# The pathophysiology of cholesterol gallstones amongst Black South African women living with HIV


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## DECLARATION

I, **Suman Mewa Kinoo**, declare as follows:

i. The research reported in this thesis, except where otherwise indicated, is my original work.

ii. This thesis has not been submitted for any degree or examination at any other university.

iii. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

iv. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

   a. their words have been re-written but the general information attributed to them has been referenced;

   b. where their exact words have been used, their writing has been placed inside quotation marks, and referenced.

v. That my contribution to the project was as follows: Collection of all demographic parameters, specimens, and consents from patients. Interpretation of analyzed data and write up of all manuscripts for publication, literature review and synthesis of this thesis.

vi. That the contributions of others to the project were as follows: Storage and analysis of data by the Department of Medical Biochemistry, UKZN. Intellectual contributors to the write up of all manuscripts were Dr Savania Nagiah, Professor Anil Chuturgoon, and Professor Bhugwan Singh.

**Signed: ……**  **…….** **Date: 31 March 2021**

**Suman Mewa Kinoo**

## DEDICATION

I dedicate this thesis to:

**My parents Verod and Lalitha Mewa Kinoo**

for all the hardships you have endured and sacrifices you have made to give me an education,

for recognizing the potential in me and encouraging me to further my studies,

for your continued love and support throughout my career

**My wife Shirelle Assaram**

for your constant love, support and motivation and constant encouragement to pursue this degree,

for the enormous sacrifices you have made allowing me time for this project even amidst the birth of our child

**My daughter Sadhvi Mewa Kinoo**

for bringing a sated joy to my life

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## PUBLICATIONS

Manuscripts submitted for this thesis under review:

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Mewa Kinoo S, Chuturgoon AA, Singh B, Nagiah S. **Hepatic expression of cholesterol regulating genes favour increased circulating low-density lipoprotein in HIV infected patients with gallstone disease: A preliminary study.** BMC infectious disease. Submission ID 4d005632-284f-4741-a5a7-6ee6dc5cfb4c. Manuscript under review.

Mewa Kinoo S, Naidoo P, Singh B, Chuturgoon AA, Nagiah S. **Dysregulation of hepatic nuclear regulators of bile acid metabolism in HIV-infected women with gallstones**. Journal of Physiology and Biochemistry. Submission ID: a7a1bef2c6ba298d. Manuscript under review.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| RNA: | Ribonucleic acid |
| DNA: | Deoxyribonucleic acid |
| miRNA: | Micro RNA |
| mRNA: | Messenger RNA |
| SNP: | Single Nucleotide Polymorphism |
| HIV: | Human immune deficiency virus |
| ART: | Anti-retroviral therapy |
| FDC: | Fixed drug combination |
| GD: | Gallstone disease |
| CVD: | Cardiovascular disease |
| BMI: | Body mass index |
| HDL: | High-Density Lipoprotein |
| LDL: | Low Density Lipoprotein |
| VLDL: | Very low-density lipoproteins |
| CCK: | cholecystokinin |
| BA: | Bile Acid |
| SR-BI: | Scavenger Receptor Class B Type I |
| ABCA1: | ATP-Binding Cassette Transporter A1 |
| ApoB: | Apolipoprotein B |
| ApoE: | Apolipoprotein E |
| LDL-R: | Low Density lipoprotein receptor |
| HMGR: | 3-Hydroxy Methyl-Glutaryl Coenzyme A Reductase |
| ACAT: | Acyl-coenzyme A Cholesterol Acyltransferase |
| SREBP: | Sterol Regulatory Element Binding Proteins |
| PPARs: | Peroxisome Proliferator-Activated Receptors |

| | |
|---|---|
| ABCG: | ATP-Binding Cassette, Subfamily G |
| SHP: | Small Heterodimeric Partner |
| LRH1: | Liver Receptor Homologue |
| HNF: | Hepatic Nuclear Factor |
| CYP7A1: | Cytochrome P450 7A1 |
| LXR: | Liver X Receptors |
| FXR: | Farnesoid X Receptor |
| NRTI: | Nucleoside reverse transcriptase inhibitors |
| NNRTI: | Non-Nucleoside reverse transcriptase inhibitors |
| PI: | Protease inhibitor |
| InSTI: | Intergrase strand transfer inhibitors |
| Nef: | Negative Regulatory Factor |

## ABSTRACT

### Background

Thirteen percent of South Africans are living with HIV and of those infected, 52% are on antiretroviral therapy (ART). ART has changed the course of this terminal illness to one of a chronic illness. However, the longer life span of people living with HIV has brought about numerous metabolic disorders particularly with change in cholesterol metabolism and risk of cardiovascular disease. Gallstone disease (GD) is also known to be triggered by cholesterol metabolism changes; thus, it is postulated that people living with HIV and ART may be at risk for developing gallstones as well. In South Africa (SA), there is evidence of an increase incidence of GD in black South Africans, a disease once with a low incidence amongst this population group which makes up over 80% of the country's population. GD also ranks as one of the world's most expensive disorders to health care systems and thus investigating a causative relationship between HIV, ART and GD has relevance to reduce the burden on our already constrained health care system in SA.

### Aim

The aim of this study was to determine differences in clinical profiles and regulators of hepatic cholesterol and bile acid metabolism in HIV+ve Black South African women on ART presenting with gallstones compared to HIV-ve Black South African women with gallstones.

### Methods

A case series study was conducted amongst all Black South African women undergoing cholecystectomy for gallstone disease over a 1-year period at King Edward VIII Hospital, Durban, SA. A total of 52 patients (34 HIV-ve and 18 HIV+ve) were assessed. Classical risk profiles (age, BMI, children, family history) and lipogram levels. (LDL-c, HDL-c, triglycerides, total cholesterol) were compared between the HIV+ve and HIV-ve women. Categorical variables were tested using either the Fisher's exact test or Pearson's Chi-square test. Means were compared using independent t-tests. For non-normally distributed data, the Mann-Whitney test was used. Statistical tests were two-sided, and p values of less than 0.05 were considered as statistically significant. Liver biopsies from five HIV+ve women and five HIV-ve women were analyzed for hepatic expression of key genes in cholesterol metabolism (*LDLr, HMGCR, ABCA1*) and transcriptional regulators of these genes (microRNA-148a, *SREBP2*) using quantitative PCR. The same five HIV+ve and five HIV-ve women were evaluated for gene expression of *CYP7A1*, *HNF1α*, *HNF4α*, *LXRb*, miR-194-5p and miR-122*_1 using RT-qPCR. Messenger RNA and miRNA levels were reported as fold change expressed as $2^{-\Delta\Delta Ct}$ (RQ min; RQ max). Fold changes >2 and <0.5 were considered significant.

### Results

The median age of HIV+ve vs HIV-ve women was 35 years and 50 years respectively (p=0.015). The HIV-ve group had a statistically significant number of patients in the overweight/obese category (BMI > 25kg/m2) compared to the normal weight category (BMI <25kg/m2) (p<0.001). The number of obese women in the HIV+ve group however did not reach statistical significance. Circulating total cholesterol was elevated in the HIV+ve group with significantly elevated LDL-c levels (3.16±0.64mmol/L) relative to uninfected women (2.10±0.74mmol/L; p=0.04). A scavenging receptor for LDL-c, LDLr was significantly decreased (0.18-fold) in this group, possibly contributing to higher LDL-c levels. Transcriptional regulator of LDLr, SREBP2 was also significantly lower (0.13-fold) in HIV+ve women. Regulatory microRNA, miR-148a-3p, was reduced in HIV+ve women (0.39-fold) with a concomitant increase in target ABCA1 (1.5-fold), which regulates cholesterol efflux. HIV+ve women displayed higher CYP7A1 [2.078-fold (RQ min: 1.278; RQ max: 3.381)], LXRb [2.595-fold (RQ min: 2.001; RQ max: 3.000)] and HNF1α [3.428 (RQ min: 1.806; RQ max: 6.507] levels. HNF4α [0.642-fold (RQ min: 0.266; RQ max: 1.55)], miR-194-5p [0.527-fold (RQ min: 0.37; RQ max: 0.752)] and miR-122*_1 [0.595-fold (RQ min: 0.332; RQ max: 1.066)] levels were lower in HIV-ve women.

**Conclusion**

HIV+ve women do not conform entirely to the normal known risk profile for GD. Black South African HIV+ve women with GD were significantly younger. Black South African HIV-ve women conform to the known risk factor of obesity with a statistically higher BMI whilst HIV+ve women do not. HIV+ve women also had fewer 1st degree relatives with GD compared to HIV-ve women, and less oestrogen exposure. HIV+ve women have a significant increase in circulating LDL-c coupled with reduced mRNA expression of hepatic *LDLr*. However, the suppression of miR-148, an epigenetic regulator of LDLr, was downregulated in the HIV+ve group. This would indicate a possible alternate pathway in the downregulation of LDLr in HIV+ve women linked with raised LDL-c and gallstone formation and will require further investigation. MiR-148a however did appear to regulate ABCA1 with an inverse relationship being observed in the HIV+ve woman. HIV+ve women displayed elevated expression of CYP7A1, HNF1α and LXRb. This could have been further influenced by ART and aging. HNF4α, which is known to cause upregulation of CYP7A1, was suppressed with upregulation of CYP7A1 and LXR, known to cause downregulation of CYP7A1 in humans as opposed to mice, also had the opposite effect in HIV+ve women. The best theoretical explanation for this will be an interruption in the enterohepatic circulation, as evident by HIV+ve patients known to have chronic inflammatory and relative malabsorptive disorders of the ileum, which may result in upregulation of CYP7A1 to produce more bile salts. However, these conclusions are drawn from a case series. Larger cohort studies are required into the effects of HIV on GD and the impact of ART on GD in order to put strategies in place to curb this disease process and reduce the morbidity from it and reduce the cost to the overburdened health system.

# CHAPTER 1: INTRODUCTION

## 1.1 Background

South Africa has a population of 57.7 million and it is estimated that 7.52 million (13,1%) are living with Human Immune deficiency virus (HIV), the highest than any other country (1). Almost 26% of the infected reside in the province of KwaZulu Natal (1). Of those infected, 3.9 million patients (52%) are on antiretroviral therapy (ART) (1).

The introduction of ART to treat HIV has changed a previously terminal illness to a chronic, manageable disease. This extended life span has seen an unprecedented number of patients living longer with HIV – this has seen the development of adverse outcomes resembling metabolic syndrome (hypertriglyceridemia, low high-density lipoprotein cholesterol, and insulin resistance), which increases risk for cardiovascular disease (CVD) (2). The pathology for this metabolic syndrome varies, but it is thought that the pathology in HIV positive (+ve) patients differ from HIV negative (-ve) patients as classical characteristics e.g. increased waist circumference occurs less frequently. The host response to HIV infection, specific ARTs, mitochondrial toxicity, and HIV-associated altered fat metabolism are factors contributing to these metabolic outcomes. As health is restored, those that are genetically predisposed, will manifest with metabolic syndrome. The vulnerability to higher incidences of CVD in the absence of known risk factors such as smoking in these HIV+ve patients is thus higher (2).

The pathogenesis of gallstone disease (GD) is well established in HIV-ve patients. However, unlike metabolic syndrome specific to HIV, gallstones specific to HIV has not been studied. Gallstone formation occurs due to supersaturation of cholesterol in bile, and this supersaturation has been attributed to risks commonly referred to the five “F”s (Female, Fat, Fertile, Forty and Family history) (3). The observed metabolic changes in HIV+ve patients may contribute to GD, as these patients are known to have increased circulating lipid levels. The evidence for this is that lipid levels rise concomitantly with HIV RNA levels independent of ART. Further, ART use increases lipid release into the bloodstream, thus both resulting in hyperlipidemia (4). This may in turn make HIV+ve patients vulnerable to higher incidences of GD without known risk factors such as the 5 “F’s, however this has not been studied before.

## 1.2 Problem Statement

The incidence of gallstones in Sub-Saharan Africans is reportedly one of the lowest in the world, to the extent of being labeled as “almost non-existent in the Bantu population of sub Saharan Africa” (5), compared to the rest of the developed world where the incidence averages about 20% (6). These clinical observations were made in small sample sizes and date back more than 30 years (7). However in a recent study by Khan et al (2020) (8) studying cholecystectomies in public sector hospitals in South Africa accounting for 82% of the country’s population which makes up 87% Black Africans (1) they showed an increase of cholecystectomies by 65% between the years 2004 to 2014 (8). The anecdotal evidence of increasing prevalence in recent times, especially in Black Africans is mostly attributed to urbanization together with urbanized diets and increasing obesity particularly amongst black African women (9) . As Khan’s study (2020) was only a review of cholecystectomy specimens, HIV results were thus lacking and no link or relation to the increase of HIV during those years of study could be drawn. GD and its complications (cholecystitis, biliary colic, gallstone pancreatitis, gallbladder cancer, and obstructive jaundice) are treated by surgical intervention, making it one of the world’s most expensive diseases (10). As a result, this places a huge economic burden and immense pressure on the already resource constrained health care system in South Africa especially with recent evidence mounting to an almost doubling cholecystectomy rate in the last decade (8). This rise in gallstone incidence is paralleled with the rise in incidence of HIV and the judicious role out of ART. HIV and ART drugs are known to affect cholesterol homeostasis, however the effect on gallstone formation has not been studied. Detection of modifiable risk factors for gallstone formation specific to our population especially linked to the recent rise in incidence is crucial in South Africa in the era of HIV and ART. This will provide evidence to guide healthy policy makers and implementers of health interventions on the most appropriate intervention for reducing gallstones and the complications associated with it to reduce the high disease burden on our resource constrained health care system in South Africa. Further, the identification of molecular mechanisms of pathology allow investigation into targeted intervention strategies and possible biomarker discovery.

## 1.3 Hypothesis

The main hypothesis is HIV+ve women on ART display different risk factors and pathological profiles to HIV-ve women presenting with gallstone disease.

## 1.4 Research Questions

### *1.4.1 Main research question*

Are there differences in the pathophysiology and risk factors of gallstone development amongst HIV +ve and HIV-ve black South African women?

### *1.4.2 Specific research questions*

1. Are there differences in clinical, biochemical and demographic parameters between HIV+ve and HIV-ve women presenting with gallstones?
2. Are genetic and epigenetic regulators of hepatic cholesterol metabolism differentially regulated in HIV+ve women presenting with gallstones compared to HIV-ve women?
3. Are genetic and epigenetic regulators of hepatic bile acid synthesis differentially regulated in HIV+ve women presenting with gallstones compared to HIV-ve women?

## 1.5 Aims, Objectives and ethical approval of this study

### *1.5.1 Main Aim*

To determine differences in clinical profiles and regulators of hepatic cholesterol and bile acid metabolism in HIV+ve Black South African women on ART presenting with gallstones compared to HIV-ve Black South African women with gallstones.

### *1.5.2 Objectives*

1. To compare the classical risk profiles (age, BMI, children, family history) and lipogram (LDL-c, HDL-c, triglycerides, total cholesterol) data between HIV+ve women with gallstone disease and HIV-ve women with gallstone disease
2. To measure the hepatic expression of genes involved in cholesterol homeostasis (LDLR, ABCA1, HMGCR) and the transcriptional regulators of these genes (SREBP2, miR-148a) in HIV+ve women with gallstones relative to HIV-ve women with gallstones
3. To measure the hepatic nuclear factors (HNF4a, HNF1a, LXR) that regulate bile acid synthesis (CYP7A1) in HIV+ve women with gallstones relative to HIV-ve women with gallstones

### *1.5.3 Ethical approval*

Full ethics approval was granted by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC), reference number BE276/16 (addendum 1). Permission to conduct the research was granted by King Edward VIII (Durban, South Africa) hospital management (addendum 2).

## 1.6 Overview of this thesis

This thesis consists of 5 chapters (including the current chapter)

### *1.6.1 Chapter 1*

This chapter presents the background, aim, objectives and significance of the study as well as providing a general outline of the thesis, its structure, the literature review and methodology.

### *1.6.2 Chapter 2*

This chapter presents the results of a case series study, comparing known risk factors of gallstone formation in HIV+ve and HIV-ve Black South African women presenting with symptomatic stones for cholecystectomy. The study sought to evaluate if the risk factors in HIV+ve women differ to that of HIV-ve women. The manuscript is presented in the form of a journal article titled "Symptomatic Gallstones and HIV in Black South African Women: Changing trends of gallstone disease?". This manuscript is currently under review at the Southern African Journal of HIV Medicine. Submission ID 1208.

### *1.6.3 Chapter 3*

This chapter presents the results of a case series study, comparing hepatic expression of cholesterol homeostasis genes and microRNA (miRNA) regulators of the selected genes in HIV+ve and HIV-ve women presenting with symptomatic gallstones. This study sought to evaluate hepatic expression of key genes in cholesterol metabolism (*LDLr*, *HMGCR*, *ABCA1*) and transcriptional regulators of these genes (microRNA-148a, *SREBP2*) in HIV+ve women on ART presenting with gallstones. The manuscript is presented in the form of a journal article titled "Hepatic expression of cholesterol regulating genes favor increased circulating low-density lipoprotein in HIV infected patients with gallstone disease: A preliminary study". This manuscript is currently under review at the BMC Infectious diseases Journal. Submission no. 4d005632-284f-4741-a5a7-6ee6dc5cfb4c

### *1.6.4 Chapter 4*

This chapter presents the results of a case series study, evaluating the expression of HNF1α, HNF4α, LXRb and miRNAs (HNF4α specific: miR-194-5p and miR-122$^{*}$_1) that regulate CYP7A1 transcription in HIV+ve Black South African women on combination antiretroviral therapy (cART) and

presenting with gallstones relative to HIV-ve women with gallstone disease. The manuscript is presented in the form of a journal article titled “Dysregulation of hepatic nuclear regulators of bile acid metabolism in HIV-infected women with gallstones”. This manuscript is currently under review at Digestive Diseases and Sciences. Submission ID: DDSJ-D-20-03193.

### *1.6.5 Chapter 5*

This chapter is a discussion of study findings, strengths and limitations as well as a conclusion which contains some observations on the findings of this study, suggests futures plans and makes recommendations for future research, based on the findings of this study.

## 1.7 Literature Review

### *1.7.1 Introduction*

The formation of cholesterol gallstones is complex and usually involves a combination of genetic and environmental factors including lifestyle. Most research into the actual cause and risk factors of gallstones are performed in Europe and some South American countries with a paucity of data in Africa. This oversight maybe in part due to the historically lower incidence amongst black South Africans (7,11) compared to the rest of the world, and part due to the focus on the four other concurrent health crisis, specifically HIV, maternal death, malnutrition and other non-communicable diseases (12). Traditionally, gallstones were a disease exclusive to Caucasians, however in South Africa, particularly amongst black South Africans evidence would suggest that there is a rapid increase in recent years (11). Urbanization together with urbanization of diet with an increase in prevalence of obesity amongst Black South Africans is currently the only anecdotal explanation for this increase, however other possible influential factors such as HIV have not been studied (8). The growing concern around metabolic disorders in HIV endemic settings further warrants evaluation of the effect of both the virus and its chronic treatment on the formation of gallstones in a population where gallstones was previously virtually non-existent. Further investigation into the epigenetic regulation of hepatic cholesterol metabolism in patients with gallstones together with a closer look at HIV and ART influence on cholesterol metabolism warrant more attention and research. The effects of HIV infection on cholesterol metabolism independent of ART, and the added effects of ART on cholesterol metabolism, poses a risk for cholesterol precipitation in bile and thus increases the risk of gallstone formation. Patients on protease inhibitors (PI's) have the greatest risk, but this does not negate the effect of first line therapy without PI's due to the direct effect of HIV and nucleoside reverse transcriptase inhibitors (NRTIs) on cholesterol homeostasis. Newer drugs such as Integrase inhibitors are promising and have less effect on cholesterol homeostasis. However, the exact mechanism of how HIV and ART directly affect gallstone formation is not known. Thus, further studies like these presented in this thesis on the effect of HIV and ART on cholesterol and bile acid metabolism might give us a better understanding and help curb this high disease burden of GD on our already strained health system.

### *1.7.2 Epidemiology of cholesterol gallstones*

The incidence of gallstones (the frequency of developing gallstones over a specific time period, usually expressed as per year) is difficult to illicit and thus there are is a lack of available data. The prevalence of gallstones (the number of individuals with gallstones at any point in time) is more commonly reported and largely based on ultrasound evaluation (13). The worldwide prevalence of gallstones varies amongst population groups affecting 10-15% of Caucasian adults in developed countries (6) and more than 85% are of the cholesterol type (14). However, reports range from as high as 70% in American Indians (15), to as low as 5% in Black Americans and sub-Saharan Africans (6). This low prevalence of gallstones

in Sub-Saharan Africa although quoted in more recent literature (5) are from studies dating back to 1989 (7). A recent review by Khan et al (2020) of cholecystectomies in South Africa would suggest that the prevalence may be much higher (8). What is constant however amongst all population groups is a female predominance mainly in the younger years (16).

### *1.7.3 Risk factors for cholesterol gallstones*

#### *1.7.3.1 Age*

Both human and mice studies have demonstrated that gallstone incidence increases with age. In humans it is more common in older age categories peaking at age 34 and continuing to rise above 75 (17). Age results in greater intestinal absorption of cholesterol, greater biliary excretion of cholesterol and reduced hepatic synthesis and biliary excretion of bile salts. Also, there is a relative hypoperfusion of the gallbladder wall resulting in gallbladder dysfunction, together with decreased gallbladder wall contraction. These findings have been demonstrated in mice by Wang et al. (2002) (18). They showed that in mice with lithogenic diets (1% cholesterol, 0,5% cholic acid and 15% butter fat), there is up regulation of HMG-CoA reductase activity and a down regulation in 7 alpha-hydoxylase (CYP7A1) activity with increasing age (18).

#### *1.7.3.2 Gender*

Oestrogen is the hormone that is linked with gallstone disease. Women of child bearing ages, pregnant women, women that are on hormone replacement therapy or had a previous history of high dose unopposed oestrogen (>50mcg) contraception, have 2-3 times higher incidence of gallstones. The high oestrogen levels in pregnancy are associated with increased cholesterol secretion in bile, whilst the high progesterone levels cause a decrease in bile salts and decreased contractility of the gallbladder wall resulting in precipitation of cholesterol (19). Males with cirrhosis of the liver have higher oestrogen levels and thus have increased incidence of gallstones (20).

#### *1.7.3.3 Weight*

Obesity and rapid weight loss are linked to increased gallstones formation. The amount of cholesterol synthesized and secreted in bile is directly proportionate to being overweight and thus it is not surprising that a direct proportionate relationship to the incidence of symptomatic gallstone per year to the body mass index (BMI) exists as well (21). In patients where BMI is adjusted for, weight cycling in these patients is also at an increased risk of gallstone formation with larger fluctuation and more weight cycles being associated with the highest risks (22). Whilst normalization of weight results in normal concentrations of cholesterol in bile, rapid weight loss either non operatively or operatively in the form of gastric bypass surgery results in gallstone formation in greater than 50 % of patients. Mechanisms

involved are increased biliary saturation secondary to increased cholesterol mobilization, increased nucleation due to changes in bile arachidonate and glycoprotein concentrations, and elevated levels of mucin and calcium in bile (23).

*1.7.3.4 Diet and Exercise*

The amount of cholesterol in bile is increased with high levels of cholesterol intake. Refined carbohydrates are also found to increase cholesterol saturation in bile. Diets low in fibre, result in low intestinal transit time promoting absorption of secondary bile acids (24). An increase in cholesterol in bile due to decreased enteral stimulation and the decrease in bile salts from an interruption in the enterohepatic circulation results in the precipitation of cholesterol in bile in patients with prolonged fasting / parenteral nutrition (25). Long-term parenteral nutrition promotes gallbladder dilatation and hypokinesia (25).

Protective diets include 2-3 cups of coffee a day, small doses of alcohol and poly- and monounsaturated fats and nuts and vegetable protein (26–28). Regular vitamin C supplementation may be protective as vitamin C deficiency in guinea pigs leads to cholesterol supersaturation (29).

Men who perform 30min of endurance type exercise 5 times a week independent of weight loss have a reduced risk of symptomatic gallstones while women with recreational activity reduces their risk of cholecystectomies (30,31). Evidence for female patients being protective against gallstone by exercising are lacking.

*1.7.3.5 Dyslipidemia*

Cholesterol is in solution in bile due to specific percentage concentrations between itself together with bile salts and lecithin. An increase in cholesterol or a decrease in bile salts and lecithin, results in cholesterol precipitation and gallstone formation (3). There is a correlation between serum total cholesterol, Apolipoprotein B, C2, C3 and the amount of cholesterol saturation in bile. Also, there is a correlation with the levels of serum high-density lipoprotein (HDL) with the amount of lecithin and bile acids in bile (32). This indicates that serum cholesterol and low-density lipoprotein (LDL) may be an attributing factor to gallstone formation while serum HDL might be protective.

*1.7.3.6 Insulin resistance*

A number of studies have linked type 2 diabetes (insulin resistance diabetes), obesity and hyperlipidemia to cholesterol gallstone formation, the so-called metabolic syndrome or syndrome X (15,33). The basic pathophysiological cause of this syndrome is insulin resistance and results in coronary heart disease, stroke, type 2 diabetes and cholesterol gallstones. In control subjects with insulin resistance/ hyperinsulinemia, and in non-insulin dependent diabetes patients with insulin treatment, an increase in biliary cholesterol secretion has been demonstrated (34). The exact role of hyperinsulinemia on gallbladder emptying and mechanisms of bile secretion has not been evaluated.

### *1.7.4 Types of gallstones*

Gallstones are classified into three categories determined by cholesterol concentration viz, a cholesterol stone, a pigment stone and a mixed stone. If the cholesterol concentration is higher than 70%, these are classified as cholesterol stones. Cholesterol stones are by far the most common accounting for up to 75% of all gallstones (35). Pigment stones (contain <30% of cholesterol) are due to crystallization of calcuim bilirubinate and can occur in 2 forms; either black or brown. Black stones arise as a result of increased bilirubin usually from haemolysis whereas brown stones occur as a result of stasis and infection, most commonly outside the gallbladder within the bile ducts (36). Biliary cholesterol super saturation is thus the main prerequisite for the formation of cholesterol gallstones and therefore stands to reason that an in depth look at the mechanism of cholesterol precipitation and the risk factors contributing toward this be reviewed.

