genotypes (Ito et al., 2014). Most of the previous studies were mono-infected with chronic HBV and not co-infected with HIV which could be a contributor to the differences in findings. Differences in ethnicity could also account for the differences in our study and studies from other countries as it has been reported in South African studies that chronic HBV is higher in black South Africans than any other ethnic groups and our studies consisted of the black ethnic group. In chronic HBV patients without HBeAg, HBV has a naturally occurring mutant that does not produce HBeAg due to a mutation in the pre-core or core promoter region of the genome (Carman et al., 1989a, Zhang et al., 2009). HBeAg-negative chronic HBV usually progresses rapidly to cirrhosis and HCC often develops. Therefore, non-invasive methods to monitor disease progression are needed. Hsa-miR-15b-5p and hsa-miR-181b-5p may serve as potential

markers in HBeAg-negative chronic HBV disease progression. We assume that most participants would have been infected as children and they stay for a long period in the immune-tolerant phase which is mediated by HBeAg status. Our results suggest that miRNA expressions of hsa-miR-15b-5p and hsa-miR-181b-5p may be associated with HBeAg production in our clinical setting and can be potentially used as biomarkers of HBV replication. The ALT is utilized as a marker for liver damage and its persistent elevation can be used to predict chronic hepatitis B disease progression, particularly greater than two times the upper limit of normal (2x ULN) levels (Iloeje et al., 2006, Tai et al., 2009). In the current study plasma levels of miRNAs were compared and no significant differences were observed between chronic hepatitis B samples with high (> 35 U/l) vs low (< 35 U/l) ALT levels. Corresponding to our results, previous studies did not show significant differences between miRNA expression levels in samples with low vs high ALT levels (Winther et al., 2013, Huang et al., 2014, Yu et al., 2015a, Li et al., 2016, van der Ree et al., 2017). Our studied miRNAs were not able to differentiate between low and high ALT levels, and there was no correlation between miRNA expression levels and ALT levels. These results suggest that the miRNA in this panel may not have the potential use as markers of active liver disease in our clinical setting.

Our study is one of the early studies in our clinical setting to look at the association of miRNAs expression levels in chronic HBV samples with high HIV viral load compared to samples with low HIV viral load. In South Africa, HBV and HIV are usually acquired and transmitted independently. In most cases of HBV-HIV co-infection, HBV typically occurs in early childhood, while HIV is normally acquired later in life, most commonly through sexual contact. No significant differences were observed in miRNA expression levels between high HIV viral load samples and low HIV viral load samples. Witwer et al. (2012), investigated miRNA expression in HIV-infected elite suppressors (patients with undetectable viral load) and viraemic patients whereby low levels of hsa-miR125b-5p in viraemic patients and high levels of hsa-miR181b-5p in viraemic patients were demonstrated compared to elite suppressors. The difference between the present study and the abovementioned study is that our study samples were coinfected with HBV and HIV while the other study was studying miRNA profiling in samples monoinfected with HIV. Our results suggest that miRNAs in the panel may not be used as markers of HIV viral replication and disease progression in our clinical setting but more studies are needed to verify our results.

Our study investigated potential relationships between circulating miRNAs and HBV viral load, HIV viral load, as well as ALT levels, and a significant positive correlation was found between hsa-miR-122-5p and HBV viral load as well as between hsa-miR-192-5p and HBV viral load. Our results are in agreement with previous studies which found a significant positive correlation between HBV viral load and the expression levels of hsa-miR-122-5p and hsa-miR-192-5p (Winther et al., 2013, van der Ree et al., 2017, Wu et al., 2019). The studies by van der Ree et al. (2017) and Wu et al. (2019) were conducted on patients that were undergoing antiviral treatment and the study by Winther et al. (2013) was conducted on patients mono-infected with chronic HBV. Our study was able to confirm these findings in samples with chronic HBV-HIV coinfection. These results suggest that hsa-miR-122-5p and hsa-miR-192-5p may play a role in HBV viral replication. Previous studies also found a significantly strong positive correlation between hsa-miR-194-5p expression levels and HBV viral load (Winther et al., 2013, Li et al., 2016, van der Ree et al., 2017). Our study did not find any correlation between HBV viral load and hsa-miR-181b-5p. In contrast, Yu et al. (2015) showed a significant positive correlation between hsa-miR181b-5p levels and HBV viral load. The differences in results may be explained by the differences in study cohorts since the participants of the Yu et al. (2015) study were mono-infected with genotype E chronic HBV. Yousefpouran et al. (2020) found a significant positive correlation between hsa-miR-122-5p and hsa-miR181b-5p expression levels and HCV viral load. These findings should be validated in our clinical setting in patients with HCV infection since HCV is also a major risk factor for HCC. Our study found no correlation between our studied miRNAs and HIV viral load. Other studies reported a significant negative correlation between HIV viral load and hsa-miR-29a-5p expression levels (Witwer et al., 2012, Moghoofei et al., 2018, Yousefpouran et al., 2020). Our results did not show the potential role that our studied miRNAs may play in viral replication, and their potential to be utilized as a prognostic marker for HIV disease progression. Therefore, there is a need for more similar studies that will validate our findings or reveal the potential relationship that may exist between HIV viral load and our studied miRNAs. Our study showed no significant correlation between ALT levels and expression levels of our studied miRNAs. Wu et al. (2019) reported a negative correlation between hsa-miR-122-5p levels and ALT, however, another study found a positive correlation between ALT and hsa-miR-122-5p (van der Ree et al., 2017). In agreement with our findings, other studies found no correlation between hsa-miR-122-5p, hsa-miR-20a-5p, and hsa-miR181b-5p and ALT levels (Ji et al., 2011, Yu et al., 2015a).