### *1.7.5 Mechanism of cholesterol gallstone formation*

#### *1.7.5.1 Supersaturation of cholesterol in bile*

Cholesterol is integral for cellular function. It plays an important role in the production of steroid hormones (including testosterone and oestrogen) and vitamin D, and supports all cell membranes in the body (37). It is also the starting point for bile salts for the breakdown and absorption of the body's ingested fats. The liver is central to regulation of cholesterol in the body. Not only does it manufacture cholesterol and thus is the source of most of the body's cholesterol, it also removes cholesterol from the body in the bile where it can be excreted in the faeces.

Bile is 97% water, 0.7% bile salts, 0.2% bilirubin and 0.51% fats (cholesterol, fatty acids, and lecithin). Cholesterol is totally insoluble in water and highly soluble in bile (38). This is achieved with micelle formation together with bile salts and lecithin. In cholesterol gallstones when cholesterol supersedes its saturation point, it results in cholesterol microcrystal formation which inevitably leads to gallstone formation (38). This can either be due to an increase in cholesterol content in bile or a decrease in bile salt and lecithin content in bile, or a combination of both. Admirand et al. (1968) eloquently demonstrated this in a triangular relationship between the three components of bile (cholesterol, bile salts and lecithin) each as a percentage of the total quantity of all three components with other components being a constant (3) (figure 1.1). They plotted a line representing the maximum solubility of cholesterol in bile with varying proportions of lecithin and bile salts (red line in figure 1.1). They found that bile of normal patients without gallstones had a certain percentage combination of the three components which kept them in micelle formation and prevented gallstone formation (plotted under the red line in figure 1) while bile from patients with gallstones had percentage combinations resulting in microcrystal and cholesterol precipitation (plotted outside the red line in figure 1.1).

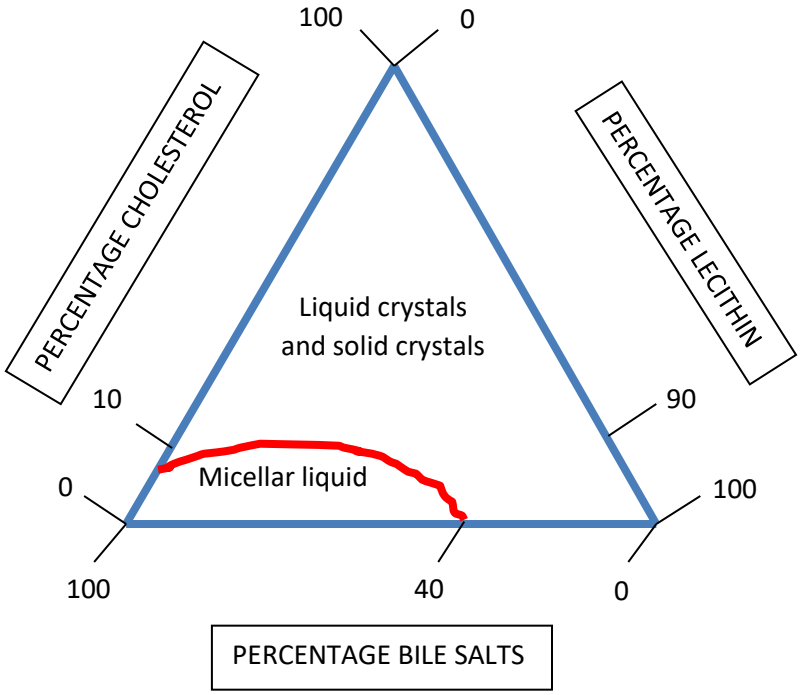


**Figure 1. 1: Admirand's triangular percentage relationship of cholesterol, bile salts and lecithin in bile. (adapted from Admirand et al 1963)**

*A combination of these 3 constituents of bile falling under the red line keeps cholesterol on solution in micelles, however combinations outside this line results in cholesterol precipitation and gallstone formation.*

*1.7.5.2 Impairment in gallbladder function*

Gallbladder contraction removes cholesterol crystals and mucus clumps, and absorbs water preventing the formation of stones. Cholesterol gallstones occur due to an impaired gallbladder wall contraction and wall inflammation. Cholesterol supersaturation in bile gets incorporated into the gallbladder smooth muscle cell impairing contractility thereby allowing more time for nucleation for cholesterol gallstone formation. Meal-induced release of cholecystokinin (CCK) from the duodenum is the principal factor driving gallbladder smooth muscle contraction. CCK-1-receptors-deficient mice result in a significantly higher prevalence of cholesterol gallstones (39). Pregnancy, obesity, rapid weight loss, diabetes mellitus, and total parenteral nutrition are known to reduce CCK release from duodenum and predisposes to cholesterol gallstone formation (40).

The gallbladder wall is exposed to detergent bile salts, unesterified cholesterol and bacteria, all of which could induce inflammation. In mice, Helicobacter subspecies causes wall inflammation playing a significant role in the pathophysiology of cholesterol gallstone formation, however their role in human gallstone pathogenesis remains to be defined despite various helicobacter species and antibodies to

*Helicobacter hepaticus* being detected at increased frequency in human gallbladders of gallstone patients (41).

*1.7.5.3 Impairment of enterohepatic circulation*

The enterohepatic circulation refers to the movement of bile acid (BA) from the liver to the small intestine and back to the liver. In the liver BA's traverse the hepatocyte and are actively secreted into canalicular bile, completing the enterohepatic cycle. Ninety-five percent of BA are absorbed in the small intestine and the remaining 5% plays an important part in microbiological transformation in the large intestine and are lost in the faeces (42). Beyond BA's role in solubilizing lipids for absorption, it has many physiological roles and thus maintaining the equilibrium of the enterohepatic circulation therefore making secretion and absorption rates critical. An increase in absorption may contribute to the development of nonalcoholic liver steatosis, whereas a malabsorption results in increased hepatic bile acid synthesis and increased intestinal luminal concentrations which may lead to bile acid diarrhea (43).

Patients with impaired intestinal mobility and a prolonged intestinal transit time are at risk for gallstones. These patients were found to have an increase in secondary hydrophobic bile salt deoxycholate and an increase in total and gram-positive anaerobes and more 7α-dehydroxylating activity in the caecum compared to normal subjects (44). Ileal disease, resection or bypass results in increased calcium and bilirubin with normal cholesterol levels, thus resulting in pigment rather than cholesterol stones (45). However chronic distal intestinal infection with *Helicobacter* species other than *H. Pylori* has shown to increase cholesterol gallstone formation by nucleating cholesterol supersaturated bile (41). Bile salt malabsorption is another factor for cholesterol gallstones. Common examples are excess dietary carbohydrates and prolonged TPN which causes ileal atrophy (46,47).

### *1.7.6 Molecular and metabolic pathogenesis of biliary cholesterol secretion and gallstone formation*

The prevalence of GD varies worldwide according to ethnic population groups which suggests that a high prevalence of GD in differing ethnic groups is due to the presence of genetic factors. Environmental factors however will still play an important role in gene expression. The liver plays a critical role in the whole-body cholesterol homeostasis. The excess cholesterol in bile required for gallstone formation as described by Admirand (3) can occur in any 1 of 3 steps in the liver; cholesterol input to liver, cholesterol production in the liver, and cholesterol output from liver. Each of these various steps might be under genetic control encoding for proteins and enzymes and/or influenced through intermediate metabolic pathways linked to a variety of environmental factors responsible for amount of cholesterol that's eventually excreted into the bile (figure 1.2; table 1.1).

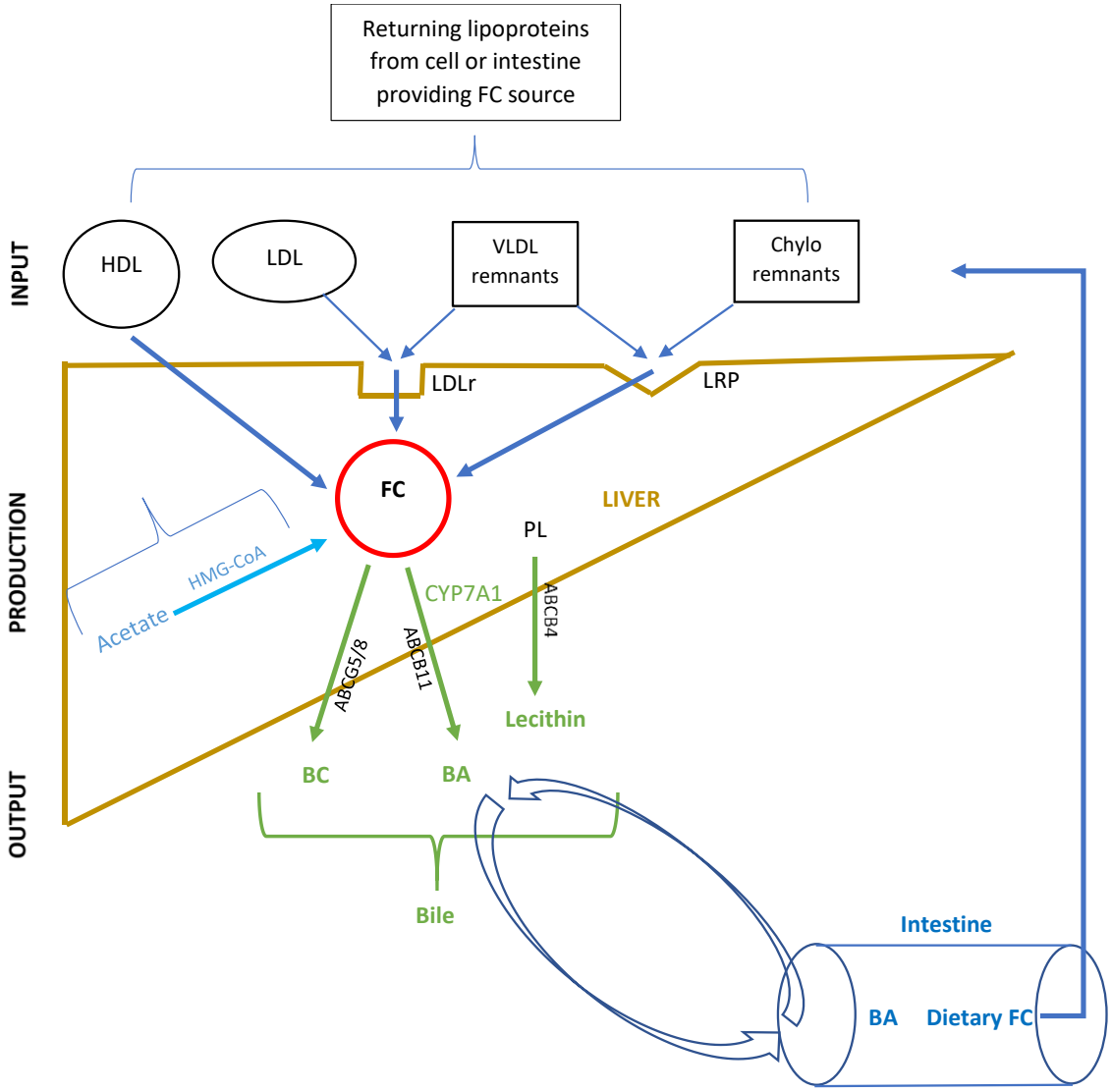


**Figure 1. 2: Representation of source of Bile cholesterol (BC), Bile Acids (BA), and Lecithin excretion in bile (prepared by author)**

*Free cholesterol (FC) in liver is from 2 major sources; liver production from acetate via HMG-CoA reductase (HMGR), and returning cholesterol to liver from peripheral and intestinal sources viz: High density lipoprotein (HDL), Low Density lipoprotein (LDL), Very Low Density lipoprotein (VLDL), and chylo remanants. These enter the liver via the LDL receptor (LDLr) and the LDL like receptor (LRP). Free cholesterol is cleaved by CYP7A1 in to bile acids. Some of the free cholesterol is excreted directed into bile as BC. It is thought HDL is a major contributor to BC. Lecithin is produced from Phosopholipids (PL). Biliary lipid secretion is mediated by different ABC transporters: ABCG5/G8 for BC, ABCB4 for PL and ABCB11 for BA*

**Table 1. 1: Coding of genes/proteins/receptors/miRNA/mRNA involved in cholesterol trafficking within the liver. (prepared by author)**

| Cholesterol | Gene/protein/ receptor/ miRNA/mRNA | Synthesis/ transport/metabolism | Function | Reference |
|---|---|---|---|---|
| **Input to liver** | *SR-BI* | HDL Transport | Plasma absorption of HDL | *Acton 1999* |
| | *ABCA1* | HDL Transport | Efflux of HDL from cell & transport | *Oram 2005* |
| | *Apo B* | LDL Transport | Ligand for LDL receptor | *Han 2000* |
| | *Apo E* | LDL Transport | Ligand for LDL receptor | *Amigo 2000* |
| | *LDL-R* | LDL Transport | Plasma absorption of LDL | *Dietschy 1993* |
| | miRNA148 | LDL-r (transcriptional) | Regulates LDL-r | *Goedeke 2015* |
| **Production in liver** | *HMGCoA-R* | Synthesis (enzyme) | cholesterol synthesis | *Ahlberg 1981* |
| | *ACAT 1&2* | Synthesis (enzyme) | Esterifies cellular cholesterol excess | *Chang 2001* |
| | *SREBP* | Synthesis (transcriptional) | FFA and choles homeostasis | *Brown 1997* |
| | *PPAR* | Synthesis (transcriptional) | Activates FFA metabolism genes | *Bertolotti 2006* |
| **Output from liver** | *CYP7A1* | BA synthesis | Regulator of BA production | *Schwarz 1998* |
| | *HNF 1&4* | BA transport | Transmembrane choles excretion | *Maher 2006* |
| | *FXR* | BA synthesis (transcriptional) | Regulator of bile acid uptake & synthesis | *Moschetta 2004* |
| | *LXR* | BA synthesis (transcriptional) | Regulator of bile acid uptake & synthesis | *Janowski 1999* |
| | *ABCG 5&8* | BA transport | Transmembrane choles excretion | *Hazard 2007* |
| | *SHP* | BA synthesis & transport | Regulator of bile acid uptake & synthesis | *Watanabe 2004* |
| | *LRH-1* | BA synthesis | Regulator of BA production | *Goodwin 2000* |
| **Lith genes** | *Lith 1* | | Bile salt export | *Wittenburg 2002* |
| | *Lith 2* | | | |

*Tabulated according to their respective level of action viz cholesterol input to liver, cholesterol production in liver and cholesterol output from liver in the form of bile acids. Scavenger Receptor Class B Type I (SR-BI). ATP-Binding Cassette Transporter A1 (ABCA1) Apolipoprotein B (ApoB). Apolipoprotein E (ApoE). Low Density lipoprotein receptor (LDL-R). MicroRNA-148a (miRNA 148a). 3-Hydroxy Methyl-Glutaryl Coenzyme A Reductase (HMGR). Acyl-coenzyme A Cholesterol Acyltransferase (ACAT). Sterol Regulatory Element Binding Proteins (SREBP). Peroxisome Proliferator-Activated Receptors (PPARs). ATP-Binding Cassette, Subfamily G, Member 5 and 8 (ABCG5/8). Small Heterodimeric Partner (SHP). Liver Receptor Homologue (LRH1)*

*1.7.6.1 Molecular and metabolic influence on cholesterol input to liver*

Lipids (cholesterol, triglycerol and phospholipids) are not soluble in water and therefore have to be transported in solution with a protein (apolipoprotein). This combination of lipids and protein for transport are called lipoproteins (48). There are 4 main types named according to their degree of lipid in relation to protein. Those with more lipids are referred to a high-density lipoprotein (HDL), those with fewer lipids are referred to as low density lipoprotein (LDL), and those with very less lipids are referred to as very low-density lipoproteins (VLDL). Those with very high lipid levels are called chylomicrons (49).

Bile and other enzymes within the intestine break down ingested fat into cholesterol and lipids. The enterocytes produce chylomicrons which transport this cholesterol and lipids via the thoracic duct, bypassing the liver, directly into the blood stream where triglycerides are extracted and utilized in muscle and stored in fat (50). The remaining cholesterol and lipids in the chylomicrons are then transported to the liver where the chylomicrons are endocytosed by the hepatocyte and cholesterol and lipids are extracted (51). These are then repackaged as VLDL in the liver and transported to muscle and fat. Here the muscle and fat further extract triglycerides from the VLDL converting it to LDL. When cells are low on cholesterol, they can now utilize cholesterol from the circulating LDL. When cells have excess cholesterol, they can package it into HDL and transport it to the liver where the excess cholesterol can be excreted into bile.

Cholesterol input to the liver occurs from returning cholesterol directly from dietary free cholesterol returning from the gut and by receptor mediated internalization of chylomicron remnants, VLDL and LDL and selective cholesterol uptake from HDL mediated by the scavenger receptor class b, type 1 (SR-BI) not depicted in figure 1.2 (52). Cholesterol obtained from HDL is selectively transferred to the hepatocyte and is used as a preferential source of cholesterol secreted into bile (53). Cholesterol is highly insoluble in water and thus high input may result in lithogenic states by the inability of hepatocytes to produce sufficient bile acids to eliminate sterols.

*1.7.6.1.1 Scavenger Receptor Class B Type I (SR-BI)*

SR-BI is a multifunctional receptor that has an affinity for HDLs and mediates uptake of cholesterol-esters selectively (54). It is known that the unesterified cholesterol secreted in bile is preferentially used from HDL via the SR-BI receptor pathway (55). This is evidenced by an overexpression of SR-BI receptor resulting in reduced plasma HDL and increased bile cholesterol secretion and an under expression of SR-BI having the opposite effect (53,56). This then suggests that SR-BI is a candidate gene in the reverse transport of cholesterol from plasma to bile in gallstone formation. However, the definitive relevance for gallstone formation has not been addressed directly and remains an area of controversy (57).

*1.7.6.1.2 ATP-Binding Cassette Transporter A1 (ABCA1)*

ABCA1 is an important regulator of reverse cholesterol transport (RCT) (58) which is the process of removing excess HDL from cells and transporting them to the liver for excretion in bile (59). Overexpression of ABCA1 in mice increases plasma HDL-cholesterol levels and hepatic delivery of HDL cholesteryl-esters and biliary cholesterol concentrations and regulators of ABCA1 have shown to prevent GD in rodents (60).

*1.7.6.1.3 Apolipoprotein B (ApoB)*

ApoB is the main protein of chylomicrons and LDL. There are two types. ApoB48 which is synthesized by the gut and ApoB100 which is synthesized by the liver. ApoB is a ligand for LDL-R. Abnormalities in ApoB are associated with changes in LDL-C which contributes to the formation of GD in certain population groups (61).

*1.7.6.1.4 Apolipoprotein E (ApoE)*

ApoE is the major protein of VLDL and minor protein of HDL. ApoE acts as a ligand for LDL. It plays a critical role in regulating biliary secretion of dietary cholesterol and diet-induced cholesterol gallstone formation in mice (62). There are three allelic variants of ApoE. ApoE2 ApoE3 and ApoE4. E2 carriers present low concentrations of LDL-C and provide protection against GD (63). E4 carriers present higher levels of LDL increase uptake of hepatic and biliary concentration of cholesterol and thus have high risk of GD (64).

*1.7.6.1.5 Low Density lipoprotein receptor (LDL-R)*

Cells have LDL-R's on their surfaces which bind to LDL and extract lipoprotein by endocytosis, and of all these cells, more than 80% of circulating plasma LDL is cleared by endocytosis at the hepatocyte (52). The LDL together with the receptor are endocytosed into the cell and then the receptor is released to be re-expressed at the cell surface before the LDL enters the lysosome to be degraded for use. Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a protein that decreases the expression of LDL-R on the surface by binding to LDL-R at the surface of the cell, prompting endocytosis into the cell together with LDL and LDL-R (65). This prevents the release of LDL-R for re-expression, thus causing LDL-R to enter the lysosome where it gets degraded together with LDL and therefore is unable to re-present itself at the cell surface. PCSK9 is also regulated by SREBP; it increases expression of LDL-R to increase LDL absorption into cell but at the same time increases PCSK9 to decrease the expression of LDL-R thus decreasing the absorption into the cell (65). Abnormalities in these regulatory elements (decreased SREBP2 and LXR, and increase in PCSK9) results in decreased LDL-R expression and decreased LDL catabolism resulting in raised LDL serum levels (66).

The hepatic LDL-R is crucial for maintaining cholesterol homeostasis. This receptor expression is regulated by SREBP and liver X receptors (LXR). Abnormalities in these regulatory elements results

in decreased LDL-R expression and decreased LDL catabolism resulting in raised LDL levels (figure 1.3). There are three forms of SREBPs i.e., SREBP 1a, -1c and -2. SREBP 1c is regulated by insulin. SREBP 1a and 2 are regulated by intracellular cholesterol concentrations (67).

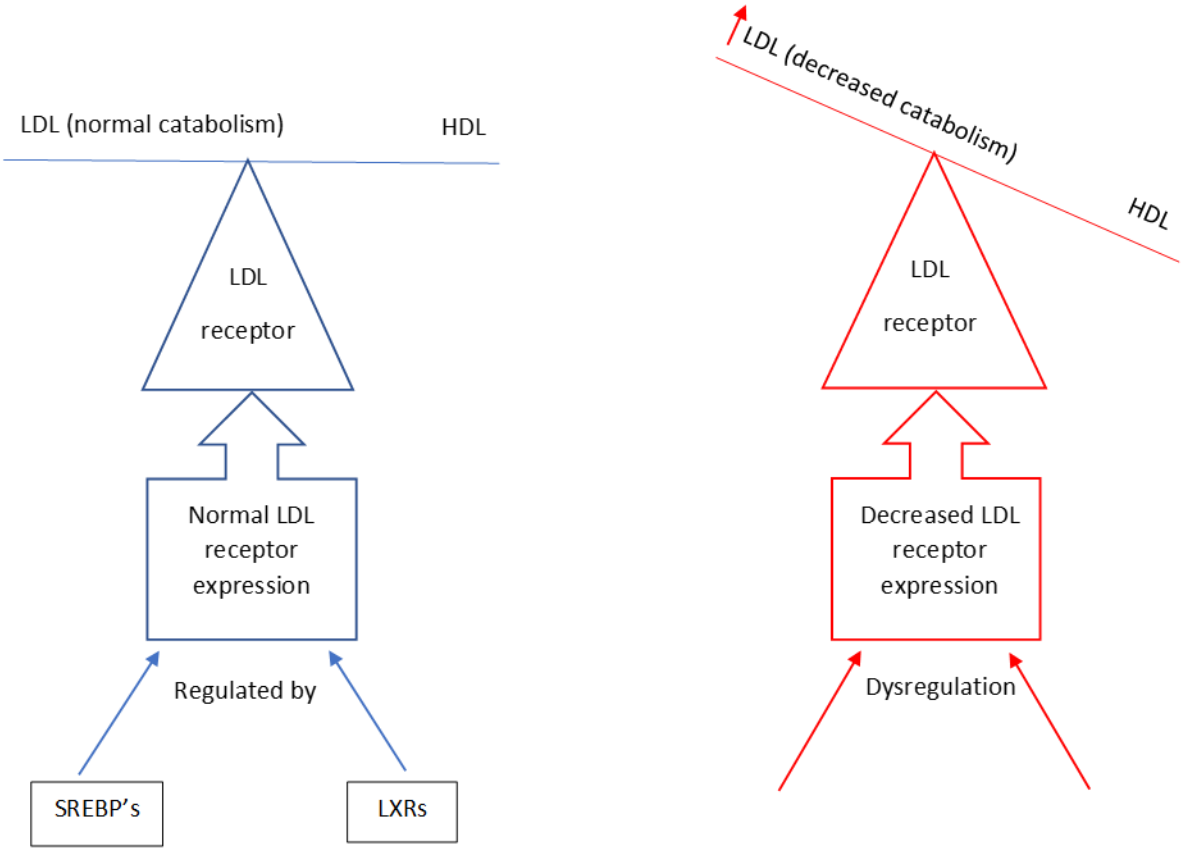


**Figure 1. 3: LDL levels during normal and decreased LDL-R expression (prepared by author)**

*Left (Blue) demonstrating normal LDL levels when there is normal LDL receptor expression. Right (Red) demonstrating abnormal high LDL levels due to decreased LDL catabolism as a result of decreased LDL receptor expression. LDL receptors are regulated by sterol regulatory element – binding proteins (SREBPs) and Liver X Receptors (LXRs)*

*1.7.6.1.6 MicroRNA-148a (miRNA-148a)*

MicroRNA's (miRNA) are small non coding RNA molecules (containing about 20-24 nucleotides) (68). They are regulatory RNA's that modulate messenger RNA (mRNA) at a post transcription and pre-translation level (figure 1.4).

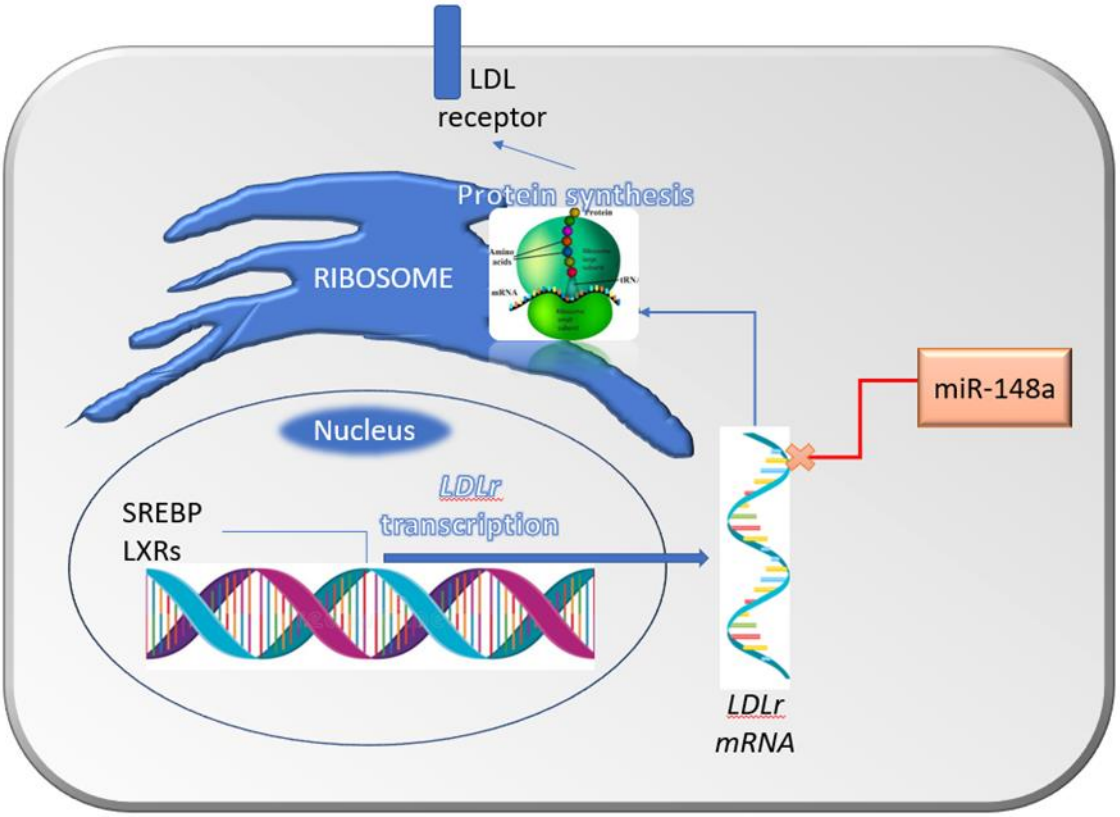


**Figure 1. 4: Post-transcriptional repression of LDL-R by microRNA-148a (miR-148a). (prepared by author)**

*Upregulation of transcription factors like SREBPs and LXRs prompt transcription of the LDL-R gene to mRNA. For LDL-R protein to be synthesized the mRNA needs to undergo translation in the ribosome. However, miR-148a is capable of binding to the LDL-R mRNA preventing the mRNA from undergoing translation. miRNA-bound mRNA is subsequently marked for degradtione*

With respect to gallstones, miRNAs may regulate the tightly controlled homeostatic mechanisms that affect bile acid synthesis and secretion; however, the role of miRNAs is still unclear. In a publication by Yang et al (2015) (69), gallbladder mucosa of patients with gallstones were analyzed for miRNA expression. Of those that were expressed, miRNA target genes whose functions and regulating pathways related to gallstones were identified and compared to miRNA expression in patients without gallstones (patients with gallbladder polyps were used for comparison). Of significance was an upregulated miR-210 which may play an important role in gallstone formation. Serum also contains miRNAs. miR-122 which is specific to liver was shown to correlate strongly with liver enzyme levels and necro-inflammatory activity (70). Numerous studies have demonstrated miRNA downregulation by HIV-1 infection (71,72), and have been implicated in changes in adipose tissue in HIV+ve patients (73). This provides enough basis for further investigating miRNA molecular mechanisms of cholesterol homeostasis and gallstone formation.

Certain miRNA's have been identified to regulate cholesterol and lipid metabolism however, the contribution to derangement in circulating cholesterol and lipid level and its association to cardio vascular disease and gallstones remains unclear (74). In a study by Wagschal et al (2015), they recognized single nucleotide polymorphism (SNP)'s in patients with abnormal circulating cholesterol and lipids and then observed which miRNA was in close proximity to those SNP's. These miRNA's were then attributed to the coding for the abnormalities (75). The findings are tabulated in table 1.2.

**Table 1. 2: Relationship of abnormal circulating cholesterol and lipid SNP's with miRNA and their associated lipid abnormality (prepared by author)**

| miRNA near SNP's | Associated abnormality in circulating lipogram | | | |
|---|---|---|---|---|
| | TC | LDL -C | HDL -C | TAG |
| miR-128-1 | X | X | | |
| miR-148a | X | X | | X |
| miR-130-b | X | | X | |
| miR-301-b | X | | | |

MiR-148a is located in a gene-poor intergenic region of human chromosome 7 and is predominantly expressed in liver. Notably, the expression of miR-148a is significantly increased in the liver of high-fat diet (HFD)-fed mice (72).

Out of 159 miRNAs identified to be highly expressed in human liver and modulated by dietary lipids, miR-148a emerged as the strongest with highest liver activity and expression in livers of HFD fed mice (76). SNP's in the promoter region of miR-148a are associated with altered LDL-C and triglyceride levels in humans (77).

LDL-R is shown to be directly regulated by miR-148a. miR-148a has 2 binding sites on LDL-R and overexpression of miR-148a markedly reduces LDL-R activity and inhibition of miR-148 significantly increased the expression of LDL-R. The inhibition of LDL-R expression by miR-148a is highly specific as the expression of other cholesterol related genes such as SREBP2 are not influenced by miR-148a overexpression (78). miR-148a has thus shown to decrease LDL binding and uptake while antagonists miR-148a increase LDL binding and uptake. Decreased LDL binding and uptake results in increased levels of circulating LDL-C and decreased levels of intracellular LDL-C (78). miR-148a expression was also shown to be modulated by SREBP1c which is in turn activated by LXR. SREBP1c is key in linking the activation of miR-148a by LXR (figure 1.5). When SREBP1c is silenced this activation of miR-148a does not occur (78). miR-148a has an opposite effect on HDL-C and decreases cholesterol efflux to ApoA1 by decreasing ABCA1 mRNA (78).

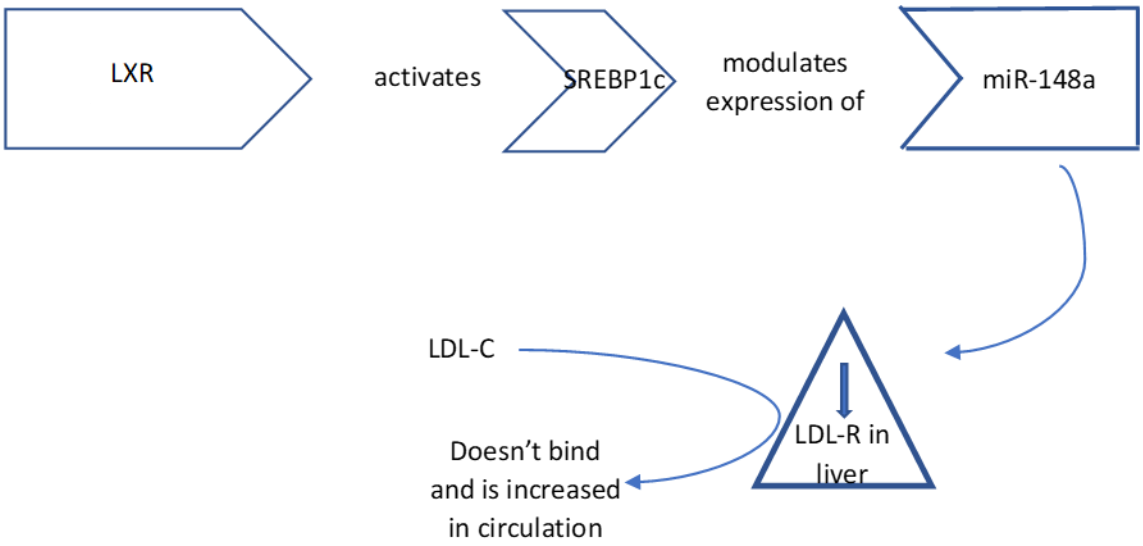


**Figure 1. 5: LDL-R downregulation by miRNA 148a (prepared by author)**

*1.7.6.2 Molecular and metabolic influence on cholesterol synthesis*

Although theoretically the increased production of cholesterol by the liver may result in cholesterol gallstone formation, results have not shown a specific pattern of abnormalities. Gallstone patients have presented with increased or normal activities of hepatic hydroxy-methyl-glutaryl-Co-A reductase (HMG-CoA reductase), the rate limiting steps of cholesterol synthesis (79).

*1.7.6.2.1 3-Hydroxy Methyl-Glutaryl Coenzyme A Reductase (HMGR)*

Cholesterol synthesis within the cell is a complex multistep process, which originates from Acetyl-CoA and has one major rate limiting step to final synthesis by HMGR enzyme which regulates the conversion of HMG-CoA to mevalonate (80). When cholesterol levels are low, there is increased gene expression for increased HMGR activity for increased cell production of cholesterol. This enzyme is regulated by sterol response element (SRE) that binds to a transcriptional activator SREBP2 within the nucleus and turns on cholesterol synthesis genes (66) . Insulin-induced gene 1(INSIG1) is a protein in the membrane of the endoplasmic reticulum (ER) (81). INSIG1 plays an important role in the SREBP-mediated regulation of cholesterol biosynthesis: by binding to the sterol-sensing domain of SCAP (SREBP cleavage activating protein) it makes the SCAP/SREBP complex stay longer in the ER, thus prohibiting SCAP from carrying activated SREBP to the golgi complex. This ultimately blocks SREBP from acting as a transcription factor for the SRE in the promoter region of the HMG-CoA-reductase gene and results in a decreased expression of HMG-CoA-reductase (82). It is activated when the concentration of cholesterol in the cell is high and binds to HMGR which results in HMGR degradation and thus reduces

the production of cholesterol. In mice, when the genes encoding for transcription of HMGR is decreased, it decreases cholesterol synthesis which corelates with the reduction in gallstone formation (83). However, in human studies, even though the saturation of gallbladder bile is higher in gallstone patients there seems to be no difference in HMGR activity between patients with and without gallstones (79).

*1.7.6.2.2 Acyl-coenzyme A Cholesterol Acyltransferase (ACAT)*

ACAT is a cellular cholesterol sensor that esterifies the excess of intracellular cholesterol and stores it in cytosolic droplets ready for secretion (84). There are 2 ACAT genes viz *ACAT1* and *ACAT2*. The *ACAT1* gene encodes a thiolase mitochondrial enzyme, whilst *ACAT2* gene encodes cytosolic acetoacetyl-CoA thiolase. ACAT genes are strongly expressed in the liver. In humans, *ACAT1* is the main cholesterol esterification gene opposed to mice where *ACAT2* is predominant (85). In both humans and mice ACAT deficiency increases cholesterol availability for biliary secretion increasing the risk for GD (86).

*1.7.6.2.3 Sterol Regulatory Element Binding Proteins (SREBP)*

SREBP controls lipid metabolism in cells (87). There are 2 types; SREBP1 present in the liver and SREBP2 which is ubiquitously present. SREBP1 is involved in activating genes in fatty acid synthesis and SREBP2 is involved in activating genes for cholesterol metabolism (87). SREBP2 is a master regulator of cholesterol synthesis in the nucleus of the cell by sensing the amount of cholesterol present in the cell and regulating the negative feedback mechanism. SREBP2 exists in the ER together with an escort protein called SREBP cleavage activating protein (SCAP). When cholesterol is in abundance in the cell the SREBP-SCAP combination binds with Insig and doesn't signal the nucleus to produce more cholesterol (88). When the cholesterol level in the cell is low, the SREBP-SCAP combination dissociates from the Insig and travels to the Golgi apparatus where it is cleaved. The SREBP2 then travels to the nucleus and binds with sterol response elements (SRE) to signal HMGR to produce more cholesterol (89). SREBP2 during times of low cholesterol also switches on LDL-R to increase cholesterol uptake (figure 1.6).

*1.7.6.2.4 Peroxisome Proliferator-Activated Receptors (PPARs)*

PPARs are nuclear receptors that activate genes involved in FA metabolism and β-oxidation. Decreased expression of PPAR has been linked to cholesterol gallstones (90).

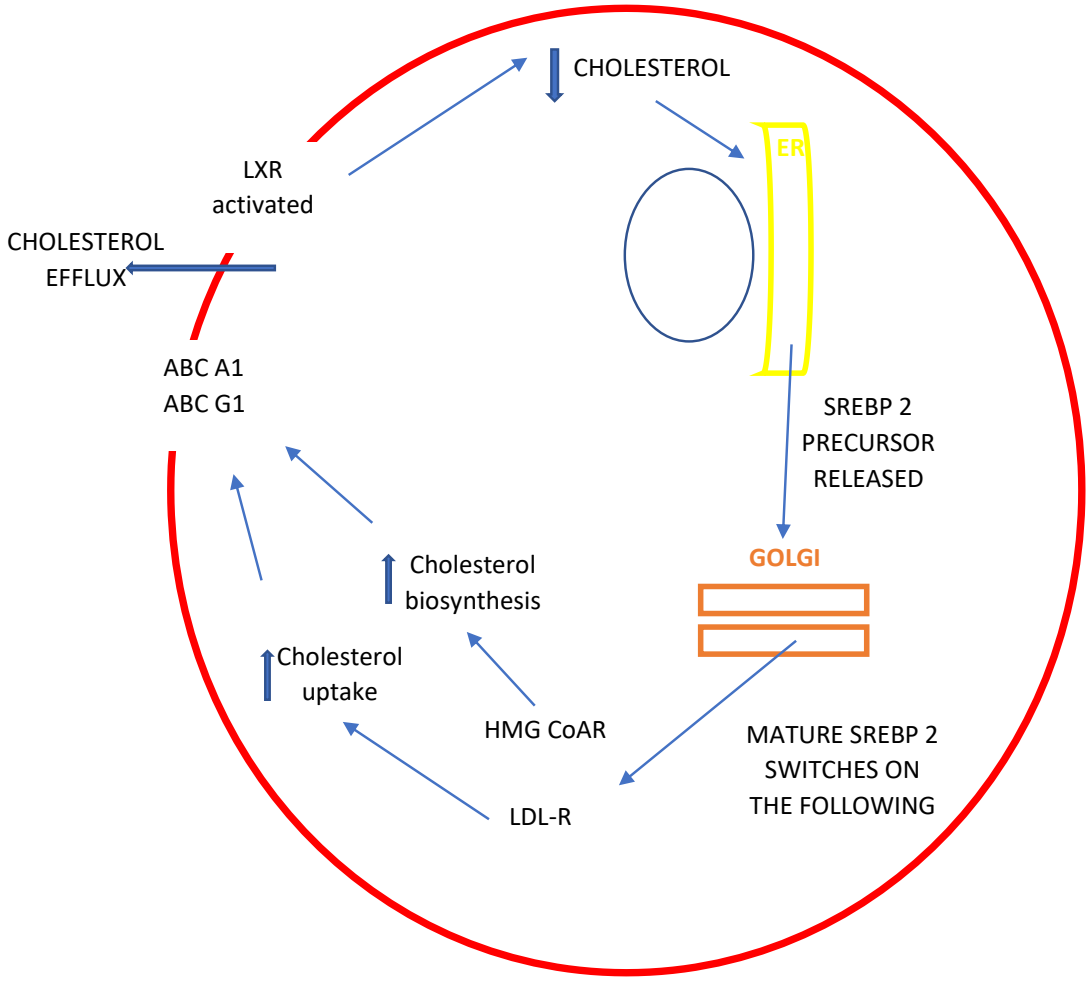


**Figure 1. 6: Diagram of cell demonstrating cholesterol homeostatic control (prepared by author)**

*SREBP2 released from Edoplasmic Reticulum (ER) and transported to Golgi apparatus to mature SREBP2 during times of low cholesterol. This switches on HMG-COA reductase increasing cholesterol biosynthesis, and LDL-R to increase cholesterol uptake. This now increased intracellular cholesterol then activates LXR and ABCA1 and ABCG1 to efflux cholesterol from cell.*

*1.7.6.3 Molecular and metabolic influence on cholesterol output from liver*

The rate limiting step in converting cholesterol into soluble bile salts for excretion into bile is catalyzed by CYP7A1 (91). Defects in the regulation of this enzyme is key to the amount of cholesterol and bile acids excreted in bile. The output of biliary lipid secretion is also mediated by different ABC transporters: ABCG5/G8 for bile cholesterol, ABCB4 for phospholipids and ABCB11 for bile acids (92). Transport of this bile usually occurs within micelles; however, when the cholesterol content in

the bile exceeds that which can be solubilized by micelles, then the excess is dispersed for transport by vesicles (higher cholesterol/phospholipid ratio and lower concentration of bile salts) (3). It is in these vesicles with a combination of biliary protein presentation that cholesterol nucleation occurs to form cholesterol monohydrate crystals (3). Bile mucin is derived from hepatocytes, gallbladder epithelium and bile duct epithelium. Mucin secretion increases especially in the gallbladder when lithogenic bile is present resulting in more viscous bile.

*1.7.6.3.1 Cytochrome P450 7A1 (CYP7A1)*

CYP7A1 is an enzyme involved in the rate limiting step of converting cholesterol to bile acid for excretion (93) (figure 1.7). When the cholesterol concentration within the hepatocyte is increased either by increased delivery to the liver or by increased production, CYP7A1 is activated via liver X receptor (LXR) to convert cholesterol into bile acid (94). When the cholesterol levels within the hepatocyte drops, SREBP is activated which downregulates CYP7A1 and thus decreases conversion of cholesterol to bile acid. Bile acid production downregulates/inhibits CYP7A1 via two pathways; (i) activation of Farnesoid X receptor (FXR) which in turn activates small heterodimer (SHP), (ii) by activating liver macrophages which activate cytokines such as TNF-alfa (95). Hepatic nuclear factor 1 (HNF-1) is a transcriptional factor for FXR (96). Liver Receptor Homologue (LRH1), is an orphan nuclear receptor that serves as a tissue-specific competence factor for bile acid synthesis. HNF in rats works via FXR and thus downregulates CYP7A1 as depicted in (figure 1.7), but in humans, HNF works directly on CYP7A1 and upregulates its expression (97). Also, in rats LXR directly binds to CYP7A1 and upregulates it, but LXR in humans does not bind to CYP7A1 (98). Human CYP7A1 doesn't seem to be influenced as much as rodent CYP7A1 by diet, so in humans CYP7A1 influence on gallstone formation may be genetically mediated (98).

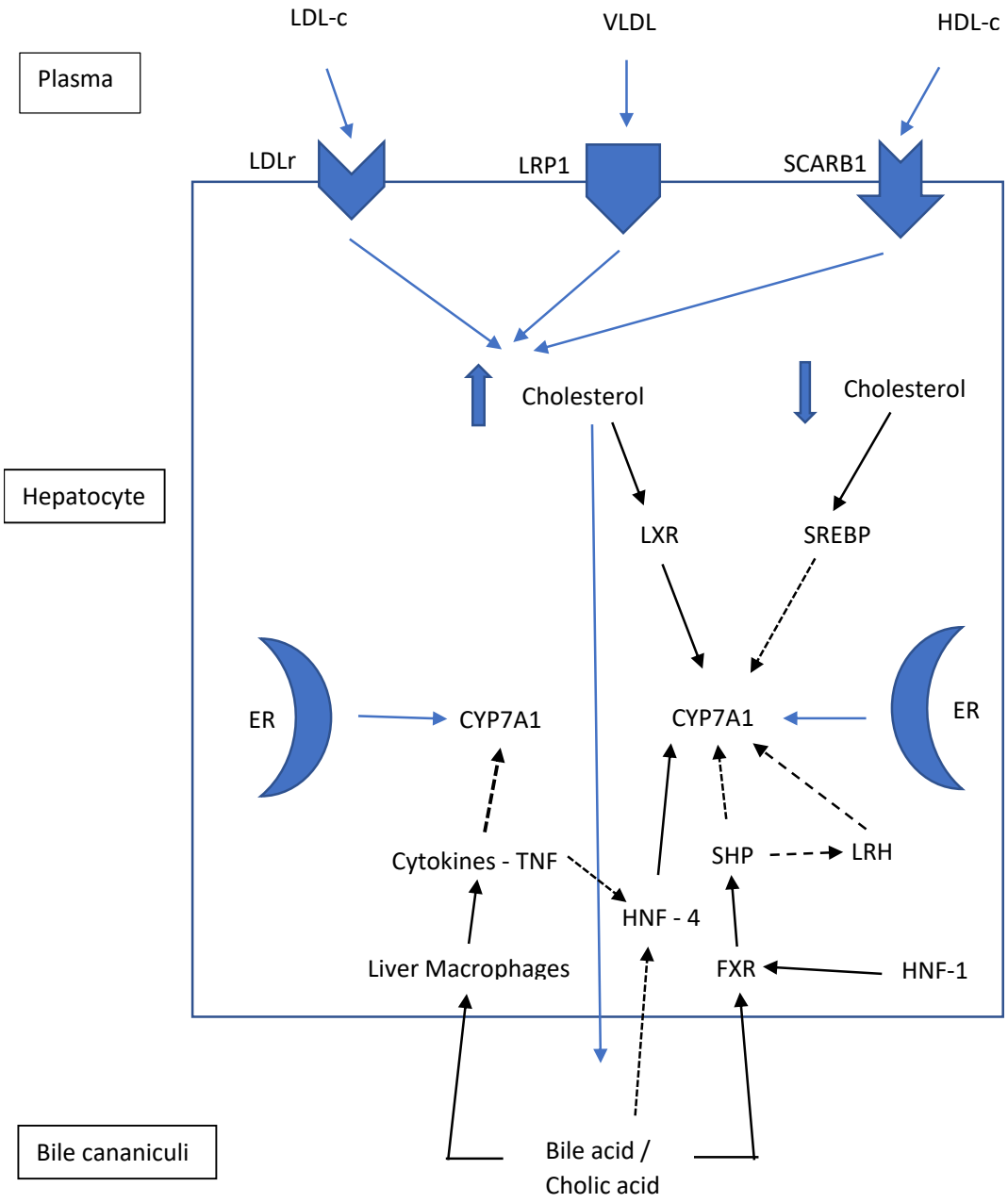


**Figure 1. 7: CYP7A1 regulation by genes and hepatic nuclear factors (prepared by author)**

*CYP7A1 is upregulated and downregulated by various genes and hepatic nuclear factors ultimately determining the amount of cholesterol converted to bile acid. Solid lines upregulation. Dotted lines represent downregulation.*

The majority of evidence dating back to as far as 1975 (99), together with more recent evidence have demonstrated that a deficiency in CYP7A1 (100), genetic variations in CYP7A1 (101) and inhibition of CYP7A1 with lipid lowering drugs like fibrates (102) result in increased cholesterol concentration secretion in bile which increases the risk of gallstone formation (96, 97).

Genetic variations are evidenced by an anecdotal report of brothers with CYP7A1 mutations in genes resulting in gallstone formation (104) and in a case control study in 232 Mexican American men with CYP7A1 having increased incidence of gallstones (105). In gallstone susceptible inbred mice, overexpression of CYP7A1 was protective against gallstone formation (106).

However, contrary to this finding in two separate reports of Chilean Hispanic and Mapuche subjects (known to have one of the highest incidences of gallstones worldwide), an increase in CYP7A1 (107) and an increase in bile acid secretion (108) resulted in increased gallstone formation. However, this has yet to be demonstrated in other population groups.

In the first scenario where CYP7A1 is downregulated, there is a decreased conversion of cholesterol to bile acid resulting in increased cholesterol secretion and decreased bile acid secretion in bile resulting in gallstones. This is in keeping with Admirands theory (3). In the second scenario where upregulation of CYP7A1 results in gallstone formation does not conform to this theory. This may be best explained by CYP7A1 being upregulated to increase bile acid secretion. A possible explanation for this is an impaired enterohepatic circulation of bile acids from malabsorptive terminal ileal disease. Alternatively, differing genetics or environmental factors may influence hepatic nuclear factors changing the expression of certain target genes.

When there is down regulation of CYP7A1 this results in decreased bile acid synthesis and increased hepatic cholesterol production. This increased hepatic cholesterol downregulates LDL-R in the liver which results in increased serum LDL-C (94).

In a study of rats and mice hepatocytes with ART PI's by Zhou et al (2006) (109) the following were found. In rat primary hepatocytes, *CYP7A1* mRNA levels were significantly decreased by atazanavir and ritonavir but increased by amprenavir (109). Similar results were obtained in mouse primary hepatocytes. Atazanavir and ritonavir also decreased CYP7A1 protein levels and bile acid biosynthesis, while amprenavir had no significant effect (109). In a separate study by Williams et al demonstrated a decrease in CYP7A1 in mice hepatocytes treated with Indinavir (110).

#### *1.7.6.3.2 Hepatic Nuclear Factors (HNF)*

Hepatic Nuclear Factors are an important group of regulatory nuclear factors in hepatocyte differentiation and hepatic metabolism. HNF-1 regulates bile acid metabolism by three mechanisms: (111). One, it is a transcriptional activator of bile acid transporters in ileal enterocytes, hepatocytes and renal tubules. Impaired HNF-1 function thus causes reduced bile acid reabsorption from the terminal ileum, kidney and decreased removal of bile acid from the portal circulation at the liver all resulting in decreased bile acid in liver (interruption of enterohepatic circulation of bile acids). Two, it is a transcriptional activator of FXR. Impaired HNF-1 function results in reduced FXR expression resulting

in decreased levels of SHP-1 which increases CYP7A1 activity and bile acid synthesis (96,111). Three, it is at transcriptional activator of hepatic bile acid binding protein which senses intracellular bile acid pool. Impaired HNF-1 function results in defective sensing of bile acid stores in hepatocytes

In rats HNF-1 binds to FXR receptors and in humans HNF-1 binds directly to CYP7A1 (97). In a study of mice, Purushotham et al (2012) demonstrated that HNF-1 alpha is a transcriptional regulator of FXR, and when HNF-1 alpha was inhibited it resulted in downregulation of FXR with resultant gallstone formation (112).

HNF-4 together with LRH-1, Chicken Ovalbumin Upstream Promoter Transcription Factor II (COUP-TFII) and Peroxisome proliferator-activated receptor gamma coactivator 1(PGC-1) are required for upregulation of CYP7A1. HNF-4 on its own is unable to upregulate CYP7A1 (113,114). Bile acids cause a feedback downregulation of CYP7A1 not only via HNF-1 and FXR but also HNF-4 which has a direct binding site on CYP7A1. Normally HNF-4 binds and upregulates CYP7A1, however there is evidence that bile acids can downregulate CYP7A1 transcription by reducing the transactivation potential of HNF-4 (115). Bacterial endotoxins, pro-inflammatory cytokines (116), TNF-alpha and interleukin -1 by macrophages in the liver (117) are known to decrease the activity of CYP7A1. De Fabainai et al (2001) demonstrated that HNF-4 is the target transcriptional factor mediating the effects of these pro-inflammatory cytokines on CYP7A1 (115).