4.2 Recommendations for future research and conclusion

This is one of the early studies investigating miRNA profiling in a South African setting, a country where HBV genotype A1 predominates. Hence, there is a need for more studies to be conducted in this setting to understand the role of miRNAs in the pathogenesis of chronic HBV infection. This study was able to show that the selected panel of miRNAs can differentiate between healthy control samples and chronic HBV samples, and that specific miRNA can potentially be used to differentiate between low versus high HBV viral load samples which were supported by previous studies, however more studies are required from our cohort clinical setting to validate our findings. This study could not differentiate between low versus high ALT levels using miRNA profiling which was supported by previous studies, therefore more future studies should investigate the association of miRNA expression with ALT levels as ALT is considered to be a marker of active disease. The association of HBeAg status with specific miRNAs should be validated by more studies in our clinical setting to better understand the role of chronic HBV sub-genotype A1 on HBeAg seropositivity and disease progression and the role that miRNAs may play in viral replication. Our study was able to correlate the expression levels of hsa-miR-122-5p and hsa-miR-192-5p with HBV viral load which measures the virus titer in the bloodstream suggesting that these miRNAs have the potential to be used as biomarkers for disease progression, but functional studies are required to investigate the role of miRNAs in chronic HBV pathogenesis in vitro in our clinical setting and more studies are needed to validate our findings.

The sample size is one of the limitations of this study and the number of replicates used which were due to the costs of reagents used for testing. Future studies should increase the sample size and the number of replicates. Other limitations of this study include the absence of HBV mono-infection study group; that this study only enrolled patients with sub-genotype Al; and the study data was not analysed based on the stage of chronic hepatitis B to better understand the role of miRNA in disease progression. Our studied miRNAs may play a role in the replication of HBV by targeting specific HBV transcripts or cellular factors. This miRNA panel can be used to distinguish patients with chronic HBV from healthy individuals in future studies. Future studies should investigate the relationship between miRNAs and their putative targets and HBV replication, as well as investigate differences in miRNA expression by genotypes as there is evidence of the presence of genotype D in South Africa. MiRNAs may serve as potential biomarkers for chronic HBV disease stages or prognostic markers for response to

antiviral treatment if they are found to have a direct or indirect role in the regulation of HBV replication. Since miRNA profiling is still a work in progress there isn't a cost effective method for testing and it would require well established laboratory for equipment and trained personnel which is a disadvantage in resource limited settings.

In conclusion, public health is at risk from millions of chronic HBV-infected individuals. HCC and cirrhosis often develop over decades, and HCC is often diagnosed much too late, leaving patients with poor prognoses and limited treatment options. For early detection of individuals at increased risk, sensitive and non-invasive methods are required that can detect subtle changes in the disease state. Through the monitoring of gene and miRNA expression in chronic HBV and liver disease, plasma or serum miRNA may be able to improve early detection. Our study was able to demonstrate the potential role of has-miR-15b-5p, has-miR-122-5p, has-miR-181b-5p, has-miR-192-5p and has-miR-193b-3p as confirmatory test markers in chronic HBV infection.

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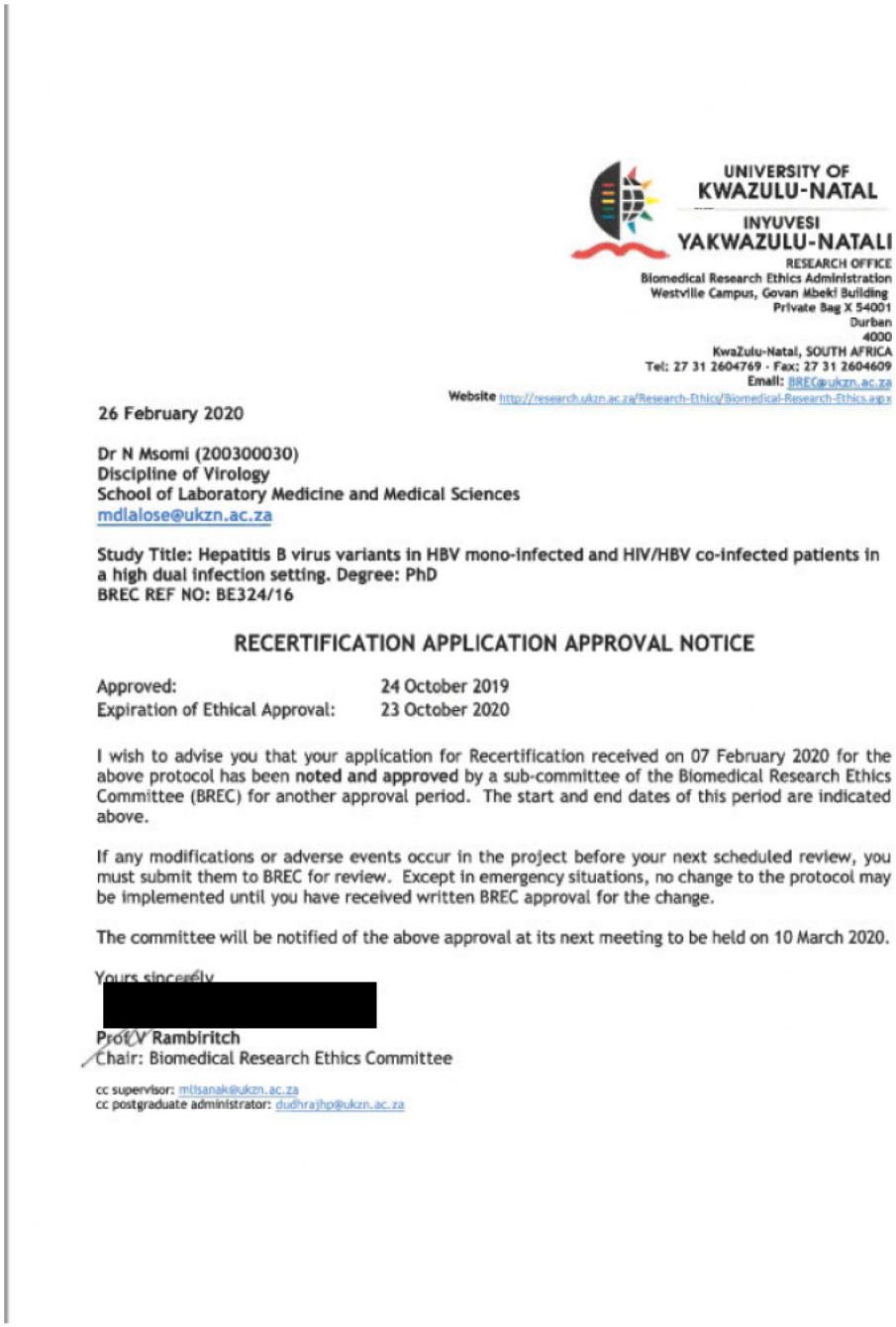
APPENDICES