*1.7.6.3.3 Farnesoid X Receptor (FXR) And Liver X Receptors (LXR)*

FXR is a bile acid (chenodeoxycholic and cholic acid) nuclear receptor that regulates transcription of genes that are involved in enterohepatic circulation of bile acids thus maintaining bile acid and cholesterol homeostasis (118). In order to bind to target genes, it forms a heterodimer with retinoid X receptor (RXR) (118).

Liver X receptors (LXR) are nuclear receptors that act as cholesterol sensors that regulates transcription of genes involved in cholesterol transport and metabolism thus maintaining cholesterol homeostasis. There are 2 types, LXR-alpha and LXR-Beta. Both also forms a heterodimer with RXR to function (119).

FXR work together to maintain cholesterol in the enterohepatic circulation. FXR downregulates, and LXR upregulates CYP7A1 (120). Mostly down regulation of CYP7A1 has been linked to increased gallstone formation, except in Chilean Mapuche Indians were up regulation increases gallstone formation (108). FXR controls the expression of ABC transporters, ABCB-11 (canalicular bile salt export pump in liver), and ABCB-4 (translocates phosphatidylcholine from inner to outer leaflet of canalicular membrane). LXR controls ABCG-5 & 8 (involved in pumping cholesterol into bile).

In the study by Moschetta et al (2004), FXR knock out mice, fed a lithogenic diet had an increased susceptibility to develop gallstones. Administration of the FXR agonist to gallstone-susceptible mice decreased the susceptibility to gallstone formation (118). This is more in keeping with the findings of the Chilean population where CYP7A1 up regulation results in gallstone formation because FXR inhibition is known to up regulate CYP7A1. They also demonstrated that the bile of the FXR knock out mice compared to mice with FXR, was more turbid and had the presence of cholesterol crystals. The phospholipid and bile salt concentrations were significantly higher; however, the cholesterol concentration was the same as mice with FXR. This therefore resulted in a higher cholesterol saturation index in the FXR knock out mice resulting in gallstones. Further to this was increased bile salt hydrophobicity in FXR knock out mice which is known to be toxic to gallbladder mucosa resulting in increased thickness, damage and inflammation to the gallbladder wall which contributes to gallstone formation (121).

*1.7.6.3.4 ATP-Binding Cassette, Subfamily G, Member 5 and 8 (ABCG5/8)*

ABCG5/8 are important genes in regulating intestinal and hepatobiliary excretion of cholesterol at the level of the enterocyte and canalicular respectively (122). In the liver they are the main regulators of cholesterol secretion of cholesterol into bile independent of bile acids (123). Mutations in these genes result in sitosterolemia, (characterized by increased plasma sterols, hypercholesterolemia, premature atherosclerosis, xanthomatosis and impaired biliary cholesterol secretion), which has been identified in Mexican American patients whom are at high risk for developing GD (124). Overexpression of ABCG5 and 8 in mice results in decreased absorption of dietary cholesterol and increased secretion of biliary cholesterol however no precipitation of cholesterol was demonstrated indicating that ABCG5 and mutations alone are not enough to cause GD (125).

*1.7.6.3.5 Small Heterodimeric Partner (SHP)*

SHP works with nuclear receptors and are also expressed in intestine and liver. SHP is upregulated by FXR which in turn reduces SREBP-1c expression which reduces CYP7A1 (126). SHP also represses LRH-1 which influences transport and synthesis of bile acids influencing the supersaturation of cholesterol (127).

*1.7.6.3.6 Liver Receptor Homologue (LRH-1)*

LRH-1 is a nuclear receptor. In addition to being expressed in the intestine and liver it is also expressed in the pancreas and ovary. It is involved in bile acid synthesis by targeting several genes including SHP and CYP7A1 (127).

*1.7.6.4 Cholesterol gallstone genes (Lith genes)*

The concept that cholesterol cholelithiasis could result from a complex interaction of environmental factors and the effects of multiple undetermined genes is well supported by epidemiologic investigations, clinical observations, and family and twin studies in humans, as well as gallstone prevalence investigations in inbred mouse models. Quantitative trait locus (QTL) analysis is a powerful genetic method for identifying primary rate-limiting genetic defects and discriminating them from secondary downstream lithogenic effects caused by mutations of the primary genes, and the subsequent positional cloning of such genes responsible for QTLs, followed by the use of manufactured mouse strains with "knockout" or "knockin" of the genes, could lead to the discovery of lithogenic actions of gallstone ( LITH ) genes (128). The successfully identification of many candidate LITH genes has results from the combined use of genomic strategies and phenotypic studies in inbred strains of mice. The orthologous human LITH genes can be identified from the mouse study because there is exceptionally close homology between mouse and human genomes, The discovery of LITH genes and more fundamental knowledge concerning the genetic determinants and molecular mechanisms underlying the formation of cholesterol gallstones in humans will pave the way for critical diagnostic and prelithogenic preventive measures for this exceptionally prevalent digestive disease.By quantitative trait loci (QTL) mapping of gallstone susceptible and resistant inbred strains of mice the localization of additional unknown gallstone genes has been identified in the genetic era of GD. A number of Lith genes have been identified viz Lith1 (chromosome 2), Lith2 (chromosome 19), Lith3 (chromosome 17), Lith4 (chromosome X) and Lith5 (chromosome 5). However, Lith1 and Lith2 are the highest associated with GD (129). This provides the genetic basis for orthologous human investigation.

### *1.7.7 Effect of HIV and ART on cholesterol metabolism*

The prevalence of HIV in South Africa is of the HIV-1 type. The majority of patients in South Africa are on a fixed drug combination (FDC) regimen (130).

FDC regimen consisted of three drugs namely; two Nucleoside reverse transcriptase inhibitors (NRTIs) drugs [Tenofovir Disoproxil Fumarate (TDF) and Emtricitabine (FTC) and one Non-nucleoside reverse transcriptase inhibitor (NNRTI) [Efavirenz (EFV)]. (130). Common second line agents include Protease inhibitors (PI) [Lopinivar/ritonavir (Aluvia) or Atazanavir/ritonavir]. A number of drugs are available for use in third-line treatment: Integrase strand transfer inhibitors (InSTIs) Dolutegravir

(DTG) and Raltegravir (RAL), the newer PI DRV and newer NNRTIs (ETR and RPV). (table 1.3). According to Southern African HIV Clinicians Society guidelines for antiretroviral therapy in adults: 2020 update (131), (DTG) based therapies are now the preferred first-line ART therapy.

**Table 1. 3: Class of antiretroviral drugs, its mechanism of action and effect on lipid metabolism (adapted from da Cunha J et al 2015)**

| Antiretroviral class | Abbreviation | Mechanism of action | Example of drug | Effects on lipids |
|---|---|---|---|---|
| **Nucleoside reverse transcriptase inhibitors** | NRTI's | Reverse transcriptase inhibition | Abacavir (ABC)<br>Didanozine (ddl)<br>Emtricitabine (FTC)<br>Lamivudine (3TC)<br>Stavudine (d4T)<br>Tenofovir (TDF)<br>Zidovudine (AZT) | ↑ Dyslipidemia<br>↑↑ Dyslipidemia<br>↑ Dyslipidemia<br>↑ Dyslipidemia<br>↑↑ Dyslipidemia<br>↑ Dyslipidemia<br>↑↑ Dyslipidemia |
| **Non-Nucleoside reverse transciptase inhibitors** | NNRTI's | Reverse transcriptase inhibition | Efavirenz (EFV)<br>Etravirine (ETR)<br>Nevirapine (NVP)<br>Rilpivirine (RPV) | ↑↑HDL,↑Dyslipidemia<br>Neutral effects<br>↑↑ HDL, ↑LDL<br>Neutral effect |
| **Protease Inhibitors** | PI's | Protease inhibition | Amprenavir/ritonavir<br>Atazanavir/ritonavir<br>Darunavir/ritonavir<br>Fosamprenavir/ritonavir<br>Indinavir<br>Lopinavir/ritonavir<br>Nelfinavir<br>Saquinavir<br>Tipranavir/ritonavir | ↑↑↑ Dyslipidemia<br>↑ Dyslipidemia<br>↑ Dyslipidemia<br>↑↑↑ Dyslipidemia<br>↑↑ Dyslipidemia<br>↑↑↑ Dyslipidemia<br>↑↑ Dyslipidemia<br>↑ Dyslipidemia<br>↑↑↑ Dyslipidemia |
| **Intergrase strand transfer inhibitors** | InSTI's | Inhibition of viral integration | Dolutegravir (DTG)<br>Elvitegravir (EVG)<br>Raltegravir (RAL) | Neutral effect<br>Neutral effect<br>Neutral effect |
| **Early inhibitors** | - | Entry inhibition | Selzentry | Neutral effect |

Cholesterol metabolism can be affected directly by the HIV-1 virus independent of ART's or as a result of ART use. The mechanisms are described vide infra.

*1.7.7.1 Effect of HIV-1 on cholesterol metabolism*

The association of HIV RNA levels with lipid levels independent of ART would suggest that the replication of the virus has a direct effect on lipid levels. As HIV RNA levels rise, the levels of HDL and LDL fall, and the levels of VLDL, cholesterol (TC), and triglyceride (Tg) levels increase (4).

Cholesterol efflux is a normal physiological process of removing excess cholesterol from cells. This is mediated by cell membrane transporter, ATP binding cassette 1 (ABCA1), which transports cholesterol mainly the lipid poor apolipoprotein A1 (apoA1) out of the cell and converts it into mature HDL for transport back to the liver. This reverse cholesterol transport is the first step in maintaining cholesterol homeostasis in the body. Upon entering the peripheral circulation, HIV-1 infects hosts lymphocytes and macrophages. In order for survival and replication of the virus within these cells it requires large amounts of cholesterol within the cell. In fact, the level of inhibition of cholesterol efflux is directly proportional to the level of viral replication within the call. It achieves this by encoding a small protein called Negative Regulatory Factor (Nef) which binds to ABCA1 and downregulates it thus preventing the efflux of apoA1 cholesterol to HDL. This results in a decreased level of HDL and macrophages that are filled with cholesterol which become referred to as foam cells responsible for large amounts of cholesterol release during apoptosis (132) (Figure 1.8).

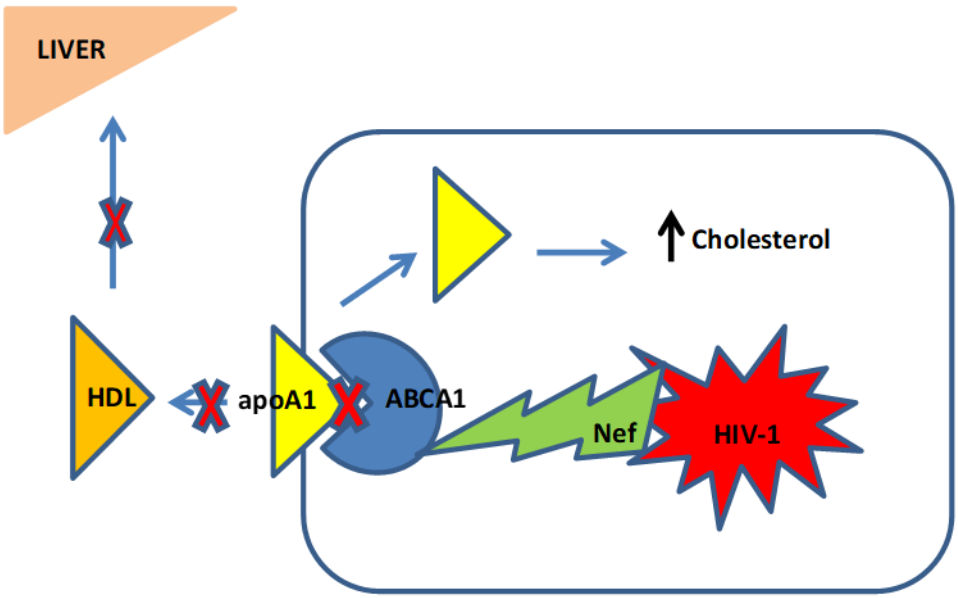


**Figure 1. 8: HIV effect on ABCA1 and ApoA1 in reverse transport of HDL (prepared by author)**

*Macrophage infected by HIV-1 encoding for protein Negative Regulatory Factor (Nef) which bind to ATP binding cassette 1 (ABCA 1) preventing it from transporting Apolipoprotein A1 (apoA1) out of the cell and thus preventing it converting to mature High Density Lipoprotien (HDL) and transport to the liver. This results in increase apoA1 and cholesterol within the macrophage*

SREBP2 is a master regulator of cholesterol synthesis in the nucleus of the cell by sensing the amount of cholesterol present in the cell and regulating the negative feedback mechanism (see section *1.7.6.2.3*). In HIV infection, HIV activates SREBP2 to activate sterol response gene TFII-I to induce the cholesterol biosynthetic pathway (133). (figure 1.9)

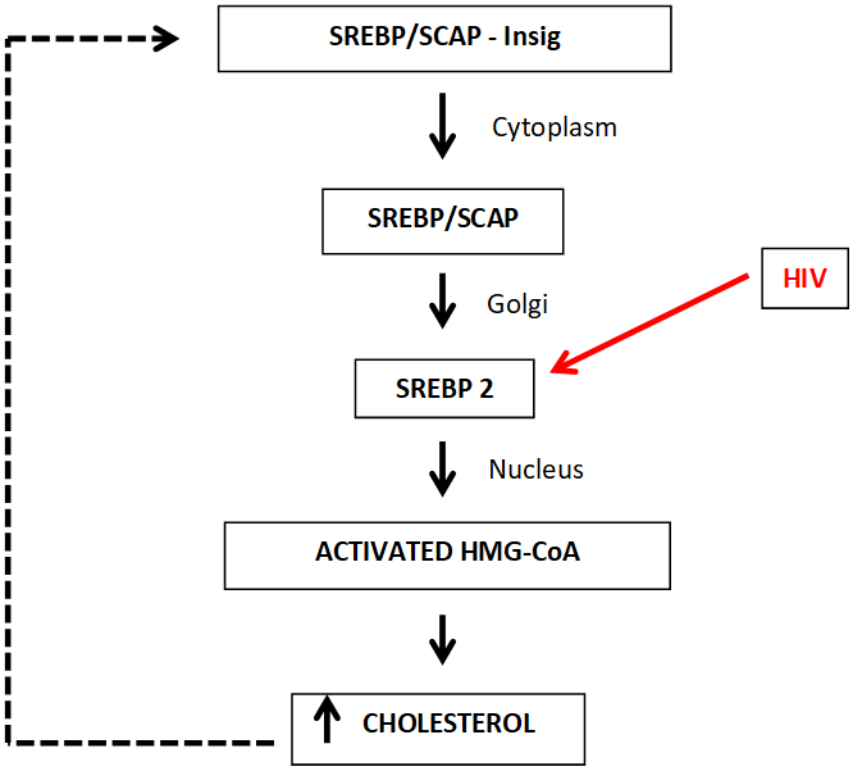


**Figure 1. 9: HIV effect on SREBP2 and HMG-CoA in cholesterol production (prepared by author)**

*Increased cholesterol in the cell causes a negative feedback preventing the cleavage of the Sterol regulatory binding protein/ SREBP cleavage activating protein (SREBP/SCAP) from insulin signaling protein (Insig), thus preventing SREBP 2 from being cleaved in the Golgi and preventing activating HMG-CoA reductase (HMG-CoA) from increasing cholesterol synthesis. However, HIV bypasses this negative feedback directly activating SREPB 2.*

*1.7.7.2 Effect of ART on cholesterol metabolism*

*1.7.7.2.1 Protease inhibitors (PIs)*

Under normal conditions, dyslipidemia is prevented by the binding of cytoplasmic retinoic acid-binding protein type 1 (CRABP-1) to intracellular retinoic acid. The retinoic acid is metabolized by cytochrome P450 (CYP450) or 3A (CYP3A) enzymes which convert retinoic acid to cis-9-retinoic acid. The cis-9-retinoic acid then binds to the retinoid X receptor-peroxisome proliferator-activated receptor γ (RXR-PPARγ). This receptor then stimulates adipocyte differentiation and inhibits apoptosis (134).

Protease inhibitor drugs bind to and inhibit the catalytic region in HIV-1 protease. The problem is that the catalytic region in HIV-1 protease and CRABP-1 are very similar and some of the drug binds to CRABP-1 thus inhibiting its function and prevents adipocyte differentiation resulting in apoptosis. The resultant adipocyte loss, decreased lipid storage and lipids released into the bloodstream cause hyperlipidemia (134) (figure 1.10).

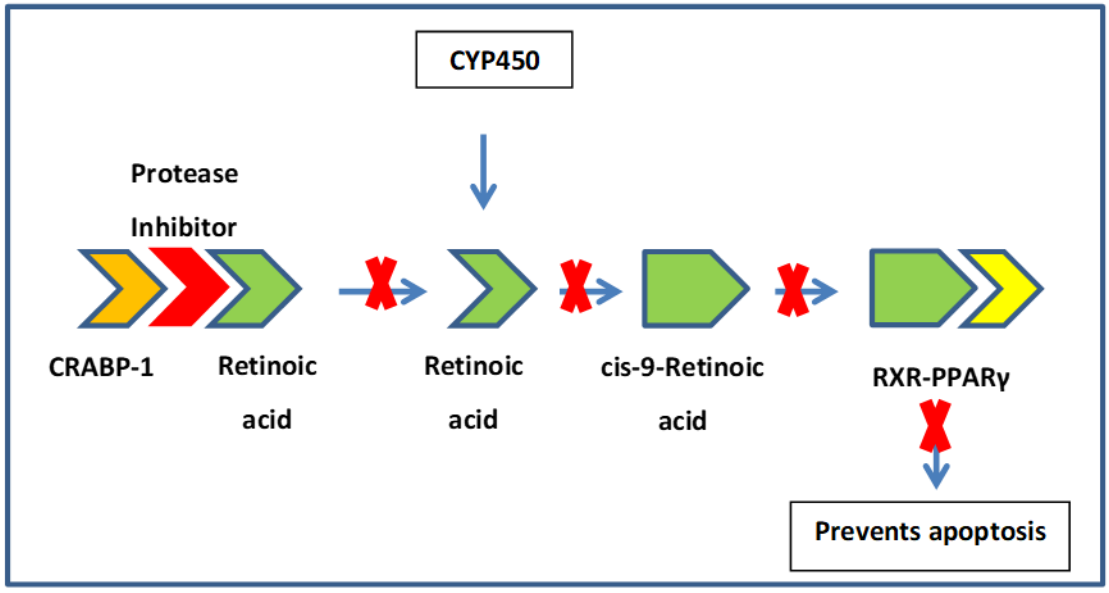


**Figure 1. 10: Protease Inhibitor effect on RXR and PPAR γ (prepared by author)**

*Protease inhibitors bind to Cytoplasmic retinoic acid-binding protein type 1 (CRABP-1) thus preventing it from binding to Retinoic acid, which prevents its conversion to cis-9-Retinoic acid, preventing the binding to retinoid X receptor-peroxisome proliferator-activated receptor γ (RXR-PPARγ) which ultimately results in increased apoptosis and hyperlipidaemia*

Another mechanism to prevent dyslipidemia, is the binding of low-density lipoprotein-receptor related protein type 1 (LRP1) to lipoprotein lipase (LPL) on the capillary endothelium. This LRP1-LPL

complex cleaves fatty acids from triglycerides resulting in free fatty acid accumulation in peripheral adipocytes (135).

The problem herein again is the similarity of the catalytic region in HIV-1 protease to LPR-1, resulting in some of the protease inhibitor drugs binding to LPR-1 and inhibiting its function. This results in the inability to store fatty acids. These fatty acids are then released into the blood stream and reach the liver where they promote synthesis of LDL and VLDL with a resultant increase in cholesterol concentration (135).

PI's increase the expression and secretion of proinflammatory cytokines, such as tumor necrosis factor-alpha, interleukin-1β and interleukin-6. These proinflammatory cytokines alter adipocyte function and decrease adiponectin. This results in decreased fatty acid break down (136). PI's also suppress proteasome-mediated degradation of SREBP's in the liver and adipocytes, thus promoting SREBP accumulation in the liver resulting in an increase in the biosynthesis of total TC (136). PI-based therapy increases the hepatic synthesis of Tg, and to a lesser extent, VLDL cholesterol (137).

In Japan, recent studies report an increased rate of cholelithiasis (9,8%) in HIV+ve population which maybe mostly related to PI treatment (138). Lin et al (2015) demonstrated that the accumulative exposure to atazanavir/ritonavir for over 2 years is associated with a 6.29-fold increase in the risk for incident cholelithiasis (139).

*1.7.7.2.2 Nucleoside reverse transcriptase inhibitors (NRTIs)*

The most widely known adverse effect of NRTIs is mitochondrial toxicity having implications for ATP production and beta-oxidation of fatty acids. DNA polymerase-γ (DNA pol-γ) is a nuclear- encoded polymerase that regulates the replication of mitochondrial DNA (mtDNA) in cells (140). As the site of cellular energy production through oxidative phosphorylation and via the mitochondrial respiratory chain, mitochondrial dysfunction leads to compromised energy production and increased free radical production (141). NRTI's inhibit DNA pol-γ, resulting in decreased mitochondrial DNA, with resultant respiratory chain dysfunction and decreased cellular energy. This decreased energy in adipocytes due to mitochondrial depletion results in metabolic disorders within the adipocytes with resultant increase in plasma lipid levels (142).

*1.7.7.2.3 Non-Nucleoside reverse transcriptase inhibitors (NNRTIs)*

NNRTI's have not been typically linked to derangements of lipid metabolism however Efavirenz has specifically shown to have some effect on lipids. At high concentrations it alters the magnitude of adipocyte differentiation and prevents murine pre-adipocytes from accumulating lipids. It increases the serum cholesterol but also increases high density lipoprotein (HDL) making the overall affect neutral (78).

*1.7.7.2.4 Intergrase strand transfer inhibitors (InSTI's)*

InSTI's have been touted as not affecting cholesterol metabolism which may have a big impact on preventing CVD and GD in these subsets of patients. However, there are growing reports of weight gain with the use of InSTI's (143–145) , especially in Black African women (146). In fact, one study showed a greater increase in abdominal fat compared to PI's (147). This is concerning considering that a large proportion of patients with CVD and GD are linked to obesity, and in particular abdominal fat.

### *1.7.8 Conclusion*

Cholesterol gallstones are formed due to the supersaturation of bile with cholesterol. The process as to how this occurs is complex and involves a combination of environmental and genetic factors summarized above. As the liver is the most important route in eliminating the bodies cholesterol via bile an in-depth study and understanding into the livers trafficking of cholesterol in terms of its input, manufacturing and secretion of cholesterol is explained above and forms the basis for any further investigation into the pathogenesis of gallstones. Despite HIV affecting cholesterol metabolism directly and from ART's as outlined above, there is a paucity of data regarding its role in the pathogenesis in gallstone formation. In this thesis study we therefore attempt to explore the possible risk factors and pathogenesis of gallstones in this subset of patients by using information obtained in this literature review in predominantly murine studies.

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## CHAPTER 2

Chapter 1 literature review provided an overview of GD, the current understanding of its pathogenesis in HIV-ve humans and murine subjects and a background on HIV and ART and how it may potentially be involved in the de-novo pathogenesis of GD. In order to seek differences in HIV+ve and HIV-ve patients with gallstones a comparative case series study was conducted.

This chapter responds to objective 1 of this study viz to compare cholesterol levels and known risk factors and demographics for cholesterol gallstone formation in HIV+ve and HIV-ve in black South African women. The chapter is presented in the form of a manuscript entitled “Symptomatic Gallstones and HIV in Black South African Women: Changing trends of gallstone disease”. This manuscript is currently under review in the Southern African Journal of HIV Medicine. Submission ID 1208.

**Symptomatic gallstones and HIV in Black South African Women: Changing trends of gallstone disease?**

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**ABSTRACT**

**Background**

The incidence of metabolic disorders iHIV endemic settings is a prevailing burden in developing countries. Cholesterol homeostasis and fat metabolism are altered by HIV and ART, thereby possibly contributing to complications such as gallstone formation.

**Objective**

To evaluate established risk factors for the formation of gallstones in HIV+ve Black South African women.

**Methodology**

A case series study was conducted amongst all Black South African women undergoing cholecystectomy for gallstone disease over a 1-year period at King Edward VIII Hospital, Durban, South Africa. Age, BMI, family history of gallstones, oestrogen exposure and lipograms were compared between HIV+ve and HIV-ve women. Categorical variables were tested using either the Fisher's exact test or Pearson's Chi-square test. Means were compared using independent t-tests. For non-normally distributed data, the Mann-Whitney test was used. Statistical tests were two-sided, and p values of less than 0.05 were considered as statistically significant.

**Results**

A total of 52 patients were assessed, 34 HIV-ve and 18 HIV+ve. The median age of HIV+ve vs HIV-ve patients was 35 years and 50 years respectively ($p=0.015$). The $HIV^{-ve}$ group had a statistically significant number of patients in the overweight/obese category (BMI > 25kg/$m^2$) compared to the normal weight category (BMI <25kg/$m^2$) ($p<0.001$). The number of obese patients in the HIV+ve group however did not reach statistical significance.

**Conclusion**

Gallstone disease amongst the Black South African HIV+ve women group were significantly younger and had fewer patients in the obese category. These findings differ from known risk factors of gallstone disease in other population groups and in Black South African HIV-ve women. This could be attributed to the metabolic alterations caused by HIV infection itself and long-term ART use. Larger cohort studie/s are required to elucidate the role of HIV and ART in cholestatic diseases.

Keywords: HIV, ART, Gallstone disease, Cholesterol gallstones, HIV induced cholesterol gallstones, ARV induced cholesterol gallstones.

## INTRODUCTION

South Africa has a population of 57.7 million and it is estimated that 7.52 million (13,1%) are living with Human Immune deficiency virus (HIV), higher than any other country (1). Almost 26% of the infected reside in the province of KwaZulu Natal (1). Of those infected, 3.9 million patients (52%) are on antiretroviral therapy (ART) (1). Most research into the actual cause and risk factors of gallstones are performed in Europe and some South American countries with a paucity of data from Africa. This oversight maybe in part due to the historically lower incidence of gallstones amongst Black South Africans (2,3) compared to the rest of the world, and to the focus on other concurrent health crisis, specifically HIV, maternal death, malnutrition and other non-communicable diseases (4).

Gallstone disease (GD) can present in a variety of ways, including biliary colic, cholecystitis, gallstone pancreatitis, obstructive jaundice and even as a risk to gallbladder cancer. The actual cost of GD to the already overburdened health care system in South Africa (SA) is not known. However, if extrapolated from developed countries, it is rated as the second highest gastro-intestinal cost burden to the health care system (5). GD occurs in up to 20% of the population in developed countries, and is reported as low as 5% in Sub Saharan Africa (6). Exclusive prevalence reports from SA are reported as low but are outdated (reports from 1987) (3). Reasons for this low prevalence reports at the time, were due to the low prevalence in Black South Africans (who constitute 80% of the population); historically GD was predominantly observed in Caucasians. Since these earlier reports, there has been an exponential increase in the incidence of GD in Black South Africans (2,3). Lifestyle factors, especially urbanization of Black South Africans and consumption of foods high in fats and low in fibre have seen a surge in high body mass index (BMI) in this population, an established risk to gallstone formation (7). This rise in gallstone incidence amongst Black South Africans is paralleled with the rise in incidence of HIV and the judicious role out of highly active ART.

The introduction of ART has changed the spectrum of disease of HIV from that of a terminal illness to a chronic, manageable disease. An unprecedented HIV positive (+ve) population with an extended life span has seen the emergence of adverse metabolic outcomes that resemble metabolic syndrome defined as a cluster of biochemical and physiological abnormalities associated with the development of cardiovascular disease and type 2 diabetes characterised by hypertriglyceridemia, low high-density lipoprotein (HDL) cholesterol, and insulin resistance, which is known to predict increased risk of cardiovascular disease (CVD)(8). There is no one common underlying pathology for this metabolic syndrome as each component has different causes, but it is thought that the pathology in HIV+ve patients differ from HIV negative (-ve) patients as increased waist circumference occurs less frequently (8). Factors contributing to these metabolic outcomes are the host response to HIV infection, specific ART drugs, and HIV-associated lipodystrophy (8). The restoration to health also allows metabolic

syndrome to manifest in those who were genetically predisposed. This makes HIV+ve patients vulnerable to higher incidences of CVD in the absence of known risk factors such as smoking (8).

Like metabolic syndrome, the pathogenesis of GD is well established. However, unlike metabolic syndrome specific to HIV, gallstones specific to HIV has not been studied. Gallstone formation occurs due to supersaturation of cholesterol in bile, and this supersaturation has been attributed to risks commonly referred to the 5 "F"s (Female, Fat, Fertile, Forty and Family history) (9). The observed metabolic changes in HIV+ve patients may contribute to GD, as these patients are known to have increased circulating lipid levels. The evidence for this is that lipid levels rise concomitantly with HIV RNA levels independent of ART. Further, ART use increases lipid release into the bloodstream, thus both resulting in hyperlipidaemia (10). This may in turn make HIV+ve patients vulnerable to higher incidences of GD without known risk factors such as obesity.

To date, there have been no studies investigating the risk factors of gallstone formation in Black South Africans since its first report of their surge in this population group in the early 1990's (11). Whether or not they conform to the same risk factors such as obesity as observed in largely Caucasian populations is yet to be determined. We compare well known risk factors for gallstone formation to validate if HIV+ve patients are in accord with these risks and compare them to our HIV-ve population in Black South African women. This report is thus the first in addressing this issue.

**METHODS**

A case series study was conducted, comparing known risk factors of gallstone formation among HIV+ve and HIV-ve Black South African women presenting with symptomatic stones for cholecystectomy. All patients over the age of 18 years undergoing a cholecystectomy for GD (including biliary cholic, cholecystitis, jaundice and gallstone pancreatitis) from King Edward VIII hospital, Durban, South Africa during January to December 2017 were included in this study.

Informed consent was received from all study participants with a standard consent form in 2 of the official main languages of SA (English and Zulu), for gathering information including voluntary counselling and testing for HIV, if not tested prior to cholecystectomy. Full ethics approval was granted by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC), reference number BE276/16. Permission to conduct the research was granted by King Edward VIII (Durban, South Africa) hospital management.

Clinical parameters of patients including, sex, age, race, BMI, family history of gallstones, lifestyle parameters (history of contraception/hormone use) and co-morbidities (HIV status, including ART use

or not) were assessed by questionnaire. Patients undergoing cholecystectomy for reasons other than gallstones where incidental gallstones were found were excluded from the study. Patients whose HIV status was unknown or who refused testing after voluntary counselling were also excluded. Haematology and serology were used for the quantification of viral loads (VL) and $CD4^+$ lymphocyte ($CD4^+$) counts for HIV+ve patients and lipograms (including triglyceride, high density lipoprotein (HDL), low density lipoprotein (LDL) and total cholesterol) were performed on all patients.

Statistical analyses were performed using R Project for Statistical Computing. Descriptive statistics such as frequencies and percentages were used to summarize categorical variables. Central tendency and dispersion of data was measured using means and standard deviations for normally distributed variables and medians and interquartile ranges for skewed variables. Associations between categorical variables was tested using either the Fisher's exact test or Pearson's Chi-square test. Similarly, with regards to the testing of associations between continuous variables: for normally distributed data; means were compared using independent t-tests; for non-normally distributed data, the Mann-Whitney test was used. Statistical tests were two-sided, and p values of less than 0.05 were considered as statistically significant.

**RESULTS**

A total of 55 Black South African women underwent cholecystectomies during the study year. Three patients refused HIV testing after voluntary counselling and were excluded (N=52). Median age of all women was 43 years (IQR 30-54). Patient demographic and clinical parameters are presented in Table 1.

**Table 2. 1: Demographics and clinical parameters of study sample**

| Baseline characteristic | HIV Negative (N=34) | HIV Positive (N=18) | p-value |
|---|---|---|---|
| **Age** | | | |
| Median (Q1-Q3) | 50 (31-58) | 35 (29-42) | p = 0.015 |
| | **Distribution (n, %)** | | |
| **BMI[a]** | | | |
| <25 | 4 (12%) | 5 (36%) | |
| ≥25 | 30 (88%) | 9 (64%) | p = 0.099 |
| **Comorbidities** | | | |
| No | 22 (65%) | 14 (78%) | |
| Yes | 12 (35%) | 4 (22%) | p = 0.331 |
| **Oestrogen Exposure[b]** | | | |
| Negative | 2 (6%) | 2 (14%) | |
| Positive | 29 (94%) | 12 (86%) | p = 0.578 |
| **Family History[c]** | | | |
| None | 23 (74%) | 13 (93%) | |
| Positive | 8 (26%) | 1 (7%) | p = 0.236 |

Data missing for: [a]four, [b]seven, [c]seven women

Thirty-four patients were HIV-ve and 18 patients were HIV+ve. Of the 18 HIV+ve patients, 14 (78%) were on ART for an average of 4.9 years.

All patients on ART had undetectable VL, with an average $CD4^+$ count of 569 cells/µl. ART naïve patients had an average VL of 89,000 copies/ml and an average $CD4^+$ count of 424 cells/µl.

HIV+ve were younger than HIV_ve women; median ages of 35 years vs. 50 years respectively (p=0.015) (Table 1 and Figure 1)

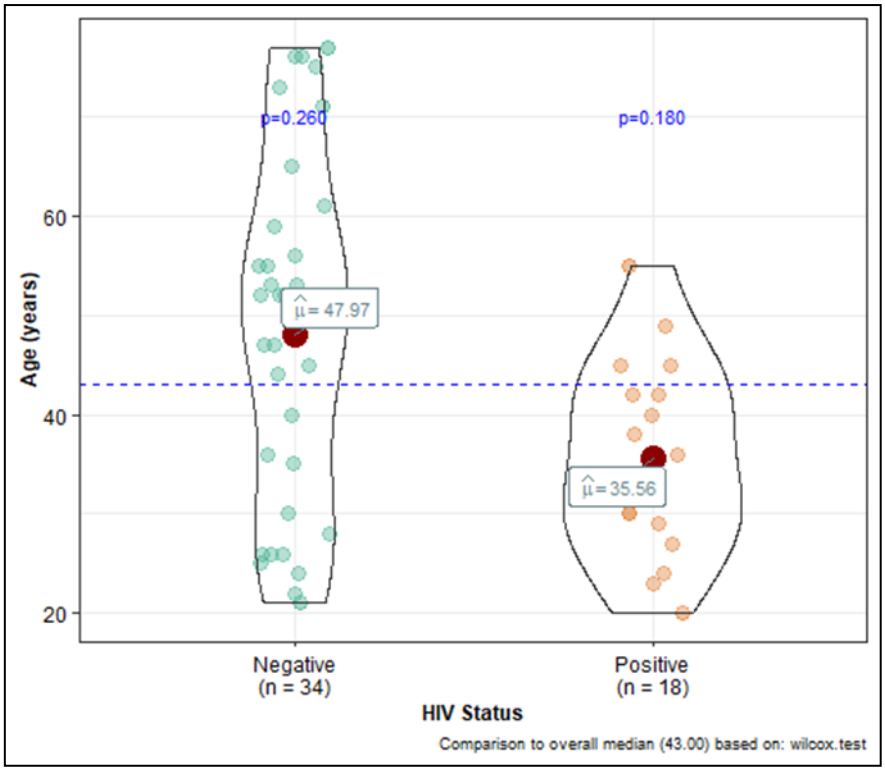


**Figure 2. 1: Comparative age in HIV-ve individuals undergoing cholecystectomy compared to HIV+ve patients; **p<0.05**

The median BMI between the HIV-ve and HIV+ve group was 33kg/m$^2$ (IQR 28-39) and 30kg/m$^2$ [IQR 24-35] respectively with no statistical significance (p=0.231). However, when separating the BMI into a normal weight category (BMI <25kg/m$^2$) and an overweight/obese category (BMI >25kg/m$^2$), the HIV-ve group showed statistically significant number of patients in the overweight/obese category compared to the normal weight category (p<0.001). However, the HIV+ve group behaved differently demonstrating no statistically significant number of patients in the overweight/obese group (Figure 2).

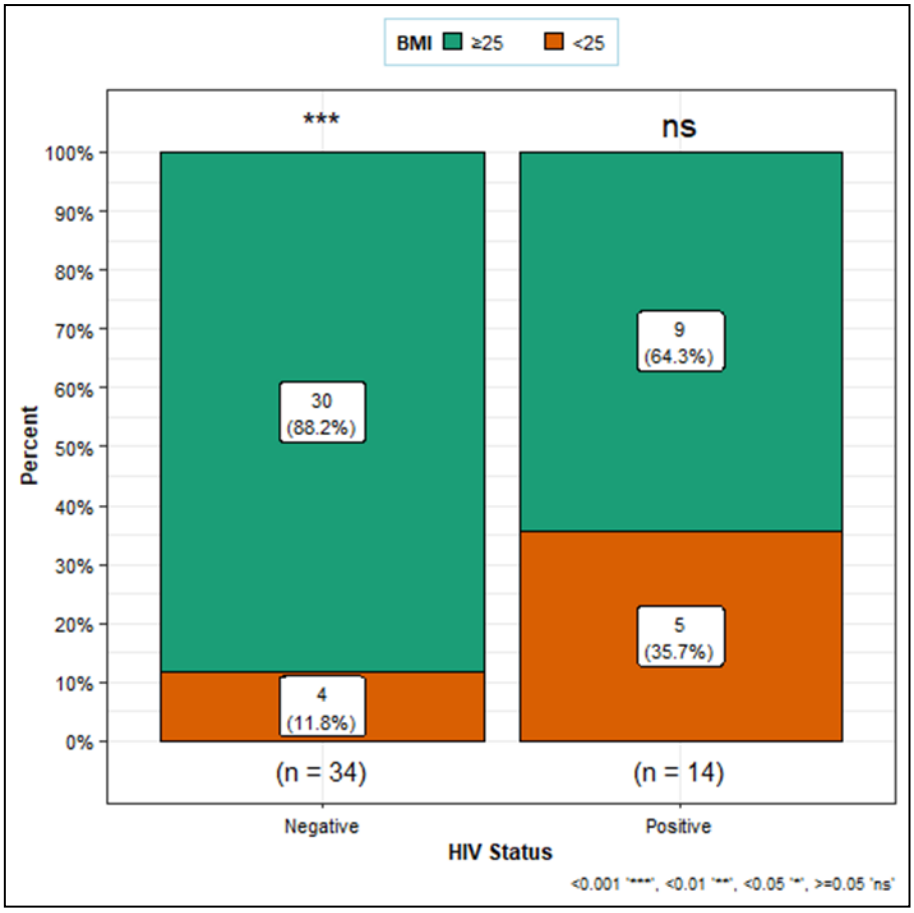


**Figure 2. 2: BMI comparison of HIV+ve and HIV-ve patients in the normal (<25), overweight/obese (>25) categories**

Seven percent (1/14) of HIV+ve women compared to 26% (8/31) of HIV-ve women had a positive family history of gallstones (Table 1).

Oestrogen exposure in the form of pregnancies and contraception was higher, 94% (29/31) among HIV-ve women compared to HIV+ve women, 86% (12/14) (table 1). The average use of oestrogen in the HIV-ve group was also higher (5years) compared to the HIV+ve group (2 years). Hypercholesterolaemia (>5mmol/l) as a co-morbidity was absent in both groups of Black South African women. The prevalence of overall comorbidities (Hypertension, diabetes mellitus, previous tuberculosis) between the groups are shown in Table 1.

Lipogram results of patients are reflected in Tables 2 and 3.

**Table 2. 2: Median lipogram values**

| Parameters | Negative (N=34) | Positive (N=18) | p-value |
|---|---|---|---|
| **Total Cholesterol (mmol/l)[a]** | | | |
| Median (Q1-Q3) | 4.73 (3.74-5.51) | 4.59 (4.18-5.47) | p = 0.766 |
| **Triglycerides (mmol/l)[b]** | | | |
| Median(Q1-Q3) | 1.12 (0.67-1.45) | 1.04 (0.84-1.33) | p = 0.670 |
| **HDL (mmol/l)[c]** | | | |
| Median (Q1-Q3) | 1.18 (0.94-1.35) | 0.94 (0.91-1.32) | p = 0.866 |
| **LDL (mmol/l)[d]** | | | |
| Median (Q1-Q3) | 2.85 (2.37-3.77) | 3.24 (2.93-3.74) | p = 0.167 |

Data missing for: [a]four, [b]five, [c]five, [d]six women

The median values of LDL were higher in the HIV+ve group compared to the HIV-ve group; HDL levels were lower among HIV+ve women; These results did not reach statistical significance (Table 2).

**Table 2. 3: Percentage of patients with abnormal lipogram values**

| Parameters (normal values) | Negative (N=34) | Positive (N=18) | p-value |
|---|---|---|---|
| **Total Cholesterol group[a]** | | | |
| Normal (<5mmol/l) | 19 (63%) | 12 (67%) | |
| Abnormal | 11 (37%) | 6 (33%) | p = 0.815 |
| **Triglycerides group[b]** | | | |
| Normal (<1.7mmol/l) | 23 (79%) | 15 (83%) | |
| Abnormal | 6 (21%) | 3 (17%) | p = 1.000 |
| **HDL group[c]** | | | |
| Normal (>1.2mmol/l) | 14 (50%) | 5 (29%) | |
| Abnormal | 14 (50%) | 12 (71%) | p = 0.175 |
| **LDL group[d]** | | | |
| Normal (<3mmol/l) | 16 (57%) | 6 (35%) | |
| Abnormal | 12 (43%) | 11 (65%) | p = 0.155 |

Data missing for: [a]four, [b]five, [c]seven and [d]seven women

The proportion of abnormal HDL and LDL levels was higher among HIV+ve women as compared to HIV-ve women; differences in the groups were not statistically significant (Table 3).

## DISCUSSION

The largest analysis of gallstone pathology amongst Black South Africans dates back to 1987, when 100 cholecystectomies were performed over a 3-year period. The study demonstrated an exponential increase in the cholecystectomy rates in Black South Africans from 1967 to 1987. Analysis of stone composition and bile composition was similar to that compared to Caucasians (3). In a comparison study of acute pancreatitis over two decades in a single institution in South Africa the incidence of gallstone pancreatitis increased by 9.7% with a 3.4% increase in Black South Africans (12). In another study undertaken in the early 1990's in King Edward VIII Hospital (Durban, SA), 82% of the 156 cholecystectomies during a 2-year period was on Black South Africans, indicating an increasing incidence of GD (11). However, since the turn of the century there is paucity of data regarding the incidence, and more importantly the effect of the HIV endemic and ART on patients presenting with GD. Well known risk factors of GD are commonly, increasing age, and what is referred to as the five ''F's'' (Female, Fat, Fertile, Forty and Family history) with a raised BMI in patients of child bearing age exposed to oestrogen, usually peaking around the age of 40 years together with a positive 1$^{st}$ degree family history of gallstones, being the biggest known risk factors for GD (3).

### Age

It is well known that advancing age is a risk for gallstone formation. In mice with lithogenic diets (1% cholesterol, 0.5% cholic acid and 15% butter fat), there was an up-regulation of HMG-CoA reductase, the rate limiting enzyme for cholesterol synthesis, with increasing age, and down-regulation in 7 alpha-hydoxylase (CYP7A1), the rate limiting enzyme in bile synthesis activity, with increasing age (13). This results in greater intestinal absorption of cholesterol, greater biliary excretion of cholesterol and reduced hepatic synthesis and biliary excretion of bile salts (13). With ageing comes a relative hypoperfusion of the gallbladder wall resulting in gallbladder dysfunction, together with decreased gallbladder wall contraction (13). The largest study of age to date was a multigeneration registry used in Sweden, where the ages of 660,732 patients with symptomatic gallstones were studied. The incidence of cases per 100,000 people began to peak between the ages of 30-34 years and continued to rise to the ages of 75 and over, indicating an increase in incidence of gallstones with increasing age (14). In our study, HIV-ve women were more likely to be older than HIV+ve women. Previous reports of GD in younger patients (under 30 years) attribute this paradigm shift to teenage pregnancy, sedentary lifestyle and raised BMI. In a report of 507 patients in Rawalpindi, 48% of patients with GD were under the age of 30 years. The study concluded that high BMI and raised socioeconomic status were associated with cholelithiasis in the younger ages (15). In a large study in USA there was a comparison of patients

presenting with GD in the Bronx area compared to other counties. HIV+ve patients and pregnant patients were excluded. The incidence of gallstones in patients under the age of 20 years did not increase over the 15-year study period in counties other than the Bronx county. However, the Bronx county demonstrated an exponential increase over time. These patients were predominately female and although not specifically analysed in this study group, the assumption drawn for this increase was related to concurrent increase in rates of teenage pregnancy and obesity in the Bronx compared to other counties (16). The reasons for these younger patients presenting with GD was different from our HIV+ve group. In these studies, the young patients correlate with known risk factors viz, raised BMI and high teenage pregnancy rates. It is reasonable to accept that these patients are presenting with gallstones at a younger age. These explanations however do not conform to our HIV+ve group who, when compared to the HIV-ve group, do not display a significantly raised BMI (infra vide). Furthermore, the exposure to oestrogen in the form of pregnancies and contraception was also lower (infra vide). It is thus reasonable to assume that the younger age of gallstone presentation in the HIV+ve population compared to the rest may be purely linked to the metabolic effects of HIV itself or ART or a combination of both.

**Weight**

Up to the late 1980's GD was a condition almost exclusive to Caucasians. Increasing reports suggesting an increase in gallstones amongst Black South African women prompted a screening study of 100 Black South African women without any gastrointestinal symptoms in Soweto, Johannesburg. The study demonstrated an incidence of 10% of gallstones amongst Black South African women (2). The BMIs of the 10% were significantly higher compared to those without gallstones, and this was attributed to urbanization and diets higher in fats and lower in fibre. This mirrors the findings in our HIV-ve group of Black South African women where a statistically significant number of the women (88%) were in the overweight/obese category (BMI >25kg/$m^2$). These findings are not surprising as there is a clear link to gallstone formation in patients that are overweight. This is explained by the amount of cholesterol synthesised and secreted in bile being directly proportionate to being overweight, resulting in a direct proportionate relationship to the incidence of symptomatic gallstone per year to the BMI existing as well (17).

Interestingly there was an absence of a significant number of overweight/obese patients (BMI >25kg/$m^2$) in the HIV+ve group. This may be best explained by weight cycling. In patients where BMI is adjusted for, weight cycling in these patients is also at in increased risk of gallstone formation with larger fluctuation and more weight cycles being associated with the highest risks (18).

Rapid weight loss in HIV infected patients is well known and this may be a risk factor for gallstone formation that is not necessarily seen only in higher BMI patients. (19). Whilst normalisation of weight results in normal concentrations of cholesterol in bile, rapid weight loss, either non-operatively or

operatively in the form of gastric bypass surgery, results in gallstone formation in more than 50% of patients. Mechanisms involved are increased biliary saturation secondary to increased cholesterol mobilization, increased nucleation due to changes in bile arachidonate and glycoprotein concentrations, and elevated levels of mucin and calcium in bile (20). Rapid weight gain after ART initiation therapy may also contribute to weight cycling in these patients predisposing them to gallstone formation (21).

Overall BMI may not be an accurate risk stratifying tool in the development of gallstones in HIV+ve patients. In a study looking at the fat distribution in HIV infected patients at different BMI's the visceral adiposity was increased in the normal BMI group (18.5 – 24.9 $kg/m^2$) and the overweight BMI group (25.0–29.9 $kg/m^2$) relative to control subjects, but not among those in the obese category (≥ 30.0 $kg/m^2$). There could be a link with the increase in visceral adiposity rather than an increase in BMI to the development of gallstones in HIV+ve patients compared to HIV-ve patients (22). Furthermore, this visceral adipose tissue results in accelerated lipolysis and is what contributes to the high prevalence of non-alcoholic fatty disease liver reported up to 30-35% of mono infected patients (patients with HIV without hepatitis B or C) (23).

In the longest follow up of body composition following ART, it was found that continued fat gain after 96 weeks of treatment was associated with the greatest risk of metabolic disorders (24). Southern African HIV Clinicians Society guidelines for antiretroviral therapy in adults: 2020 update (25), now recommend Integrase strand transfer inhibitors (InSTIs) Dolutegravir (DTG) based therapies as the preferred first-line ART therapy. InSTI's have been touted as not affecting cholesterol metabolism which may have a big impact on preventing CVD and GD in these subsets of patients. However, there are growing reports of weight gain with the use of InSTI's (26–28), especially in Black African women (29). One study showed a greater increase in abdominal fat compared to PI's (30); this is concerning considering that a large proportion of patients with CVD and GD are linked to obesity, and in particular abdominal fat.

**Genetics**

The hypothesises of family history and GD dates back to as early as the mid 1900's (31). Since then, numerous studies observing familial traits together with genetic mapping have been undertaken to prove this hypothesis. In a study of 4581 patients in Copenhagen, Denmark, cross sectional ultrasound observation demonstrated a 2:1 positive 1st degree family history of gallstones (32). In an attempt to obviate inaccuracies in reporting family histories by the use of case–control studies with limited sample sizes, a multigeneration registry was used in Sweden. It is the largest, unbiased study of family history of GD using a multigeneration registry of 15.8 million people studied over 83 years from 1931 to 2015; 660,732 people were identified with gallstones. Familial cases accounted for 36% of all gallstones, 50.9% had a parental family history, 35.1% had a sibling history and 14.0% had a parental and sibling

history (14). The familial risk of developing gallstones is 2-4 times normal. There is also growing evidence of a genetic influence (33). Because of the homology between mice and human chromosomes, much of the genetic studies have been undertaken in mice. What has been identified are 25 lithogenic or *Lith* genes in mice gallstone gene mapping. The sterol export pumps ATP-Binding Cassette transporter gene 5 (*ABCG5*) and the ATP-Binding Cassette transporter Gene 8 (*ABCG8*) regulate biliary cholesterol absorption and excretion and are closely related to lith 9 gene in chromosome 17 in mice which are strong candidates for gallstone formation. In humans' variants of gene ABCG 5/8, namely ABCG5-R50C, and ABCG8-D19h, have increased risk of gallstone formation in Indians, Chinese, Chileans and Germans, independent of age, sex and BMI (34). Our HIV-ve group of patients with a 1$^{st}$ degree family history of GD was 26% compared to the Swedish study of 36%, and our HIV+ve group only revealed a 1$^{st}$ degree family history of 7%. This low prevalence of 1$^{st}$ degree family history of gallstones in HIV+ve patients is another risk that deviates from known risk factors of gallstone formation. This therefore again points that the risk of gallstones in HIV+ve patients may well be related to HIV or the use of ART itself, rather than known risks such as genetic influence contributing to gallstone formation.

**Oestrogen**

Oestrogen is the hormone that is linked with gallstone disease, with a 2-3 times higher incidence of gallstones in women of childbearing ages, pregnant women, women that are on hormone replacement therapy or had a previous history of high dose oestrogen (>50mcg) unopposed contraception. The high oestrogen levels in pregnancy are associated with increase cholesterol secretion in bile, whilst the high progesterone levels cause a decrease in bile salts and decreased contractility of the gallbladder wall resulting in precipitation of cholesterol (35). Males with cirrhosis of the liver also have increased incidence of gallstones due to the higher oestrogen levels (36). In this observational study the overall combined oestrogen exposure rates of those patients presenting with symptomatic gallstones was 94% and 86% in the HIV-ve and HIV+ve groups respectively. The pregnancy rates indicated by the number of children in the HIV+ve group (61%) was also lower than the HIV-ve group (79%). Although not statistically significant, this may well point to oestrogen being less of a player in the pathogenesis of gallstone formation in the HIV+ve group compared to our HIV-ve patients.