APPENDIX A



UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI
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Biomedical Research Ethics Administration
Westville Campus, Govan Mbeki Building
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Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za
Website http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

26 February 2020

Dr N Msomi (200300030)
Discipline of Virology
School of Laboratory Medicine and Medical Sciences
mdlalose@ukzn.ac.za

Study Title: Hepatitis B virus variants in HBV mono-infected and HIV/HBV co-infected patients in a high dual infection setting. Degree: PhD
BREC REF NO: BE324/16

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: 24 October 2019
Expiration of Ethical Approval: 23 October 2020

I wish to advise you that your application for Recertification received on 07 February 2020 for the above protocol has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 10 March 2020.

Yours sincerely

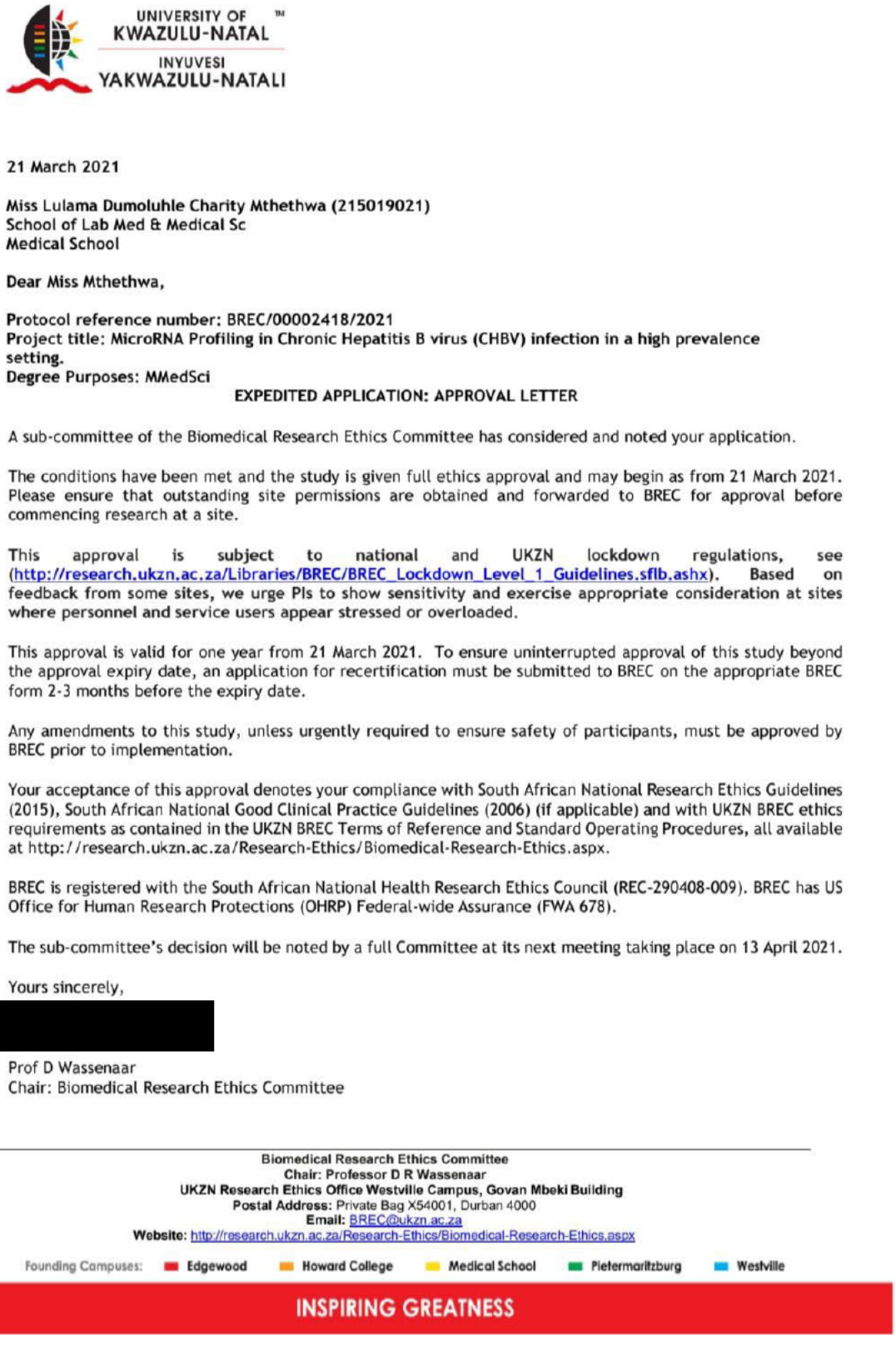
[redacted]

Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

cc supervisor: mlisanak@ukzn.ac.za
cc postgraduate administrator: dudhrajhp@ukzn.ac.za

Figure 1: The previous study's ethical clearance.

APPENDIX B



UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

21 March 2021

Miss Lulama Dumoluhle Charity Mthethwa (215019021)
School of Lab Med & Medical Sc
Medical School

Dear Miss Mthethwa,

Protocol reference number: BREC/00002418/2021
Project title: MicroRNA Profiling in Chronic Hepatitis B virus (CHBV) infection in a high prevalence setting.
Degree Purposes: MMedSci

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 21 March 2021. Please ensure that outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is subject to national and UKZN lockdown regulations, see (http://research.ukzn.ac.za/Libraries/BREC/BREC_Lockdown_Level_1_Guidelines.sflb.ashx). Based on feedback from some sites, we urge PIs to show sensitivity and exercise appropriate consideration at sites where personnel and service users appear stressed or overloaded.

This approval is valid for one year from 21 March 2021. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 13 April 2021.

Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
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Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

INSPIRING GREATNESS

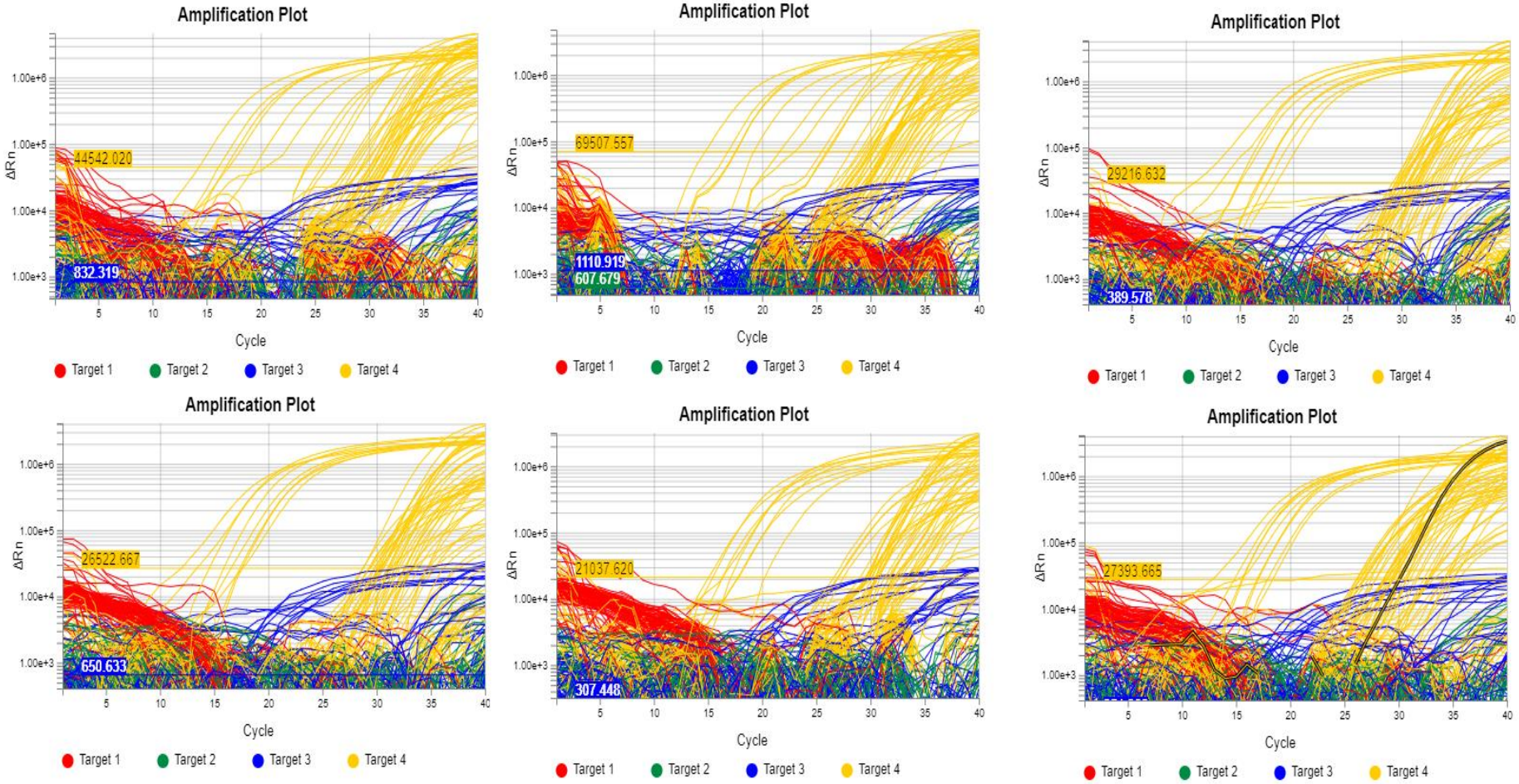
Figure 1: The current study's ethical clearance.

APPENDIX C

Table 1: Mature miRNA **sequences**

MiRNA **target name**	**Mature** miRNA **sequence (5'-3')**	**Catalogue number**	**Assay ID**
hsa-miR-15b-5p	UAGCAGCACAUCAUGGUUUACA	4427975	000390
hsa-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUAG	4427975	000580
hsa-miR-29a-3p	UAGCACCAUCUGAAAUCGGUUA	4427975	002112
hsa-miR-122-5p	UGGAGUGUGACAAUGGUGUUUG	4427975	002245
hsa-miR-125b-5p	UCCCUGAGACCCUAACUUGUGA	4427975	000449
hsa-miR-181b-5p	CUCACUGAACAAUGAAUGCAA	4427975	462578_mat
hsa-miR-192-5p	CUGACCUAUGAAUUGACAGCC	4427975	000491
hsa-miR-193b-3p	AACUGGCCCUCAAAGUCCCGCU	4427975	002367
hsa-miR-194-5p	UGUAACAGCAACUCCAUGUGGA	4427975	000493
U6 snRNA (Endogenous control)	GTGCTCGCTTCGGCAGCACA TATACTAAAATTGGAACGATA CAGAGAAGATTAGCATGGCC CCTGCGCAAGGATGACACGC AAATTCGTGAAGCGTTCCATA TTTT	4427975	001973