**Dyslipidaemia**

Cholesterol is in solution in bile due to the specific percentage concentrations between itself together with bile salts and lecithin. An increase in cholesterol or a decrease in bile salts and lecithin, results in cholesterol precipitation and gallstone formation (9). There is a correlation between serum total cholesterol, apolipoprotein B, C2, C3 and the amount of cholesterol saturation in bile. Also, there is a correlation with the levels of serum HDL with the amount of lecithin and bile acids in bile (37,38). This

indicates that serum cholesterol and LDL may be an attributing factor to gallstone formation while serum HDL might be protective**,** both of which were markedly abnormal in our study population. Both HIV itself and ART are known to effect cholesterol metabolism. The association of HIV RNA levels with lipid levels independent of ART would suggest that the replication of the virus has a direct effect on lipid levels. As HIV RNA levels rise, the levels of HDL and LDL fall, and the levels of very low-density lipoprotein (VLDL), cholesterol (TC), and triglyceride (TG) levels increase (10). The added effects of ART are known to further alter cholesterol metabolism by various mechanisms. Patients on protease inhibitors (PI's) have the greatest risk, but others as in our patient population on first line therapy without PI's are still at risk due to the direct effect of HIV on cholesterol homeostasis.

## CONCLUSION

The formation of cholesterol gallstones is complex and usually involves a combination of genetic and a host of environmental factors including lifestyle. Most research into the actual cause and risk factors of gallstones are performed in the West with a paucity of data from Africa largely due to the lower incidence compared in Africa to the rest of the world.

Whilst Black South Africans are now being exposed to most known risks such as urbanized diets and obesity, it not surprising that the patients in our study presenting with GD show clear risks that are linked to those in the western world for a disease that is rife amongst Caucasians. However, what is not widely appreciated is whether these risks apply to our HIV+ve population. From these observations, these patients do not conform entirely to the normal known risk profile. Black South African HIV+ve women with symptomatic gallstones were significantly younger.

Black South African HIV-ve women conform to the known risk factor of obesity with a statically higher BMI whilst HIV+ve women do not.

HIV+ve women also had fewer 1$^{st}$ degree relatives with GD compared to HIV-ve women, and less oestrogen exposure. It is not clear whether it is the HIV disease process itself or the role of ART in the pathogenesis of GD in people living with HIV. This requires further investigation as GD has a huge cost implication on our already overburdened health care system. Larger cohort studies on the impact of HIV and ART on liver function, cholesterol metabolism and their influence on cholesterol saturation in bile may enlighten us further.

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## COMPETING INTERESTS

The authors declare no potential conflict of interest.

## AUTHORS CONTRIBUTIONS

S.M.K. conceptualized the study, collected samples, analysed generated data and was primary author in writing of the manuscript

S.N. assisted with project conceptualization and design, performed laboratory experiments, statistical analysis and gave critical feedback on the manuscript

B.S. and A.C. conceived the original idea, supervised the project and provided critical feedback on the manuscript

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## CHAPTER 3

Chapter 2 discussed the current stance on GD in Black South African women. From this study it appears that HIV+ve group do not conform entirely to the normal known risk profile. Black South African HIV+ve women with symptomatic gallstones were significantly younger. Black South African HIV-ve women conform to the known risk factor of obesity with a statically higher BMI whilst HIV+ve women do not. HIV+ve women also had fewer 1st degree relatives with GD compared to HIV-ve women, and less oestrogen exposure. It is not clear whether it is the HIV disease process itself or the role of ART in the pathogenesis of GD in people living with HIV. Although serum lipogram levels were not significantly different, as a result of these differing environmental risk factors, a closer look into the genetic differences in cholesterol trafficking is required at a level of the liver. Guided by the results obtained in Chapter 2, a case-series design comparing hepatic expression of cholesterol homeostasis related miRNA in HIV+ve and HIV-ve patients presenting with symptomatic gall stones was conducted.

Chapter 3 presents a study sought to evaluate the hepatic expression of genes involved in cholesterol homeostasis (LDLR, ABCA1, HMGCR) in Black South African HIV+ve women presenting with gallstones relative to HIV-ve women with gallstone disease. Further, transcriptional regulators of these genes (SREBP2, miR-148a) were evaluated. This is in keeping with objective 2. Chapter 3 is presented in the form of a manuscript entitled “Hepatic expression of cholesterol regulating genes favour increased circulating low-density lipoprotein in HIV infected patients with gallstone disease: A preliminary study”. The manuscript is currently under review at the BMC Infectious Disease Journal. (Submission ID 4d005632-284f-4741-a5a7-6ee6dc5cfb4c).

**Hepatic expression of cholesterol regulating genes favour increased circulating low-density lipoprotein in HIV infected patients with gallstone disease: A preliminary study**

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**ABSTRACT**

**Background**

HIV endemic populations are displaying higher incidence of metabolic disorders. HIV and the standard treatment are both associated with altered lipid and cholesterol metabolism, however gallstone disease (a cholesterol related disorder) in Sub-Saharan African populations is rarely investigated.

**Methods**

This study sought to evaluate hepatic expression of key genes in cholesterol metabolism (*LDLr*, *HMGCR*, *ABCA1*) and transcriptional regulators of these genes (microRNA-148a, *SREBP2*) in HIV+ve women on antiretroviral therapy presenting with gallstones. Liver biopsies from HIV+ve women(n=5) and HIV-ve women (n=5) were analysed for miR-148a and mRNA expression using quantitative PCR.

**Results**

Circulating total cholesterol was elevated in the HIV+ve group with significantly elevated LDL-c levels(3.16±0.64mmol/L) relative to HIV-ve group (2.10±0.74mmol/L; p=0.04). A scavenging receptor for LDL-c, *LDLr* was significantly decreased (0.18-fold) in this group, possibly contributing to higher LDL-c levels. Transcriptional regulator of *LDLr*, *SREBP2* was also significantly lower (0.13-fold) in HIV+ve women. Regulatory microRNA, miR-148a-3p, was reduced in HIV+ve women (0.39-fold) with a concomitant increase in target *ABCA1* (1.5-fold), which regulates cholesterol efflux.

**Conclusion**

Collectively these results show that HIV+ve women on antiretroviral therapy display altered hepatic regulation of cholesterol metabolizing genes, reducing cholesterol scavenging and increasing cholesterol efflux.

Key words: Gall stone, Cholelithiasis, HIV, cholesterol, LDLr, ABCA1, miR-148a

## BACKGROUND

Gallstones account for more than 95% of biliary tract diseases in developed countries affecting 20% of the population (1). In South Africa, gallstones were considered an affliction predominantly in Caucasians, however there has been an exponential increase in gallstone disease in black South Africans over the past few decades (2,3). Metabolic disorders in developing countries are a growing concern, paralleled with an increase in human immune deficiency virus (HIV) and a consequent judicious roll out of anti-retroviral therapy (ART). Whilst a significant amount of work has linked HIV and ART to altered fat and cholesterol metabolism contributing to metabolic syndrome-like effects, diabetes and cardiovascular disease (4–7), no studies have looked at gallstone disease in this population.

Gallstones can be categorised into 3 types - cholesterol, pigment or mixed, dependent on cholesterol concentration. Cholesterol stones account for ~80% of all gallstones (8) and is a result of cholesterol superseding its saturation point in bile, causing cholesterol microcrystal formation, inevitably leading to gallstone formation (9). Demographic, environmental and genetic factors may all contribute to altered cholesterol homeostasis (10), presenting complex molecular events that favour gallstone formation. Cholesterol homeostasis is maintained by various endogenous responses involving cholesterol synthesis, transport and excretion (11,12).

Cholesterol biosynthesis is regulated by the rate-limiting enzyme of the mevalonate pathway, 3-hydroxy-3-methyl-glutaryl-CoA (HMGCoA) reductase (HMGCR) (13). Transcription of *HMGCR* is controlled by transcription factor sterol regulatory element-binding proteins (SREBP1/2) which in turn is regulated by liver X receptors (LXRα/β), both of which act as biological cholesterol sensors (14,15). The central role of LXR and SREBP transcription factors on lipid and cholesterol metabolism make these prime targets in treating metabolic disorders. Cellular regulation of cholesterol flux also plays a key role in maintaining circulating cholesterol levels - Low-density lipoproteins (LDL) are scavenged by LDL receptors (LDLr) while a major determinant of high-density lipoproteins (HDL) is efflux pumps belonging to the ATP-binding cassette (ABC) family including ABCA1. The genes coding for these proteins are considered lithogenic and are implicated in gallstone progression which can be altered during HIV infection (16). Transcriptional regulation of gene expression offers an opportunity to identify molecular events influencing disease phenotype.

MicroRNAs (miRNAs), short non-coding RNA (18-24 nucleotides) are epigenetic modulators of gene expression. MicroRNA prevent translation of target messenger RNA (mRNA) by binding via base complementarity. Various miRNAs have been associated with metabolic disorders through their regulatory function on cholesterol and lipid metabolism genes (17). The role of miRNA in gallstone disease is unclear, however some miRNAs have been identified as regulators of genes involved in gallstone formation. MicroRNA-122a and miR-144a directly inhibit cholesterol 7 alpha hydroxylase (*CYP7A1*) influencing the bile acid pool (12). Yang et al. (2015) performed an integrated analysis of

miRNA/mRNA networks in gallstones, identifying 17 differentially regulated miRNAs (18). This study showed miRNAs that regulated lipoprotein signalling and ABC transporters were significantly altered in gallstone patients. MicroRNA-148a is a miRNA that is highly expressed in liver and associated with altered LDL-c and triglyceride levels in humans (19). *LDLr* is shown to be directly regulated by miR-148a and is linked to gallstone formation in high fat diet fed mice (20).

Dysregulated miRNA in HIV infection is more well established than miRNA associated with biliary tract disorders, with several papers linking altered miRNAs to disease progression, metabolic outcomes, treatment responsiveness and biomarkers (21,22). Despite the established link of HIV and ART to metabolic disorders, there is a dearth of knowledge on gallstone disease in this population. In Japan, studies report an increased rate of cholelithiasis (9,8%) in HIV+ve patients on protease inhibitor (PI)-inclusive ART (23). Lin et al (2015) demonstrated that the accumulative exposure to atazanavir/ritonavir for over 2 years is associated with a 6.29-fold increase in the risk for incident cholelithiasis (24). Despite newer integrase inhibitor (II) ART drugs showing lower lipid abnormalities than previously used PI-based ART, abnormalities in lipid concentration still occur, and this may be reflective either of the viral effects itself, chronic ART use or the persistent inflammation and immune activation in these HIV infected patients (25). The present study sought to evaluate the hepatic expression of genes involved in cholesterol homeostasis (LDLR, ABCA1, HMGCR) in Black South African HIV+ve women presenting with gallstones relative to HIV-ve women with gallstone disease. Further, transcriptional regulators of these genes (SREBP2, miR-148a) were evaluated.

**METHODS**

This study utilized a case-series design comparing hepatic expression of cholesterol homeostasis related miRNA in HIV+ve and HIV-ve women presenting with symptomatic gall stones. Ethical approval was obtained from the University of KwaZulu Natal. Biomedical Research Ethics Committee (BREC – BE276/16). Patients undergoing cholecystectomy for gall stone disease (biliary cholic, cholecystitis, jaundice and gall stone pancreatitis) at King Edward VIII Hospital, Durban, KwaZulu Natal, South Africa from January – December 2017 were recruited to evaluate known risk factors of gall stone formation in HIV+ve and HIV-ve patients. In total 52 patients gave informed consent (standard consent form in two official main languages English and isiZulu) for retrieval of a liver biopsy and recording of clinical parameters of patients including age, race, BMI, family history of gall stones and comorbidities (HIV, hypertension, diabetes, hypercholesterolemia).

Following the analysis of clinical parameters, five HIV negative (control) and five HIV positive (cases) were selected for miRNA and downstream target analysis. Inclusion criteria included women of Black African ethnicity between the ages of 18-50, presented with symptomatic cholelithiasis, were all on contraceptive for more than one month, and did not have a family history of gallstones. Patients with co-morbidities (diabetes mellitus, arterial hypertension, hypercholesterolemia, chronic inflammatory

disorders), receiving chronic therapy for the above-mentioned co-morbidities and HIV positive patients on preventative TB therapy were excluded. All HIV positive patients were on fixed dose combination therapy with CD4 counts above 500 cells/mm3 and undetectable viral loads."

The selection of 5 samples for molecular analysis was based on the limited accessibility of molecular testing following the inclusion process .

*RNA extraction*

Briefly, liver tissue (1cmx1cm) was submerged in RNAlater® Stabilization Reagent (Qiagen, Hilden, Germany) in 2ml cryovials at collection and stored at -80ºC until RNA extraction. The stabilization buffer was decanted, and tissue samples were rinsed in 0.1M phosphate saline buffer (PBS) prior to RNA extraction via the Qiazol extraction method as per the manufacturer's instructions. Crude RNA was quantified using the Nanodrop 2000 spectrophotometer (Thermofisher Scientific, Waltham, MA, Waltham, Massachusetts, USA) and 1µg of RNA was used for complementary DNA (cDNA) synthesis. The miScript RT II Kit (Qiagen) was used for miRNA quantification and the QuantiTect Reverse Transcription kit (Qiagen) was used for mRNA quantification as per the manufacturer's instructions.

*MicroRNA-148a and mRNA quantification*

Hepatic expression of microRNA-148a in patients was measured using a miScript Primer Assay specific for human miR-148a-3p (Qiagen) as per the manufacturer's instructions. Briefly, a reaction volume of 25µl consisting of template cDNA, 2X QuantiTect SYBR Green PCR Master Mix, 10X miScript Universal Primer, 10X miScript Primer Assay and nuclease free water was prepared in a MicroAMP™ 96-Well Base plate (Applied Biosystems, Foster City, CA, USA) and sealed. Thermocycler conditions for quantitative PCR were as follows - Initial denaturation (95ºC, 15 min) followed by 40 cycles of denaturation (94ºC, 15 s), annealing (55ºC, 30 s) and extension (70ºC. 30 s, fluorescence reading). House-keeping gene RNU6 was concurrently quantified for normalization of gene expression. Thermocycler conditions and data capturing were performed using the Applied Biosystems Viia7 Real-Time PCR System.

Messenger RNA quantification was performed using the PowerUp™ SYBR™ Green Master Mix (ThermoFisher) system as per the manufacturer's instructions. A reaction volume of 10µl was made up consisting of 5µl PowerUp® SYBR® Green Master Mix, 1µM of sense and antisense primers and nuclease free water. Thermocycler conditions were as follows: 50ºC 2 min, initial denaturation (95ºC, 15 s), and 40 cycles of denaturation (95ºC, 15 s), annealing (Table 2, 15 s), and extension (72ºC, min). Housekeeping genes *GAPDH* and *18S* were concurrently quantified for normalization of results. Thermocycler conditions and data capturing were performed using the Applied Biosystems Viia7 Real-Time PCR System.

**Table 3. 1: Primer sequences and annealing temperatures for quantitative PCR**

| GENE | PRIMER SEQUENCE | ANNEALING TEMPERATURE ($^oC$) |
|---|---|---|
| *LDLR* forward<br>*LDLR* reverse | 5'-GAGAGCTTGTGCCGAGATGTG-3'<br>5'- CCGCAGTTGTTAGTGCCATCA-3' | 58 |
| *SREBP2* forward<br>*SREBP2* reverse | 5'- CCTGGGAGACATCGACGAGAT-3'<br>5'- TGAATGACCGTTGCACTGAAG-3' | 54 |
| *HMGCR* forward<br>*HMGCR* reverse | 5'- TGATTGACCTTTCCAGAGCAAG-3'<br>5'- CTAAAATTGCCATTCCACGAGC-3' | 53 |
| *ABCA1* forward<br>*ABCA1* reverse | 5'-GGAAGAACAGTCATTGGGACAC-3'<br>5'- GCTACAAACCCTTTTAGCCAGT-3' | 58 |

*Statistical analysis*

Comparisons of clinical parameters between HIV-ve and HIV+ve women presenting with gall stones were made by performing a Mann-Whitney U Test using Prism 7 statistical software (GraphPad Prism Inc, La Jolla, CA, USA). P values <0.05 were considered statistically significant.

Quantitative PCR analysis of miRNA and mRNA levels was performed using QuantStudio 7 Pro Real-Time PCR Systems Software (Thermofisher). MicroRNA and mRNA levels were reported as fold change expressed as $2^{-\Delta\Delta Ct}$ (RQ min; RQ max). Fold changes >2 and <0.5 were considered significant.

**RESULTS**

Analysis of clinical parameters are summarized in Table 1. The HIV+ve group was significantly older than the HIV-ve group. The results show that the HIV+ve group had a lower BMI with overall higher levels of total cholesterol, triglycerides, high density lipoprotein cholesterol (HDL-c) and significantly higher low-density lipoprotein cholesterol (LDL-c).

**Table 3. 2: Clinical parameters of patients presenting with gall stones (HIV-ve vs HIV+ve)**

| | **CONTROL**<br>**N = 5** | **HIV**<br>**N = 5** | **P-VALUE** |
|---|---|---|---|
| **AGE** | 29.60±5.41 | 39.60±6.189 | 0.0267 |
| **BMI** | 34.06±5.980 | 32.63±10.84 | 0.807 (ns) |
| **TOTAL CHOLESTEROL (mmol/L)** | 3.640±1.107 | 4.882±0.9883 | 0.0986 (ns) |
| **TRIGLYCERIDES (mmol/L)** | 0.7640±0.3886 | 0.8980±0.4909 | 0.6457 (ns) |
| **HDL (mmol/L)** | 1.192±0.2821 | 1.328±0.5758 | 0.6526 (ns) |
| **LDL (mmol/L)** | 2.096±0.7410 | 3.160±0.6403 | 0.0419 |

*ns: no significance

**microRNA-148-3p and target gene quantification**

Gene targets of miR-148-3p were confirmed using TargetScan prediction software confirming complementary base pairing with *ABCA1* and *LDLr* (Figure 1A). Quantitative PCR results for miR-148a-3p showed significantly lower hepatic expression in HIV+ve individuals [0.396-fold (RQ min:0.297; RQ max: 0.527); Figure 1B]. Analysis of target gene *ABCA1* showed higher mRNA levels [1.541-fold (RQ min: 1.266; RQ max: 1.875); Figure 1C] while *LDLr* mRNA levels were significantly reduced in HIV+ve patients [0.181-fold (RQ min: 0.07; RQ max: 0.467); Figure 1D].

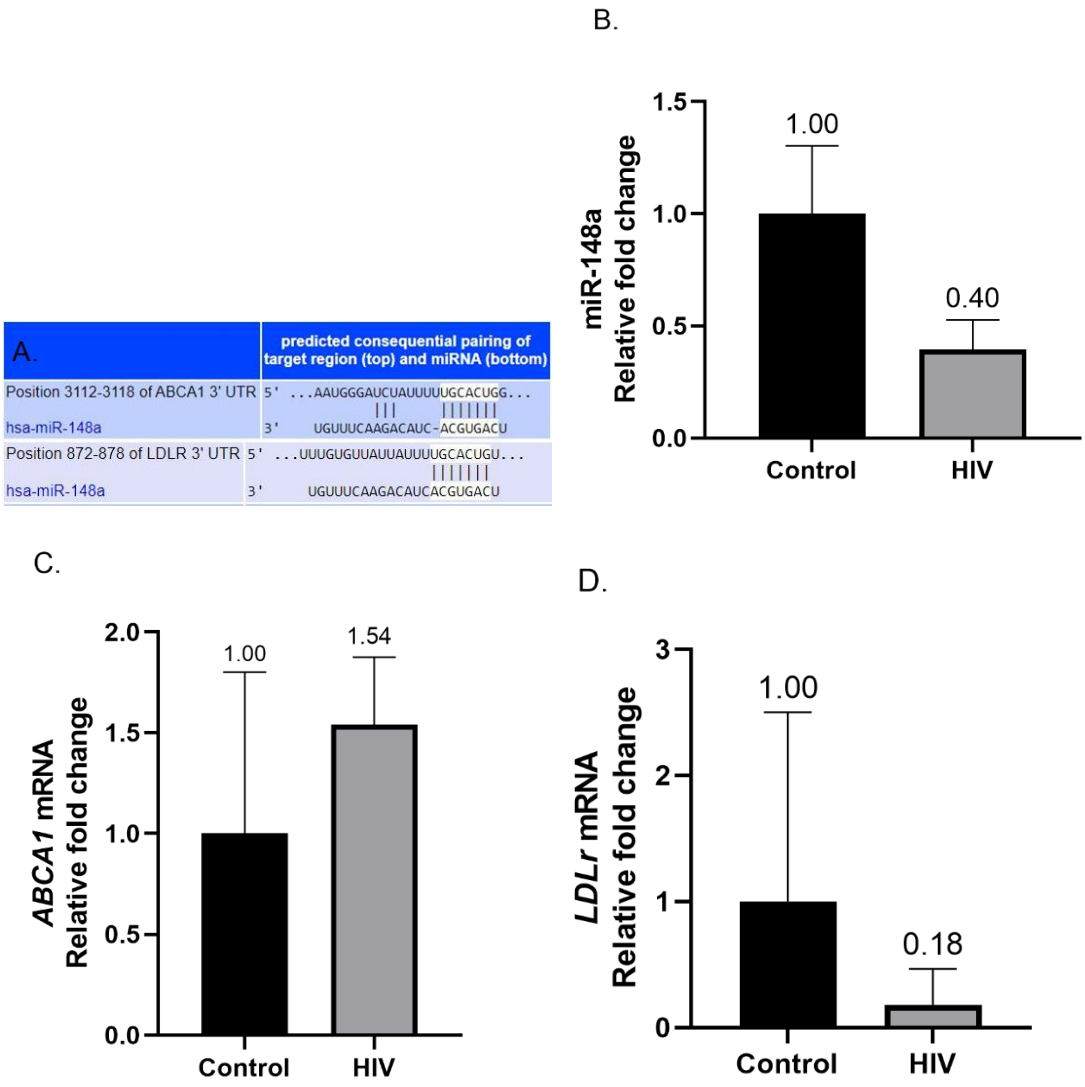


**Figure 3. 1: MicroRNA-148-3p is a regulator of cholesterol metabolism through its binding capacity on ABCA1 and LDLr (A). Hepatic expression is significantly lower in HIV positive patients (B) with an inverse observation made with ABCA1 mRNA levels (C). *LDLr* mRNA levels were significantly lower in HIV infection (D).**

### Transcriptional regulation of LDLR via SREBP2

Following analysis of miR-148a-3p and target genes *ABCA1* and *LDLr*, alternate transcriptional regulation of *LDLr* was investigated to justify the significantly lower mRNA levels observed in HIV infected individuals. Quantitative PCR results showed significantly lower *SREBP2* (transcriptional regulator of *LDLr*) mRNA levels in HIV positive patients presenting with gall stones compared to HIV-ve women [0.127-fold (RQ min: 0.088; RQ max: 0.184); Figure 2].

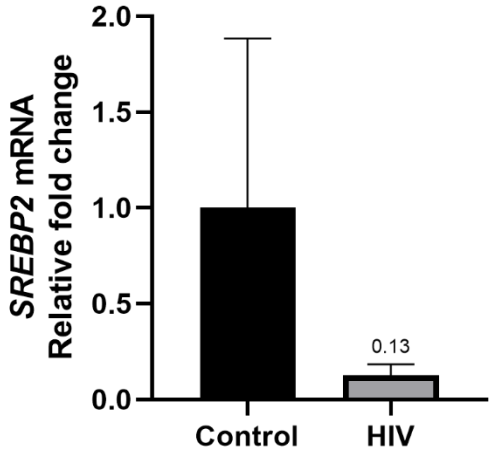


**Figure 3. 2: Quantitative PCR analysis of SREBP2 mRNA showed significantly lower expression in HIV positive patients relative to HIV negative controls**

**Cholesterol metabolism**

HIV+ve women showed higher levels of total cholesterol, therefore the gene responsible for cholesterol metabolism was quantified. *HMGCR* mRNA levels were lower in the HIV+ve group, albeit insignificantly [0.595-fold RQ min: 112; RQ max: 1.172); Figure 3].

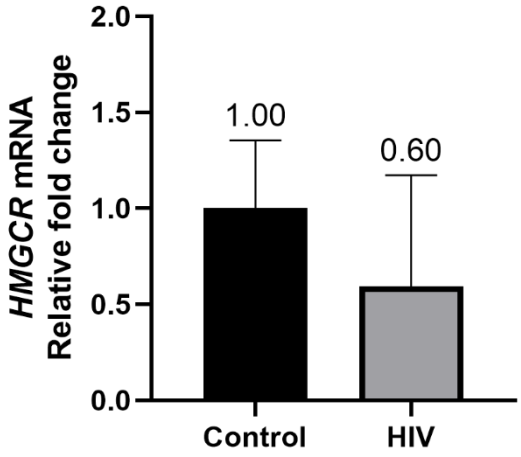


**Figure 3. 3: Quantitative PCR analysis showed 0.6-fold change in *HMGCR* mRNA levels in HIV+ve women relative to HIV-ve women**

## DISCUSSION

Urbanised diets and the consequent rise in obesity amongst Black South Africans has led to gallstones becoming more prevalent amongst this population group in recent years. Established risk factors for gallstones – over forty years of age, female, pregnancy, overweight, high-fat diet, and being sedentary, are all lifestyle or demographic dependent (10). Little is known on the implications of chronic infectious diseases like HIV on gallstone disease risk. Our unpublished data evaluating risk factors in HIV+ve women (n=18) showed that these patients do not conform to these risk factors, with this population being significantly younger to uninfected patients (26). The present study sought to investigate molecular events that may contribute to this phenotype by measuring the hepatic expression of cholesterol metabolism genes that influence gallstone formation. Biological response to a high fat diet, the rate of cholesterol absorption, hepatic cholesterol metabolism and the concentration of cholesterol excreted in bile differs between patients with and without gallstones (27). Raised LDL-c in circulation is strongly implicated in lithogenic states. Lowered HDL-c levels are also implicated in gallstone formation but data regarding this finding has been conflicting in reports (28).

Mounting evidence suggests the resultant abnormalities are linked to dietary and genetic factors. LDL-c is cholesterol that is available for delivery and cellular uptake; the circulating concentration is linked directly to dietary cholesterol consumption and associated with both cardiovascular disease and cholesterol gallstone formation (11,29). HDL-c, excess cholesterol transported from cells to the liver for excretion in a process called reverse cholesterol transport, is considered the “healthier” cholesterol due to protective properties against atherosclerosis. Lower HDL-c is linked to poorer cardiovascular outcomes and is also implicated in cholesterol gallstone formation (30). Elevated activity of cholesteryl ester transfer protein (CETP) in patients with gallstones may lower HDL-c and increase LDL-c by transferring the lipoprotein from HDL to LDL (31).