APPENDIX D


Amplification Plot
ΔRn
Cycle
44542.020
832.319
Target 1
Target 2
Target 3
Target 4
Amplification Plot
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1110.919
607.679
Amplification Plot
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389.578
Amplification Plot
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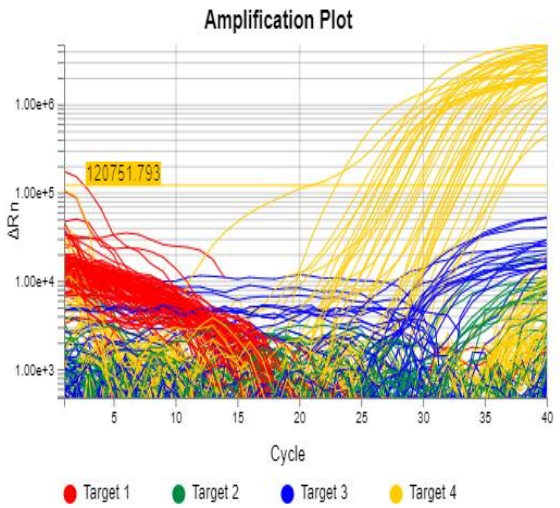
650.633
Amplification Plot
21037.620
307.448
Amplification Plot
27393.665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ΔRn
Cycle
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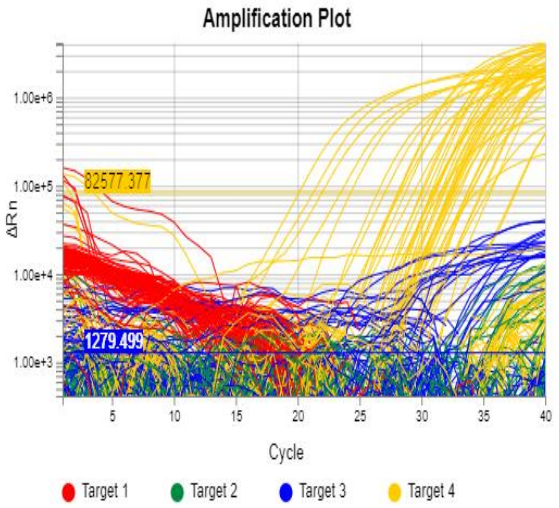
Target 4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ΔRn
Cycle
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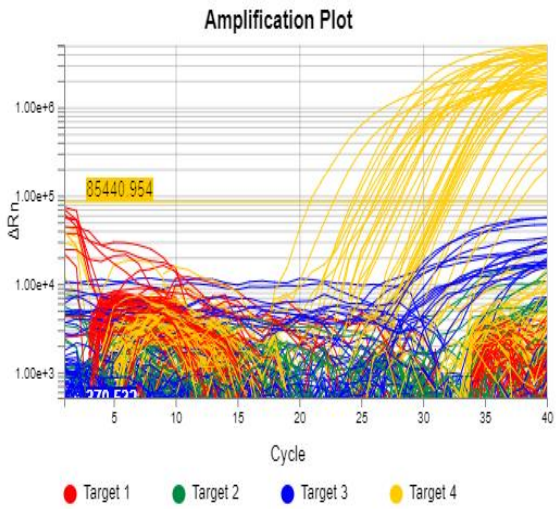
Target 3
Target 4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ΔRn
Cycle
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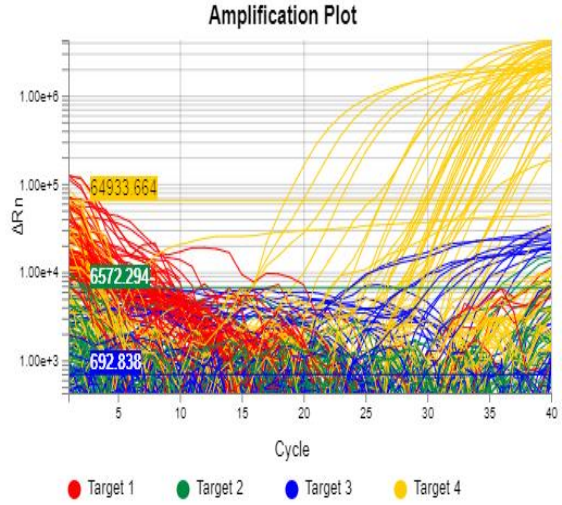
Target 4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692.838
ΔRn
Cycle
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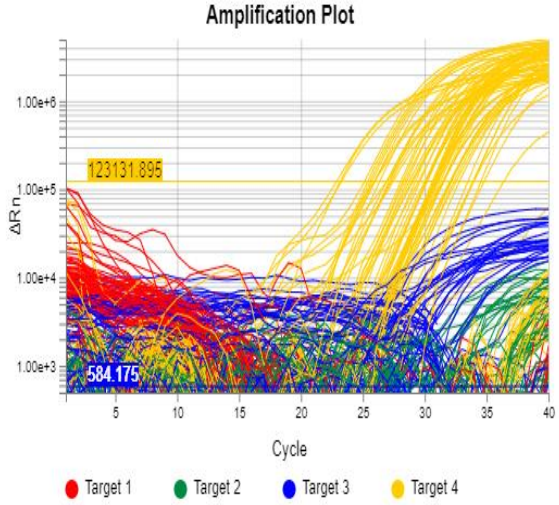
Target 3
Target 4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584.175
ΔRn
Cycle
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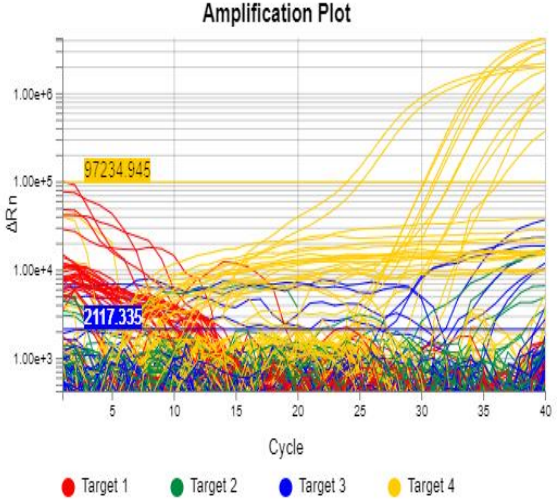
Target 2
Target 3
Target 4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ΔRn
Cycle
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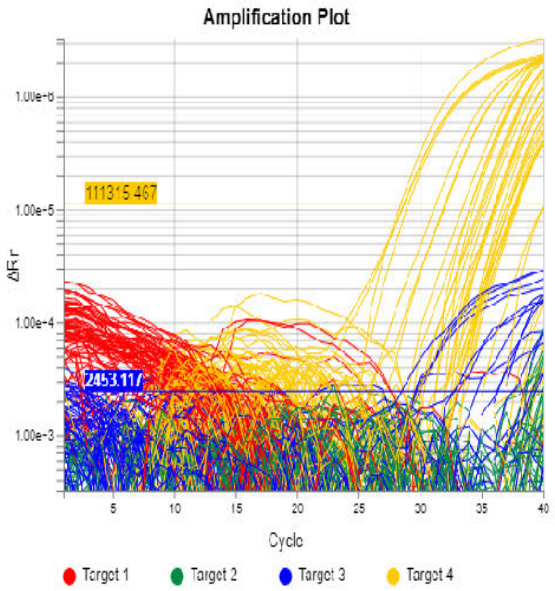
Target 3
Target 4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ΔRn
Cycle
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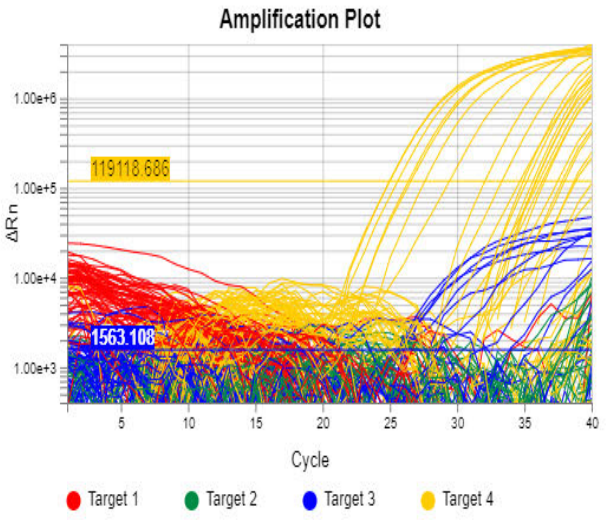
Target 2
Target 3
Target 4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1563.108
ΔRn
Cycle
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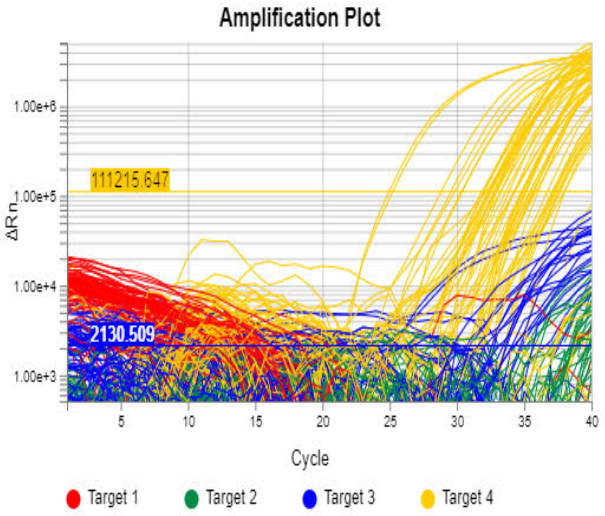
Target 2
Target 3
Target 4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2130.509
ΔRn
Cycle
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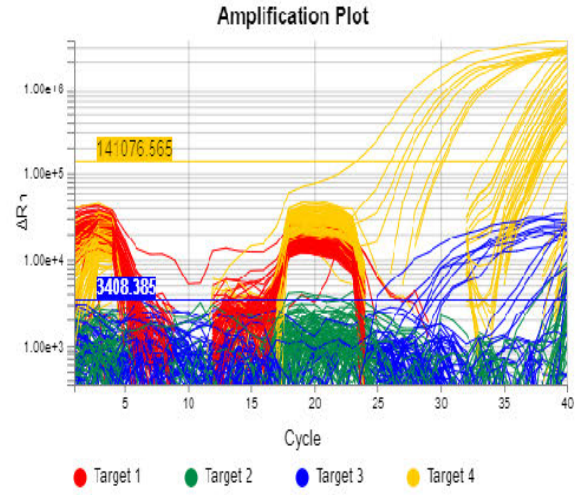
Target 2
Target 3
Target 4