A high circulating LDL-c will result in higher concentrations of cholesterol being delivered to the liver for excretion resulting in supersaturation of bile, inevitably leading to gallstone formation. This is best explained by Admirand’s triangular relationship between cholesterol, bile salts and lecithin, where biliary cholesterol hypersecretion supersedes the concentration of bile salts and lecithin with resultant precipitation and formation of cholesterol stones (9). The cholesterol hypersecretion is due to excess free cholesterol pooling in the liver either due to increased HMGCR activity or larger volumes of cholesterol returning to the liver. Cholesterol is returned to the liver via one of 4 pathways; via the LDLr, via apoE-rich lipoproteins through the LDL receptor-like protein (LRP), via $HDL_2$ free cholesterol "exchange," or via nonreceptor-mediated LDL uptake (32).

Altered cholesterol metabolism, favouring LDL-c is complicit in gallstone formation, among other metabolic disorders such as cardiovascular disease. Although HIV and ART are linked to metabolic disorders leaning toward this phenotype, little is known of the effects of HIV on gallstone formation.

In HIV+ve patients, low levels of HDL-c are congruent with high viral loads, linking this metabolic profile directly to the virus itself independent of ART (33). The use of ART reverses these effects. LDL-c levels however appear similar to HIV-ve women, but rises dependant on the use of PI-ART (34). In HIV+ve men however, there is an inverse relation with LDL-c and HIV RNA levels independent of ART, and this may well be a major contributing factor to men being relatively protected against gallstones as indicative of the low incidence of gallstones in HIV+ve men (35).

These findings are not dissimilar to that in our study group where all patients were women and all on ART. The use of ART may explain the significantly higher LDL-c, and the slight increase in HDL-C in the HIV+ve group compared to the HIV-ve group (Table 1). Altered circulating LDL-c would prompt cellular homeostatic responses. As HIV+ve women in our study displayed higher LDL-c, we evaluated the first line of hepatic LDL-c uptake. Located on the cell membrane, LDLr is responsible for hepatic absorption of LDL, with higher expression reducing circulating LDL-c levels. The expression of *LDLr* is regulated by various regulatory mechanisms including transcription factors (SREBP2 and LXR) and epigenetic regulators like miR-148a. SREBP transcription factors regulate the biosynthetic pathway of cholesterol and *LDLr* by stimulating transcriptional genes containing sterol response elements (36). There are 3 isoforms; *SREBP1a, SREBP1c* and *SREBP2. SREBP2* is highly expressed in liver amongst other cells. Abnormalities in these regulatory elements (decreased *SREBP2* and *LXR*) results in decreased *LDLr* expression and decreased LDL catabolism resulting in raised LDL-c serum levels (37).

HIV-1 infection of $CD4^+$ T cells stimulates cholesterol biosynthesis via *SREBP2* sterol response gene (protein TFII-I) activation for enhanced HIV-1 transcription and HIV-1 replication (38). HIV-1 transcription is thus modulated by LDL-c, since uptake of LDL-c results in feedback inhibition of *SREBP2*- dependent proteins such as TFII-I (38). The hepatic impact of this in HIV+ve patients with gallstones in not known. However, *LDLr* levels in mononuclear cells of both the blood and liver have been found to be down-regulated in HIV+ve patients with lipodystrophy compared to HIV-ve controls and HIV+ve patients without lipodystrophy, independent of PI-ART (39). The pathogenesis of this lipodystrophy by down-regulation of *LDLr* may not be dissimilar to that of gallstone pathogenesis in HIV+ve patients as these mimics our findings in this study.

Cholesterol reverse transport is also an important determinant in cholesterol homeostasis. In this regard, the ABC family of efflux pumps plays a significant role in cellular efflux of cholesterol. ABCA1 is a major regulator of cellular reverse cholesterol transport by transporting cholesterol, mainly the lipid poor apoA1, out of the cell and converts it into mature HDL for transport back to the liver. Overexpression of *ABCA1* in transgenic mice results in a lithogenic state by increasing plasma HDL-c levels, hepatic delivery of HDL cholesteryl-esters and biliary cholesterol concentrations (40). Its lithogenic role is further accentuated in gallbladder epithelial cells, where *ABCA1* is regulated by *LXR* and *RXR* and modulates biliary cholesterol concentrations and its excretion (41).

*ABCA1* expression can be epigenetically regulated by miR-148a. Inhibition of miR-148a increases *ABCA1* mRNA levels resulting in increased cholesterol efflux to ApoA1, thus increasing plasma HDL-c (20).

The effect of HIV on hepatic *ABCA1* expression has not been studied, however in order for survival and replication of the virus within lymphocytes, it requires large amounts of cholesterol within the cell. It achieves this by directly inhibiting *ABCA1*. The level of inhibition of cholesterol efflux is directly proportional to the level of viral replication within the cell. It achieves this by encoding a small protein called Negative Regulatory Factor (Nef) which binds to *ABCA1* and downregulates it thus preventing the efflux of apoA1 cholesterol to HDL (5).

Besides its regulatory role of *ABCA1*, miR-148a is considered a central miRNA in cholesterol and fat metabolism. MicroRNA-148a is located in a gene-poor intergenic region of human chromosome 7 and is predominantly expressed in liver (20). Notably, the expression of miR-148a is significantly increased in the liver of high-fat diet (HFD)-fed mice (20). Out of 159 miRNAs identified to be highly expressed in human liver and modulated by dietary lipids, miR-148a emerged as the strongest with highest liver activity and expression in livers of HFD fed mice (42). Lastly, we assessed cholesterol biosynthesis in HIV+ve women via *HMGCR* levels. *HMGCR*, the rate limiting enzyme in the mevalonate pathway is regulated by *SREPB2* which up-regulates cholesterol synthesis genes when cholesterol levels are low (43). Our study showed the HIV+ve group having lower *HMGCR* mRNA levels (Figure 3) which may be due to cholesterol levels being higher in this group.

The most obvious limitation of the study is the small sample number. Ideally the study would include samples from patients without gallstone disease and patients not on ART. These demographics proved difficult to collect in sufficient numbers during the patient recruitment period. Another limitation was the large average age gap between the HIV negative and HIV positive groups, which is likely to affect the observed results. The study could be enhanced by correlating the expression of these genes in the liver with circulating levels of the genes and lipogram data – these statistics would be more powerful with a bigger sample size. Furthermore, discrepancies in the duration of ART in the patients may lead to variability

**CONCLUSION**

Cholesterol homeostasis is complex, and dysregulation of the regulatory processes involved can lead to cholesterol gallstone formation. The impact of a chronic infectious disease, like HIV, needs to be considered in the context of rising incidence of metabolic disorders in developing countries. Our findings show a significant increase in circulating LDL-c in the HIV+ve group coupled with reduced mRNA expression of hepatic *LDLr*. However, the suppression of miR-148, an epigenetic regulator of *LDLr*, was downregulated in the HIV+ve group. This would indicate a possible alternate pathway in the downregulation of *LDLr* in HIV+ve patients linked with raised LDL-c and gallstone formation and

will require further investigation. MiR-148a however did appear to regulate *ABCA1* with an inverse relationship being observed in the HIV+ve patients.

**LIST OF ABBREVIATIONS**

| | |
|---|---|
| ABC | Adenosine triphosphate binding cassette |
| ABCA1 | Adenosine triphosphate binding cassette A1 |
| ART | Antiretroviral therapy |
| BMI | Body mass index |
| cDNA | Complementary DNA |
| CETP | Cholesterol ester transfer protein |
| CYP7A1 | Cholesterol-7 alpha-hydroxylase |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| HDL | High density lipoprotein |
| HDL-c | High density lipoprotein cholesterol |
| HMG CoA | 3-hydroxy-3-methyl-glutaryl-CoA |
| HMGCR | 3-hydroxy-3-methyl-glutaryl-CoA Reductase |
| HIV | Human Immunodeficiency Virus |
| LDL | Low density lipoprotein |
| LDL-c | Low density lipoprotein cholesterol |
| LDLr | Low density lipoprotein receptor |
| LRP | Low density lipoprotein receptor like protein |
| LXR | Liver X Receptor |
| miRNA | microRNA |
| mRNA | Messenger RNA |
| PBS | Phosphate saline buffer |
| PI | Protease inhibitor |

RNA Ribose nucleic acid

SREBP Sterol regulatory element binding protein

## DECLARATIONS

- **Ethics approval and consent to participate**

Ethical approval was obtained from the University of KwaZulu Natal. Biomedical Research Ethics Committee (BREC – BE276/16). Patients undergoing cholecystectomy for gall stone disease (biliary cholic, cholecystitis, jaundice and gall stone pancreatitis) at King Edward VIII Hospital, Durban, KwaZulu Natal, South Africa from January – December 2017 were recruited to evaluate known risk factors of gall stone formation in HIV positive and negative patients. In total 96 patients gave informed consent (standard consent form in two official main languages English and isiZulu) for retrieval of a liver biopsy and recording of clinical parameters of patients including age, race, BMI, family history of gall stones and comorbidities (HIV, hypertension, diabetes, hypercholesterolemia).

- **Consent for publication**

Availability of data and material

- **Competing interests**

None


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- **Authors' contributions**

S.M.K. conceptualized the study, collected samples, analysed generated data and was primary author in writing of the manuscript

S.N. assisted with project conceptualization and design, performed laboratory experiments, statistical analysis and gave critical feedback on the manuscript

B.S. and A.C. conceived the original idea, supervised the project and provided critical feedback on the manuscript


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## CHAPTER 4

Chapter 3 demonstrated that HIV+ve Black South African women with GD have a higher circulating LDL-c with a reduced mRNA LDLr hepatic expression compared to their HIV-ve counterparts with GD. However, MiR-148 a known epigenetic regulator of LDLr was also downregulated indicating a possible alternate pathway in the downregulation of LDLr in HIV+ve patients. Using this obtained information of decreased LDL input of cholesterol to hepatocytes in Chapter 3, focus was shifted into looking into the cholesterol output of the hepatocyte.

Chapter 4 presents a study sought to evaluate the expression of HNF1α, HNF4α, LXRb and miRNAs (HNF4α specific: miR-194-5p and miR-122*_1) that regulate CYP7A1 transcription in HIV-infected Black South African women on antiretroviral therapy (ART) and presenting with gallstones relative to HIV negative women with gallstone disease. This is in keeping with objective 3. Chapter 4 is presented in the form of a manuscript entitled "Dysregulation of hepatic nuclear regulators of bile acid metabolism in HIV-infected women with gallstones". The manuscript is currently under review at the Journal of Physiology and Biochemistry. (Submission ID: a7a1bef2c6ba298d).

**Dysregulation of hepatic nuclear regulators of bile acid metabolism in HIV-infected women with gallstones**

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## ABSTRACT

### Background

Gallstone formation occurs when the equilibrium between cholesterol and bile acid is altered. HIV infected patients on antiretroviral therapy (ART) are prone to hypercholesterolemia, which contributes to metabolic disorders. Although cholesterol homeostasis has been studied extensively in HIV, little is known regarding the bile acid synthesis pathway which is initiated by cholesterol 7-alpha hydroxylase (CYP7A1) and regulated by hepatocyte nuclear factors (HNF1α, HNF4α and LXRb).

### Aim/ Objective

To evaluate the expression of HNF1α, HNF4α, LXRb and miRNAs (HNF4α specific: miR-194-5p and miR-122*_1) that regulate CYP7A1 transcription in HIV+ve Black South African women on ART and presenting with gallstones relative to HIV negative patients with gallstone disease.

### Methods

Black African Women (n = 52) presenting with gallstone disease were stratified based on HIV status. The gene expression of *CYP7A1*, *HNF1α*, *HNF4α*, *LXRb*, miR-194-5p and miR-122*_1 was determined using RT-qPCR. Messenger RNA and miRNA levels were reported as fold change expressed as $2^{-\Delta\Delta Ct}$ (RQ min; RQ max). Fold changes >2 and <0.5 were considered significant.

### Results

HIV+ve women were older in age (p = 0.0267) and displayed higher low-density lipoprotein cholesterol (LDL-c) (p = 0.0419), *CYP7A1* [2.078-fold (RQ min: 1.278; RQ max: 3.381)], *LXRb* [2.595-fold (RQ min: 2.001; RQ max: 3.000)] and *HNF1α* [3.428 (RQ min: 1.806; RQ max: 6.507] levels. *HNF4α* [0.642-fold (RQ min: 0.266; RQ max: 1.55)], miR-194-5p [0.527-fold (RQ min: 0.37; RQ max: 0.752)] and miR-122*_1 [0.595-fold (RQ min: 0.332; RQ max: 1.066)] levels were lower in HIV+ve women.

### Conclusion

HIV+ve women with gallstone disease displayed higher LDL-c levels and increased markers for bile acid synthesis, which was evident by the elevated expression of *CYP7A1*, *HNF1α* and *LXRb*. This could have been further influenced by chronic use of ART.

## INTRODUCTION

The advent of antiretroviral therapy (ART) in human immunodeficiency virus (HIV) treatment was one of the most significant advances in modern medicine. The HIV epidemic went from a public health crisis to a chronic treatable disease as ART markedly extended the life expectancy of infected individuals. While the large-scale rollout of ART has made a positive impact on the outcomes of HIV morbidity and mortality, there now emerges an unprecedented population of people aging with HIV (1–3). This has led to a new phenomenon - an "epidemic within an epidemic", with disorders of metabolism becoming increasingly prevalent in HIV+ve individuals on ART. These range from acute to chronic adverse effects with the most common being cardiovascular disease, lipodystrophy, diabetes and metabolic syndrome (4–8). Altered glucose metabolism and hypercholesterolemia are hallmarks of HIV patients on chronic ART (6,8,9). Kato et al. (2020) reported higher low-density lipoprotein (LDL) levels in HIV+ve patients on ART relative to ART naïve patients (6). Elevated circulating LDL cholesterol (LDL-c) contributes to several of the metabolic syndrome like symptoms observed in people on ART. Despite established prevalence of metabolic syndrome and altered cholesterol homeostasis in this population, there is a dearth of knowledge on gallstone disease, a disease closely linked to both these factors (10).

Cholesterol gallstones, the result of biliary cholesterol superseding it saturation point causing cholesterol microcrystal formation (11), account for ~80% of all gallstones in western populations (12). The aetiology of gallstone formation involves an interplay between genetic and environmental factors. Risk factors include obesity, female sex, high oestrogen levels, aging, diabetes and metabolic syndrome (13,14). Historically, gallstone disease was most prevalent in North America, South America, some European populations and India (15); however, the rapid rate of urbanization, high fat diets and the influence of HIV on non-communicable diseases has seen a paradigm shift, warranting more investigation into developing countries and Sub-Saharan Africa (14). Data collected in South Africa suggest a steady increase in gallstone disease in Black African people over the past ten years (16,17). Considering the HIV endemic setting, very little is known on HIV+ve patients on ART with gallstone disease (18).

Gallstones are the result of a shift in equilibrium of the triad of cholesterol, bile acid and lecithin, favouring bile to exist in a lithogenic state rather than liquid state (11). Bile acid synthesis is initiated by the rate limiting enzyme cholesterol 7-alpha hydroxylase (CYP7A1), which catalyses the conversion of cholesterol to 7-alpha-hydroxycholesterol (19). The CYP7A1 enzyme is regulated via multiple pathways including a negative feedback loop from the hepato-enteric circulation of bile acids (20). As CYP7A1 regulates the first step of bile acid synthesis, it has been extensively investigated in relation to gallstone formation. Several studies have evaluated genetic variations in the *CYP7A1* gene in relation to gallstone disease risk with stronger links found to genetic predisposition in males compared to

females (21–24), while a deficiency in the enzyme is associated with gallstone formation (25,26). Considering that women are disproportionately affected by gallstone disease and the demographic at highest risk for new HIV infections in South Africa, alternative molecular mechanisms need to be investigated.

The transcription of the *CYP7A1* gene is regulated by transcription factors in response to fluctuations in bile acid and cholesterol. Hepatocyte nuclear factors (HNFs) are a group of transcription factors predominantly expressed in the liver that maintain metabolic homeostasis through the regulation of genes involved in glucose, cholesterol and fatty acid metabolism (27). Hepatocyte nuclear factor 1 alpha (HNF1α) and hepatocyte nuclear factor 4 alpha (HNF4α) are HNFs that respond to bile acid levels: HNF4α binds directly to *CYP7A1* (28) while HNF1α binds to CYP7A1 regulators - hepatic bile acid binding protein and Farnesoid X receptor (FXR) (29). MicroRNAs (miRNAs), namely miR-194-5p and miR-122*_1 is chiefly regulated by HNF4α in the liver (30,31). Liver X receptor α/β (LXR α/β) is closely related to FXRs and binds to the liver x receptor element (LXRE) of LXR target genes. Among these target genes are regulators of reverse cholesterol transport, the most prominent being ATP-binding cassette (ABC-) G1, ABCG5 and ABCG8. The activity of these cellular efflux pumps determines cholesterol flux, thus regulating CYP7A1 activity in response to hepatic cholesterol concentration (32,33).

Nuclear factors like HNFs and LXRs are key upstream regulators of hepatic cholesterol metabolism and bile acid synthesis. Dysregulation of these transcriptional regulators leads to pathogenic outcomes that underlie metabolic disorders. This present study sought to evaluate the expression of hepatic nuclear factors (HNF1α, HNF4α and LXRb) and miRNAs (HNF4α specific: miR-194-5p and miR-122*_1) that regulate CYP7A1 transcription in HIV+ve Black South African women on ART and presenting with gallstones relative to HIV-ve women with gallstone disease.

## METHODS

### Patient recruitment

This study utilized a case-series design comparing hepatic expression of cholesterol regulating genes in HIV+ve and HIV-ve women presenting with symptomatic gallstones. Ethical approval was obtained from the University of KwaZulu Natal (UKZN) Biomedical Research Ethics Committee (BREC) (BE276/16). Patients undergoing cholecystectomy for gallstone disease (biliary cholic, cholecystitis, jaundice and gall stone pancreatitis) at King Edward VIII Hospital, Durban, KwaZulu-Natal, South Africa from January – December 2017 were recruited. In total 52 Black South African women gave informed consent (standard consent form in two official main languages, i.e., English and isiZulu) for retrieval of a liver biopsy and recording of clinical parameters of patients including age, race, BMI, family history of gall stones and comorbidities (HIV, hypertension, diabetes, hypercholesterolemia).

Following the analysis of clinical parameters in all subjects, five HIV-ve and five HIV+ve women were selected for mRNA analysis to identify hepatic nuclear factors that were differentially regulated. All patients selected were of Black African ethnicity and female with no co-morbidities (diabetes and hypertensive statin therapy). All HIV+ve patients were on fixed dose combination (FDC) therapy with CD4 counts above 500 cells/$mm^3$ and undetectable viral loads. FDC regimen consisted of 3 drugs namely; two Nucleoside reverse transcriptase inhibitors (NRTIs) drugs [Tenofovir Disoproxil Fumarate (TDF) and Emtricitabine (FTC) and one Non-nucleoside reverse transcriptase inhibitor (NNRTI) [Efavirenz (EFV)]. Common second line agents used were Protease inhibitors (PI) [Lopinivar/ritonavir (Aluvia) or Atazanavir/ritonavir]. None of the patients were on Integrase strand transfer inhibitor (InSTI) [Dolutegravir (DTG)] based therapies.

**RNA extraction and Real Time Quantitative PCR (RT-qPCR)**

Liver tissue (1cm x 1cm) was submerged in RNAlater® Stabilization Reagent (Qiagen) in 2ml cryovials at collection and stored at -80ºC until RNA isolation. RNA was extracted using a chloroform-based method using Qiazol lysis buffer (Qiagen) as per the manufacturer's instructions. The purity and concentration of the crude RNA was assessed using the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). The crude RNA was standardised to a concentration of 1,000ng/µl and stored at -$80^0$C until further use.

Thereafter, 1µg of the standardised RNA sample was used for complementary DNA (cDNA) synthesis using PCR. The miScript RT II kit (Qiagen) (miRNA gene expression studies) and QuantiTect Reverse Transcription kit (Qiagen) (gene expression studies) was used to make cDNA as per the manufacturer's instructions.

Messenger RNA quantification (*CYP7A1*, *HNF1α*, *HNF4α* and *LXRb*) and miRNA quantification (miR-194-5p and miR-122*_1) was performed using the Applied Biosystems Viia7 Real-Time PCR System (Thermo Fisher Scientific).

MiRNA expression studies was performed using the miScript™ SYBR™ Green PCR kit (Qiagen, Hilden, Germany) and specific miScript Primer Assays for the above-mentioned miRNAs (Qiagen) according to the manufacturer's instructions. Human RNA, U6 small nuclear 2 (*RNU6-2*) was used as a housekeeping gene.

Messenger RNA gene expression studies was performed using the PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) and specifically designed primer sequences for the above-mentioned genes (Inqaba Biotec) according to the manufacturer's instructions. The sense and antisense primer sequences for the above-mentioned genes are shown in Table 1 together with their annealing temperature. Housekeeping genes *GAPDH* and *18SrRNA* were concurrently quantified for normalization of results.

**TABLE 4. 1: Primer sequences and annealing temperatures for RT-qPCR**

| Gene | Primer sequence | Annealing temperature (ºC) |
|---|---|---|
| *HNF1α* sense | 5'- ACCAAGCCGGTCTTCCATACT -3' | 58 |
| *HNF1α* antisense | 5'- GGTGTGTCATAGTCGTCGCC -3' | |
| *HNF4α* sense | 5'- CACGGGCAAACACTACGGT -3' | 55 |
| *HNF4α* antisense | 5'- TTGACCTTCGAGTGCTGATCC -3' | |
| *LXRb* sense | 5'- AGAAGATTCGGAAACAACAGCA -3' | 53 |
| *LXRb* antisense | 5'- GCTGGATCATTAGTTCTTGAGCC -3' | |
| *CYP7A1* sense | 5'- GAGAAGGCAAACGGGTGAAC -3' | 54 |
| *CYP7A1* antisense | 5'- GGATTGGCACCAAATTGCAGA -3' | |

**Statistical analysis**

Comparisons of clinical parameters between HIV-ve and HIV+ve women presenting with gall stones were done by performing a Mann-Whitney U Test. All data were analysed using the GraphPad Prism 7 statistical software package. A $p<0.05$ were considered statistically significant.

RT-qPCR analysis of mRNA levels was performed using the QuantStudio 7 Pro Real-Time PCR Systems Software (Thermo Fisher Scientific). Messenger RNA and miRNA levels were reported as fold change expressed as $2^{-\Delta\Delta Ct}$ (RQ min; RQ max). Fold changes >2 and <0.5 were considered significant.

**RESULTS**

**Clinical characteristics of patients**

Analysis of clinical parameters are summarized in Table 2. The HIV+ve group was significantly older than the HIV-ve group (p = 0.0267). The results show that the HIV+ve group had a lower BMI with overall higher levels of total cholesterol, triglycerides, high density lipoprotein cholesterol (HDL-c) and significantly higher low-density lipoprotein cholesterol (LDL-c) (p = 0.0419).

**TABLE 4. 2: Clinical parameters of patients presenting with gall stones stratified based on HIV status**

| Parameters | HIV-ve | HIV+ve | p-value |
|---|---|---|---|
| Age (years) | 29.60±5.41 | 39.60±6.189 | *0.0267 |
| BMI (kg/m$^2$) | 34.06±5.980 | 32.63±10.84 | 0.807 |
| Total Cholesterol (mmol/L) | 3.640±1.107 | 4.882±0.9883 | 0.0986 |
| Triglycerides (mmol/L) | 0.7640±0.3886 | 0.8980±0.4909 | 0.6457 |
| HDL-c (mmol/L) | 1.192±0.2821 | 1.328±0.5758 | 0.6526 |
| LDL-c (mmol/L) | 2.096±0.7410 | 3.160±0.6403 | *0.0419 |

**Hepatic *CYP7A1, LXRb, HNF1α* and *HNF4α* gene expression**

RT-qPCR analysis showed that hepatic mRNA levels of *CYP7A1* were significantly higher in women with HIV and gallstone disease relative to women with gallstone disease alone [2.078-fold (RQ min: 1.278; RQ max: 3.381)] (Figure 1). Transcriptional regulators of *CYP7A1* were subsequently quantified: *LXRb* [2.595-fold (RQ min: 2.001; RQ max: 3.000)] (Figure 2A) and *HNF1a* [3.428-fold (RQ min: 1.806; RQ max: 6.507] (Figure 2B) mRNA levels were concomitantly higher in the HIV+ve women compared to the HIV-ve group. Hepatic *HNF4a* mRNA levels were lower in HIV+ve women [0.642-fold (RQ min: 0.266; RQ max: 1.55)] (Figure 2C).