Amplification Plot
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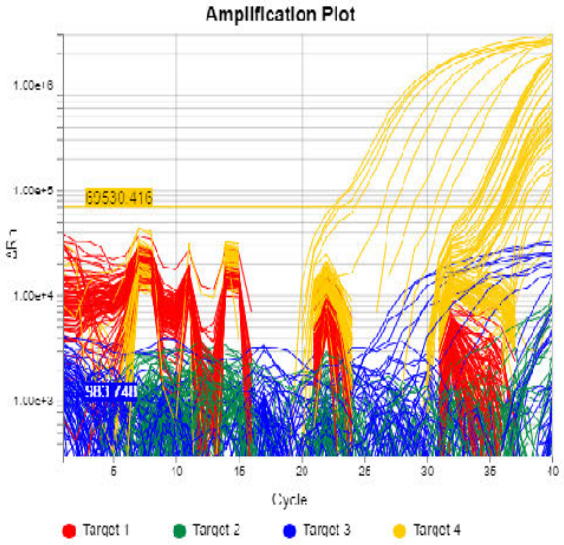
3408.385
ΔRn
Cycle
Target 1
Target 2
Target 3
Target 4


Amplification Plot
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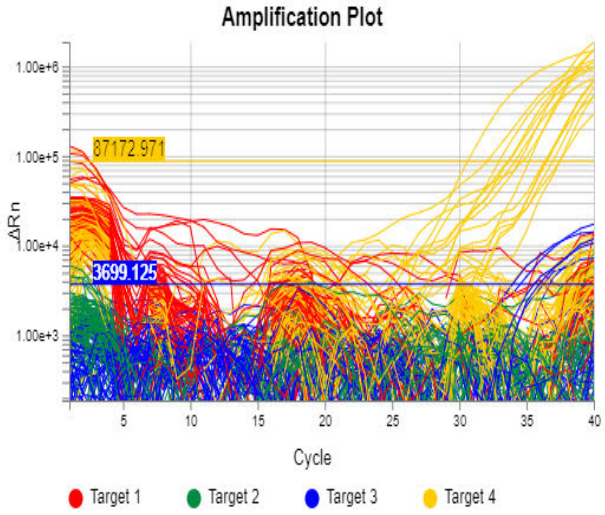
ΔRn
Cycle
Target 1
Target 2
Target 3
Target 4


Amplification Plot
87172.971
3699.125
ΔRn
Cycle
Target 1
Target 2
Target 3
Target 4

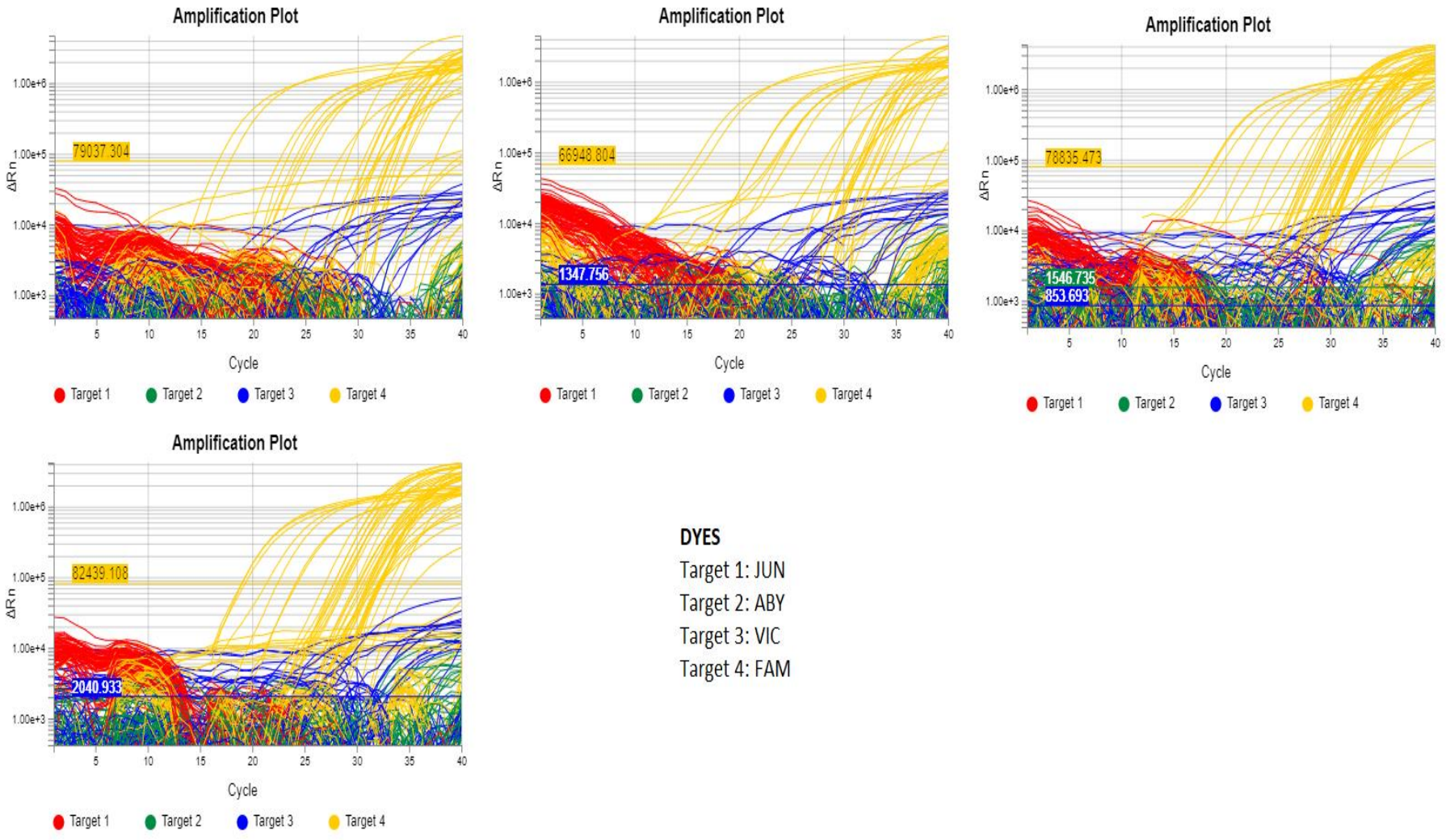


Figure 1: Amplification plots for miRNA expression profiles for the studied panel.

APPENDIX E

Table 1: Comparisons of miRNA expression levels between different age groups and gender of the study participants

	Healthy controls (n=23)						**CHBV samples (n=50)**					
	Age group			Gender			Age group			Gender		
	≤31	>31	p-value	Female	Male	p-value	≤37	>37	p-value	Female	Male	p-value
Number	15	8		16	7		27	23		21	29	
hsa-miR-15b-5p	-0.18 (1.6)	0.12 (1.71)	0.83	-0.41 (1.51)	0.57 (1.85)	0.77	1.76 (0.71)	2.08 (1.07)	0.16	1.77 (0.65)	2.02 (1.09)	0.35
hsa-miR-20a-5p	0.24 (0.84)	-0.08 (1.54)	0.97	-0.22 (1.28)	0.32 (0.79)	0.28	1.68 (1.16)	1.99 (1.41)	0.19	1.77 (0.89)	1.88 (1.7)	0.22
hsa-miR-29a-3p	0.03 (1.01)	0.12 (0.91)	0.43	0.02 (1.04)	0.14 (0.51)	0.82	2.49 (1.11)	2.58 (0.95)	0.83	2.51 (0.75)	2.49 (1.49)	0.69
hsa-miR-122-5p	-0.05 (1.07)	-0.08 (1.40)	0.47	0.02 (0.90)	-0.05 (0.34)	0.41	3.42 (1.84)	3.43 (1.68)	0.89	3.44 (1.45)	3.24 (1.98)	0.85
hsa-miR-125b-5p	0.13 (0.81)	0.04 (0.36)	0.78	0.007 (0.79)	0.13 (0.34)	0.92	2.39 (0.47)	2.43 (1.02)	>0.99	2.43 (0.57)	2.39 (1.54)	0.80
hsa-miR-181b-5p	-0.05 (0.64)	0.20 (1.36)	0.24	0.01 (0.92)	0.06 (0.69)	0.82	2.22 (0.75)	2.36 (1.02)	0.25	2.22 (0.93)	2.29 (1.26)	0.45
hsa-miR-192-5p	0.13 (0.92)	0.26 (0.75)	0.39	0.13 (0.77)	0.19 (0.62)	0.72	2.46 (1.78)	2.41 (1.48)	0.63	2.46 (1.37)	2.37 (2.04)	0.89
hsa-miR-193b-3p	0.28 (1.03)	0.17 (0.67)	0.78	0.09 (1.43)	0.49 (0.63)	0.22	0.96 (1.60)	1.40 (1.71)	0.38	1.19 (1.18)	0.98 (2.06)	0.79
hsa-miR-194-5p	-0.24 (1.62)	0.27 (0.54)	0.32	-0.05 (1.25)	0.28 (1.40)	0.45	1.84 (1.10)	2.01 (1.08)	0.54	1.95 (0.91)	1.87 (1.14)	0.82

CHBV, chronic hepatitis B virus

Relative expression levels (=2^- (ΔCt of target miRNA– arithmetic mean of ΔCt for the control group)) are shown as median, the expression values are relative to the endogenous control expression level. The *p*-values are greater than 0.05.