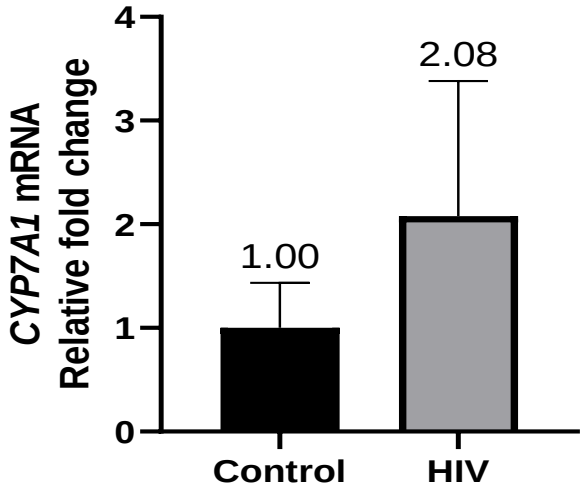


**Figure 4. 1: Hepatic *CYP7A1* transcript levels were significantly higher (>2-fold) in HIV+ve women.**

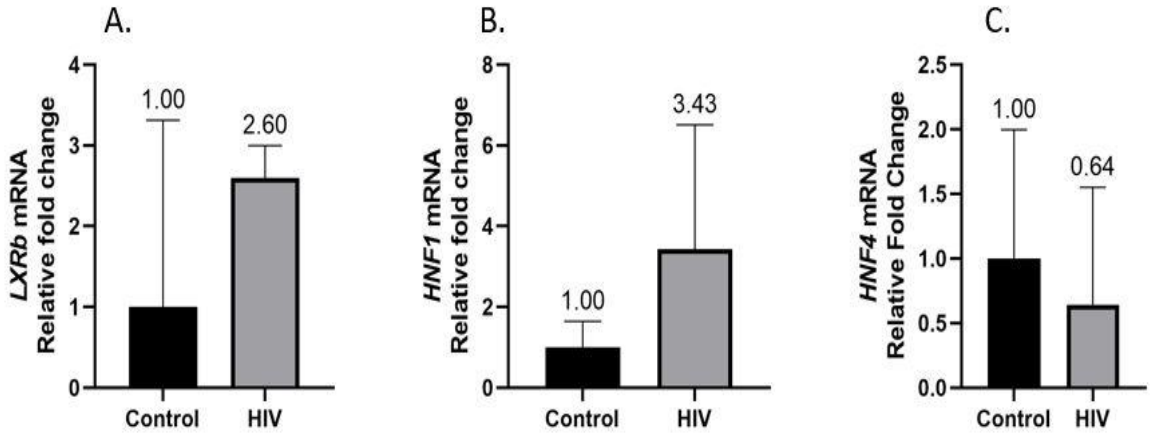


**Figure 4. 2: CYP7A1 regulators *LXRb* (A) and *HNF1α* (B) mRNA levels were significantly higher in HIV positive patients with gallstone disease (>2-fold). Hepatic *HNF4α* mRNA levels were lower in HIV infected women.**

**Hepatic miR-194-5p and miR-122*_1 gene expression**

Hepatic miRNAs regulated by HNF4α were quantified by RT-qPCR. Both miR-194-5p [0.527-fold (RQ min: 0.37; RQ max: 0.752)] (Figure 3A) and miR-122*_1 [0.595-fold (RQ min: 0.332; RQ max: 1.066)] (Figure 3B) were observed at lower concentrations in HIV+ve women with gallstone disease compared to HIV-ve women.

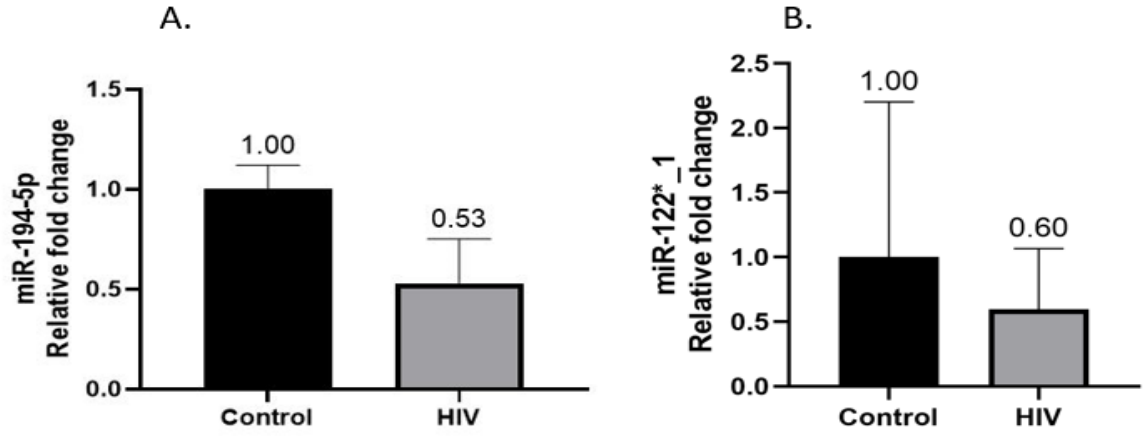


**Figure 4. 3: Hepatic expression of both miR-194-5p and miR-122*_1 was lower in HIV+ve women with gallstone disease relative to the HIV-ve group.**

## DISCUSSION

Gallstone disease is triggered by several risk factors, including female sex, aging, obesity, high oestrogen levels and dyslipidaemia (13, 14). Within the context of an African population, age, waist circumference and LDL-c were risk factors for gallstone disease in Sudanese individuals (34). In Black South African women, BMI and continuous exposure to a western lifestyle (characterised by high fat intake and low dietary fibre intake) were the main risk factors for gallstone disease (16).

Long term use of ART has been linked to a number of metabolic diseases, however, there is a paucity of data on their association with gallstone disease in black South Africans (9–12). It has been demonstrated in other populations that the accumulative exposure to atazanavir/ritonavir for over 2 years is associated with a 6.29-fold increase in the risk for incident cholelithiasis (38), whilst other studies report an increased rate of cholelithiasis (9,8%) in HIV+ve patients on protease inhibitor (PI)-inclusive ART (39). HIV-infected patients on ART are more susceptible to hypercholesterolemia (6, 9). Despite newer integrase inhibitor (II) antiretroviral drugs showing lower lipid abnormalities than previously used PI-based ART, abnormalities in lipid concentration still occur, and this may be reflective either of the viral effects itself, chronic ART use or persistent immune activation in HIV infection (40).

Atazanavir, ritonavir and indinavir significantly decreased *CYP7A1* mRNA levels in rodent hepatocytes, indicating an effect of ART on the bile acid synthesis pathway (41,42). A substantial amount of evidence dating back as far as 1975 (43), together with more recent evidence have demonstrated that a deficiency in CYP7A1 (44), genetic variations in the *CYP7A1* gene (45), and inhibition of CYP7A1 with lipid lowering drugs like fibrates (46), result in increased cholesterol excretion in bile which increases the risk of gallstone formation (22,26). Relative to Admirand's theory, lower CYP7A1 activity compromises the conversion of cholesterol to bile acid resulting in increased cholesterol excretion and decreased bile acid excretion favoring gallstone formation (11). In Chilean Hispanic and Mapuche subjects (known to have one of the highest incidences of gallstones worldwide), an increase in *CYP7A1* expression (47) and an increase in bile acid excretion (48) resulted in higher incidence of gallstone formation. Our study showed that HIV+ve women on ART displayed higher hepatic *CYP7A1* mRNA (Figure 1), suggesting a possible response to impaired enterohepatic circulation of bile acids in HIV+ve women (49). This finding warranted investigation into transcriptional regulators of CYP7A1 namely HNF1α, HNF4α and LXRb.

The regulation of CYP7A1 by HNF1α has been studied extensively in mice, and found to downregulate CYP7A1 indirectly by binding to FXR receptors (29,50). In a murine study, HNF1α was found to be a transcriptional regulator of FXR, and when HNF1α was inhibited it resulted in downregulation of FXR with resultant gallstone formation (51). Moschetta et al. (2004) reported that FXR knockout mice that were fed a lithogenic diet displayed an increased susceptibility to developing gallstones. Administration

of the FXR agonist to the gallstone-susceptible mice decreased the susceptibility to gallstone formation (52). Similar findings were reported elsewhere that FXR upregulation decreased gallstone formation in mice (53). However, in a study by Chen et al. (1999) they demonstrated that unlike rodents where HNF1α regulates CYP7A1 by binding to FXR, in humans HNF1α binds directly to CYP7A1 and increases its transcriptional activity thus having the opposite effect to that in rodents (54). Similar to the findings from Chen et al. (1999), in our study HIV+ve women with gallstones displayed increased expression of *CYP7A1* and *HNF1α* compared to their HIV-ve counterparts with gallstones, indicating that HIV may have an effect on HNF1α and CYP7A1.

Another hepatic nuclear factor, HNF4α, binds directly to CYP7A1. Bile acids cause a negative feedback loop, resulting in down regulation of CYP7A1 not only via HNF1α and FXR but also HNF4α, which has a direct binding site on CYP7A1. Normally HNF4α binds and upregulates CYP7A1, however there is evidence that bile acids can downregulate CYP7A1 transcription by reducing the transactivation potential of HNF4α (28). In our findings however amongst HIV+ve women with gallstones, *HNF4α* was reduced in comparison to HIV-ve women, yet *CYP7A1* was upregulated. This demonstrates that HNF4α on its own is not a regulator of CYP7A1 in the pathogenesis of gallstones in HIV+ve women. This mirrors other studies that demonstrated that HNF4α together with LRH-1, COUP, TFII and PCG-1α are required for upregulation of CYP7A1. HNF4α on its own is not capable of this (55,56). Bacterial endotoxins, pro inflammatory cytokines (57), TNF-α and interleukin-1 produced by macrophages in the liver (58) are known to decrease the activity of CYP7A1. De Fabiani et al. (2001) demonstrated that HNF4α is the target transcriptional factor mediating the effects of these pro inflammatory cytokines on CYP7A1 (28). HIV+ve patients undergo a phenomenon of persistent inflammatory response which may account for the decrease in *HNF4α* levels (59).

HNF4α is a key regulator of hepatic microRNAs miR-122 and miR-194. MiR-122 is found in abundance in the liver and plays an important role in regulating metabolic pathways including fatty acid synthesis and cholesterol biosynthesis (31). MiR-194 play a key role in hepatic cell functions (30). In our study, *HNF4α* expression was lower in the HIV-infected group which correlated with the decreased expression of miR-194-5p and miR-122*_1. This suggests that HNF4α may have more of an impact on microRNA expression in the liver that the CYP7A1 mediated bile acid synthesis pathway.

LXR agonists have been shown to bind to LXRE on mouse *CYP7A1* causing its upregulation (60). LXRE binds both LXRa and LXRb however LXRa binds stronger than LXRb thus LXRa knock out mice exhibit more cholesterol accumulation. However human CYP7A1 does not contain LXRE and is thus shown not to be upregulated by LXRa agonists and seems to be more affected by diet induced hypercholesterolemia due to its inability to convert cholesterol into bile salts (61). Goodwin et al. (2003) demonstrated a downregulation of CYP7A1 by LXR in response to cholesterol loading (62). Again,

there is implication that regulation of the entero-hepatic circulation may be a result of this as the compensation mechanism to decrease cholesterol absorption (by decreasing bile acid production) following a high cholesterol diet however in humans this compensatory mechanism has been shown to be inefficient (63). In our study, the findings are in keeping with that of mice studies where LXRb increase co-existed with an upregulation of CYP7A1, indicating that LXRb might well be a regulator of CYP7A1 and gallstone formation in these group of patients compared to HIV-ve women with gallstones. Again, this may be due to underlying dysfunction of enterohepatic terminal ileum absorption in HIV+ve patients causing LXRb to increase CYP7A1 in an attempt to produce more bile acid for absorption (64).

## CONCLUSION

In summary, HIV+ve women with gallstone disease displayed higher LDL-c levels and increased bile acid synthesis which was evident by the elevated expression of *CYP7A1*, *HNF1α* and *LXRb*. This could have been further influenced by ART and aging. HNF4α, which is known to cause upregulation of CYP7A1, was supressed with upregulation of CYP7A1 and LXR, known to cause downregulation of CYP7A1 in humans as opposed to mice, also had the opposite effect in HIV-infected women.

The best theoretical explanation for this will be an interruption in the enterohepatic circulation, as evident by HIV+ve patients known to have chronic inflammatory and relative malabsorptive disorders of the ileum, which may result in upregulation of CYP7A1 to produce more bile salts. This is the most probable explanation for gallstone formation in HIV+ve patients, however will have to be validated with further trials and bigger sample size.

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## AUTHOR CONTRIBUTIONS

S.M.K. conceptualized the study, collected samples, analysed generated data and was primary author in writing of the manuscript

S.N. and PN assisted with project conceptualization and design, performed laboratory experiments, statistical analysis and gave critical feedback on the manuscript

B.S. and A.C. conceived the original idea, supervised the project and provided critical feedback on the manuscript

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# CHAPTER 5: SYNTHESIS, SUMMARY, CONCLUSION AND RECOMMENDATIONS

This chapter presents a summary of the research findings, and assesses the strengths and limitations of this study. It also presents the conclusions, recommendations for improvement of existing management and offers suggestions for further research.

## 5.1 Background

The main aim of this study was to determine differences in clinical profiles and regulators of hepatic cholesterol and bile acid metabolism in HIV positive Black South African women on ART with GD compared to HIV negative Black South African women with GD. The significance of this study cannot be denied for several reasons; the incidence of HIV is highest amongst Black South African women particularly in KZN (1), the incidence of GD amongst Black South African women in the country is increasing (2,3), GD ranks as one of the world's most expensive diseases (4), and ART is associated with lipid abnormalities which is the major driver for GD (5). The upsurge of GD in women of this population group living with HIV together with the impact of this high burden of GD on an already overstrained health system therefore gains importance and relevance by demonstrating possible links in order to identify research gaps and suggest novel ideas for future research. The findings will unravel areas where research is lacking as well as indicate possible preventative measures for developing GD and domains in clinical practice that need improvement or refinement to manage these patients with GD. Ultimately, the conclusions drawn will benefit the millions living with HIV in SA particularly those with GD.

## 5.2 Key Findings of the Study

The formation of cholesterol gallstones is complex and usually involves a combination of genetic and a host of environmental factors including lifestyle. Most research into the actual cause and risk factors of gallstones are performed in the West and parts of Europe with a paucity of data in any part of Africa due the lower incidence compared to the rest of the world (6). Whilst Black South Africans are now being exposed to most known risks such as urbanized diets and obesity (7), it not surprising that the patients in this study presenting with GD, show clear risks that are linked to those in the Western world in a disease that is rife amongst Caucasians. However, what is unknown is whether these risks apply to our HIV+ve population. From these observations, these patients do not conform entirely to the normal known risk profile. Black South African HIV+ve women with symptomatic gallstones were significantly younger. Black South African HIV-ve women conform to the known risk factor of obesity with a statically higher BMI whilst HIV+ve women do not. HIV+ve women also had fewer 1st degree relatives with GD compared to HIV-ve women, and less oestrogen exposure.

Cholesterol homeostasis is complex, and dysregulation of the regulatory processes involved can lead to cholesterol gallstone formation. The impact of a chronic infectious disease, like HIV, needs to be considered in the context of rising incidence of metabolic disorders in developing countries.

LDL-c is cholesterol that is available for delivery and cellular uptake; the circulating concentration is linked directly to dietary cholesterol consumption and associated with both cardiovascular disease and cholesterol gallstone formation (8). LDLr is shown to be directly regulated by miR-148a and is linked to gallstone formation in high fat diet fed mice (9)

Our findings show a significant increase in circulating LDL-c in the HIV+ve group coupled with reduced mRNA expression of hepatic LDLr. However, the suppression of miR-148, an epigenetic regulator of LDLr, was downregulated in the HIV+ve group. This would indicate a possible alternate pathway in the downregulation of LDLr in HIV positive patients linked with raised LDL-c and gallstone formation and will require further investigation. MiR-148a however did appear to regulate ABCA1 with an inverse relationship being observed in the HIV positive patients.

CYP7A1 is an enzyme involved in the rate limiting step of converting cholesterol to bile acid for excretion (10). When the cholesterol concentration within the hepatocyte is increased either by increased delivery to the liver or by increased production, CYP7A1 is activated via LXR to convert cholesterol into bile acid (11).

HIV+ve women with gallstone disease displayed higher LDL-c levels and increased bile acid synthesis which was evident by the elevated expression of CYP7A1, HNF1α and LXRb. This could have been further influenced by ART and aging. HNF4α, which is known to cause upregulation of CYP7A1, was supressed with upregulation of CYP7A1 and LXR, known to cause downregulation of CYP7A1 in humans as opposed to mice, also had the opposite effect in HIV+ve women. The best theoretical explanation for this will be an interruption in the enterohepatic circulation, as evident by HIV+ve patients known to have chronic inflammatory and relative malabsorptive disorders of the ileum, which may result in upregulation of CYP7A1 to produce more bile salts.

### 5.3 Strengths of the Study

With the information gained from a case series, it has the advantage of being used to generate hypotheses that lead to focussed studies of a stronger designs. As our study of GD in HIV +ve patients was a first of any kind of study of this nature it will be helpful in refining new techniques or treatment protocols that can be studied in more advanced trials. Our study required less time and financial resources than case control, cohort studies or randomised-controlled trials. We believe that this case series was of high-quality as outlined by having several of the key criteria for a high-quality case series (12); clear study objective/question, well-defined study protocol, explicit inclusion and exclusion

criteria for study participants, specified time interval for patient recruitment, consecutive patient enrolment, clinically relevant outcomes, and a prospective outcome data collection (12).

### 5.4 Limitations of the Study

Overall, the study has made significant findings. Nonetheless, there are some limitations that must be acknowledged. Conclusions drawn from clinical profiles are from a case series rather than a case control setting. Case series represents an observational study that reports on data from a subject group without a comparison population. In the hierarchy of evidence, it represents level IV evidence (13,14). This is due to lack of control subjects, making case series prone to bias. Most of the evidence of regulators of hepatic cholesterol and bile acid metabolism that have been compared to are from murine rather than human studies. Sample sizes were relatively small. Missing data despite not being small numbers could possibly influence the results obtained.

### 5.5 Conclusions

This study demonstrates the current stance of GD amongst Black South African women living with HIV in the era of ART. It also illustrates the importance of the deficiencies in knowledge and their implications for the future. The findings of this study in this population group with HIV differs from the findings of that to other population groups and the same population group living without HIV. What this study demonstrated are key areas in the differences and possible plausible causes at a cellular level, however will require much more research to draw concrete conclusions. ART seems to be a mighty player in these differences as well, and the universal access to ART for all HIV-infected South Africans will likely result in an increase in GD amongst other diseases. Thus, this implies that GD as a possible side effect of ART should be investigated with better emphasis as well especially if the incidence and prevalence amongst HIV+ve patients with GD is high. The impact on the current health infrastructure that this increasing disease will have is unknown. There is shortfall in the knowledge and statistics on the incidence and prevalence of GD in general and amongst those living with HIV with and without ART. There is thus a deficiency in evidence on the morbidity and mortality of HIV-infected patients with GD as well. More studies are required into the effects of HIV on GD and the impact of ART on GD in order to put strategies in place to curb this disease process and reduce the morbidity from it and reduce the cost to the overburdened health system.

### 5.6 Recommendations

Based on the study conclusions, the following recommendations are suggested:

1. GD registries should be established which will assist the much-needed statistics on the morbidity and mortality of both HIV-related and non-related GD as well the ART effects.
2. Larger studies be undertaken to validate the findings of this study that HIV+ve patients indeed have different risk profiles and pathophysiology's to the development of GD
3. The effect of ART specifically on gallstones formation needs to be studied.

### 5.7 Future Studies

Based on the study findings, the following areas of research are recommended:

1. Larger population-based Cohort studies using patients without GD as a control and compare them to HIV+ve and HIV-ve patients with GD.
2. The effect of abdominal fat and waist circumference vs BMI in HIV+ve patients as a risk factor for gallstone disease.
3. Cohort studies looking at the baseline ultrasounds in those patients without gallstones upon ART initiation and following up the presence or absence of GD with time.
4. Close monitoring of newer DTG drug initiation and the link of weight gain and GD.
5. Studies looking at alternate pathways in evaluating the downregulation of LDL-r in HIV +ve patients.
6. Entero-hepatic circulation and the effect on CYP7A1 to validate if there is a relative malabsorption of bile salts causing an upregulation of CYP7A1.

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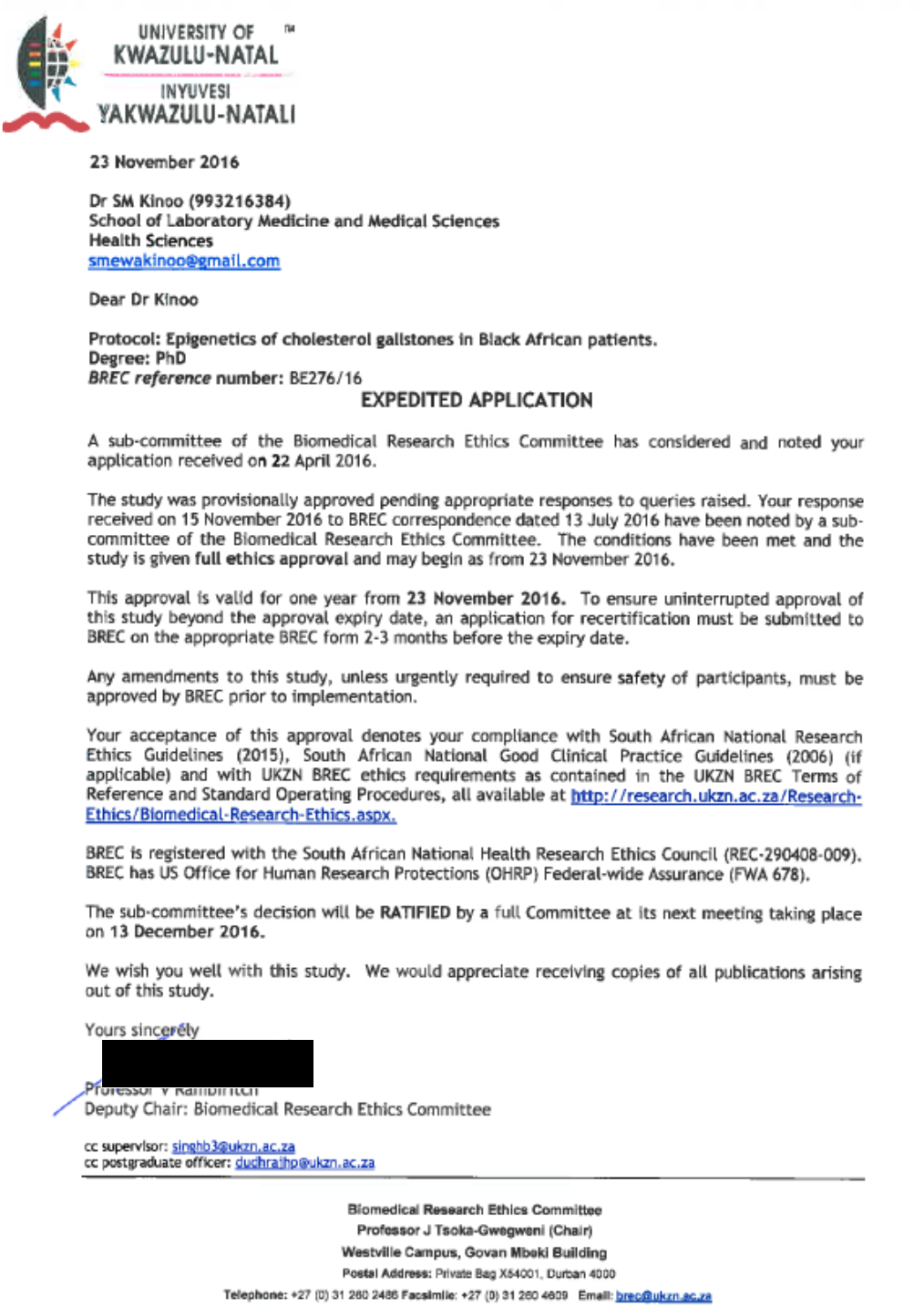
# CHAPTER 6: ADDENDUMS

## 6.1 Ethics Approval



UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

23 November 2016

Dr SM Kinoo (993216384)
School of Laboratory Medicine and Medical Sciences
Health Sciences
smewakinoo@gmail.com

Dear Dr Kinoo

**Protocol: Epigenetics of cholesterol gallstones in Black African patients.**
**Degree: PhD**
***BREC reference* number:** BE276/16

**EXPEDITED APPLICATION**

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on **22** April 2016.

The study was provisionally approved pending appropriate responses to queries raised. Your response received on 15 November 2016 to BREC correspondence dated 13 July 2016 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have been met and the study is given **full ethics approval** and may begin as from 23 November 2016.

This approval is valid for one year from **23 November 2016.** 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 **RATIFIED** by a full Committee at its next meeting taking place on **13 December 2016.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

[redacted]

Professor V Ramburuth
Deputy Chair: Biomedical Research Ethics Committee

cc supervisor: singhb3@ukzn.ac.za
cc postgraduate officer: duchrathp@ukzn.ac.za

**Biomedical Research Ethics Committee**
**Professor J Tsoka-Gwegweni (Chair)**
**Westville Campus, Govan Mbeki Building**
**Postal Address:** Private Bag X54001, Durban 4000
**Telephone:** +27 (0) 31 260 2486 **Facsimile:** +27 (0) 31 260 4609 **Email:** brec@ukzn.ac.za

NOTE: Protocol name differs from title name of PhD study. Title reworded to better suit the protocol. There was no deviation from the original protocol or ethics approval for the study.

**6.2 Site Approval**

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

OFFICE OF THE HOSPITAL CEO
KING EDWARD VIII HOSPITAL

Private Bag X02, CONGELLA, 4013
Corner of Rick Turner (Francois Road) & Sydney Road
Tel: 031 3603853 Fax 031-2061457 Email:
www.kznhealth.gov.za

Ref.: KE 2/7/1/(43/2016
Enq.: Mrs. R. Sibiya
Research Programming

8 September 2016

Dr. SM Kinoo (993216384)
School of Laboratory Medicine and Medical Sciences
Westville Campus
**UNIVERSITY OF KWAZULU-NATAL**

**Dear Dr. Kinoo**

**Protocol: "Epigenetics of cholesterol gallstones in Black African patients"**
**Degree: PhD, BREC Ref. No.: BE276/16**

Permission to conduct research at King Edward VIII Hospital is provisionally granted, pending approval by the Provincial Health Research Committee, KZN Department of Health.

Kindly note the following:-

- The research will only commence once confirmation from the Provincial Health Research Committee in the KZN Department of Health has been received.
- Signing of an indemnity form at Room 8, CEO Complex before commencement with your study.
- King Edward VIII Hospital received full acknowledgment in the study on all Publications and reports and also kindly present a copy of the publication or report on completion.

*The Management of King Edward VIII Hospital reserves the right to terminate the permission for the study should circumstances so dictate.*

Yours faithfully

[redacted] SUPPORTED/NOT SUPPORTED 13:09:2016

**MR. RR CHETTY**
**CLINICAL HEAD: SURGERY** DATE

[redacted] SUPPORTED/~~NOT SUPPORTED~~

15/09/2016

**DR. SA MOODLEY**
**ACTING SENIOR MEDICAL MANAGER** DATE

Fighting Disease, Fighting Poverty, Giving Hope