APPENDIX F

Table 1: Comparison of miRNA **expression levels between different groupings of clinical biomarkers.**

	Study group			**HBV Viral load (Log10 IU/ml)**			**HBeAg status**			**ALT Levels (U/l)**			**HIV viral load (Log10 IU/ml)**		
	CHBV patients	Healthy controls	*P* value	≤3	>3	*P* value	Positive	Negative	*P* value	≤35	>35	*P* value	≤3	>3	*P* value
Number	50	23		32	18		37	13		26	24		30	20	
hsa-miR-15b-5p	1.9 (0.91)	-0.18 (1.6)	**<0.0001**	1.89 (0.94)	1.97 (0.94)	0.75	1.76 (0.69)	2.45 (1)	**0.0054**	1.89 (0.88)	1.97 (1.04)	0.41	1.9 (0.79)	1.92 (0.91)	0.75
hsa-miR-20a-5p	1.83 (1.38)	0.24 (1.27)	**<0.0001**	1.78 (0.94)	2.49 (1.74)	0.26	1.76 (0.9)	2.69 (1.54)	0.07	1.98 (1.05)	1.77 (1.75)	>0.99	1.78 (1.17)	1.93 (1.52)	0.76
hsa-miR-29a-3p	2.51 (1.06)	0.03 (0.76)	**<0.0001**	2.43 (0.99)	2.69 (1.42)	0.08	2.40 (0.87)	2.74 (1.14)	0.07	2.55 (0.85)	2.46 (1.19)	0.89	2.50 (1.29)	2.58 (1.19)	0.79
hsa-miR-122-5p	3.42 (1.64)	-0.05 (0.53)	**<0.0001**	2.56 (1.31)	3.74 (1.18)	**0.0001**	3.43 (1.49)	2.56 (2.22)	0.88	3.33 (1.61)	3.47 (1.85)	0.88	3.43 (1.92)	3.26 (2.46)	0.69
hsa-miR-125b-5p	2.41 (0.88)	0.10 (0.6)	**<0.0001**	2.35 (0.75)	2.51 (1.53)	0.19	2.31 (0.81)	2.65 (1.35)	0.14	2.46 (0.51)	2.38 (1.78)	0.75	2.51 (0.74)	2.32 (0.71)	0.48
hsa-miR-181b-5p	2.22 (0.85)	0.06 (0.75)	**<0.0001**	2.23 (1)	2.22 (0.98)	0.99	2.22 (0.85)	2.58 (1.06)	**0.03**	2.22 (1.06)	2.22 (1.23)	0.40	2.22 (1.20)	2.22 (0.67)	0.98
hsa-miR-192-5p	2.44 (1.64)	0.13 (0.74)	**<0.0001**	1.94 (1.34)	3.07 (1.72)	**0.0003**	2.37 (1.39)	2.98 (2.31)	0.22	2.46 (1.43)	2.25 (1.9)	0.39	2.35 (1.84)	2.67 (1.52)	0.69
hsa-miR-193b-3p	1.14 (1.46)	0.18 (0.72)	**<0.0001**	0.82 (1.08)	2.14 (1.97)	**0.0002**	1.02 (1.52)	1.14 (1.62)	0.49	1.04 (1.42)	1.54 (1.84)	0.73	1.06 (2.67)	1.32 (1.69)	0.31
hsa-miR-194-5p	1.93 (1.08)	0.22 (1.21)	**<0.0001**	1.78 (0.95)	2.01 (1.75)	0.08	1.84 (1.16)	2.17 (0.91)	0.20	1.85 (0.90)	2.06 (1.37)	0.16	1.83 (1.19)	2.01 (1.08)	0.46

CHBV, chronic hepatitis B virus; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine transaminase; HIV, human immunodeficiency virus. Relative expression levels (=2^- (ΔCt of target miRNA– arithmetic mean of ΔCt for the control group)) are shown as median, the expression values are relative to the endogenous control expression level. The bold p-values are significant (<0.05)

APPENDIX G

Table 1: Assessment of confounding effects in microRNA expression

Model		Study participants	
		Standardized Coefficients Beta	p-values
1	**hsa-miR-15b-5p**		
	AGE	0.07	0.43
	Gender	-0.10	0.24
	HBsAg status	0.70	**<0.001**
2	**hsa-miR-20a-5p**		
	AGE	0.04	0.66
	Gender	-0.15	0.06
	HBsAg status	0.71	**<0.001**
3	**hsa-miR-29a-3p**		
	AGE	0.06	0.46
	Gender	-0.06	0.45
	HBsAg status	0.74	**<0.001**
4	**hsa-miR-122-5p**		
	AGE	0.06	0.42
	Gender	0.03	0.71
	HBsAg status	0.82	**<0.001**
5	**hsa-miR-125b-5p**		
	AGE	0.04	0.63
	Gender	-0.07	0.36
	HBsAg status	0.74	**<0.001**
6	**hsa-miR-181b-5p**		
	AGE	0.12	0.13
	Gender	-0.07	0.40
	HBsAg status	0.71	**<0.001**
7	**hsa-miR-192-5p**		
	AGE	-0.01	0.94
	Gender	-0.03	0.74
	HBsAg status	0.72	**<0.001**
8	**hsa-miR-193b-3p**		
	AGE	0.02	0.82
	Gender	-0.05	0.65
	HBsAg status	0.50	**<0.001**
9	**hsa-miR-194-5p**		
	AGE	0.08	0.33
	Gender	-0.03	0.75

	HBsAg status	0.71	**<0.001**