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 positive patients on antiretroviral therapy presenting with gallstones. Liver biopsies from HIV positive patients (cases: n=5) and HIV negative patients (controls: n=5) were analysed for miR-148a and mRNA expression using quantitative PCR.

Results: Circulating total cholesterol was elevated in the HIV positive group with significantly elevated LDL-c levels(3.16±0.64mmol/L) relative to uninfected controls (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 positive patients. Regulatory microRNA, miR-148a-3p, was reduced in HIV positive patients (0.39-fold) with a concomitant increase in target ABCA1 (1.5-fold), which regulates cholesterol efflux.

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

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 are 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 positive 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).

Methods
The present study sought to evaluate the hepatic expression of genes involved in cholesterol homeostasis (LDLR, ABCA1, HMGCR) in Black South African HIV positive patients presenting with gallstones relative to HIV negative patients with gallstone disease. Further, transcriptional regulators of these genes (SREBP2, miR-148a) were evaluated.

This study utilized a case-series design comparing hepatic expression of cholesterol homeostasis related miRNA in HIV positive (case) and HIV negative (control) patients presenting with symptomatic gall stones. Ethical approval was obtained from the University of KwaZulu Natal Biomedical Research Ethics Committee (BREC – BE276/16) and all subsequent methodologies were conducted in accordance with the guidelines provided by the Declaration of Helsinki. 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).

Following the analysis of clinical parameters, five HIV negative (control) and five HIV positive (cases) were selected for miRNA and downstream target analysis. All patients selected were of Black African ethnicity and female with no co-morbidities (diabetes, hypertensive, statin therapy). All HIV positive patients were on fixed dose combination therapy with CD4 counts above 500 cells/mm$^3$ and undetectable viral loads.

**RNA extraction**

Briefly, liver tissue (1 cmx1cm) was submerged in RNAlater® Stabilization Reagent (Qiagen, Hilden, Germany) in 2 ml cryovials at collection and stored at -80°C 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, USA) and 1 µg 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 25 µl 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 (95°C, 15 min) followed by 40 cycles of denaturation (94°C,
15 s), annealing (55°C, 30 s) and extension (70°C, 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 10 µl was made up consisting of 5 µl PowerUp® SYBR® Green Master Mix, 1 µM of sense and antisense primers and nuclease free water. Thermocycler conditions were as follows: 50°C 2 min, initial denaturation (95°C, 15 s), and 40 cycles of denaturation (95°C, 15 s), annealing (Table 2, 15 s), and extension (72°C, 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 2
Primer sequences and annealing temperatures for quantitative PCR

| GENE      | PRIMER SEQUENCE                  | ANNEALING TEMPERATURE (ºC) |
|-----------|----------------------------------|-----------------------------|
| LDLR forward | 5’-GAGAGCTTGTGCCGAGATGTG-3’     | 58                          |
| LDLR reverse | 5’- CCGCAGTTGTTAGTGCCATCA-3’   |                             |
| SREBP2 forward | 5’- CCTGGGAGACATCGACGAGAT-3’  | 54                          |
| SREBP2 reverse | 5’- TGAATGACCCTGTCGAGAAG-3’   |                             |
| HMGCR forward | 5’- TGATTGACCTTCCAGAGAAG-3’   | 53                          |
| HMGCR reverse | 5’- CTAAAATTGCCATTCCACGAGC-3’ |                             |
| ABCA1 forward | 5’- GGAAGAAGCAGTCATTGGGACAC-3’ | 58                          |
| ABCA1 reverse | 5’- GCTACAAACCCCTTGTAGCCAGT-3’ |                             |

Statistical analysis

Comparisons of clinical parameters between HIV negative (control) and HIV positive patients (cases) 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 positive group was significantly older than the HIV negative controls. The results show that the HIV positive 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 1
Clinical parameters of patients presenting with gall stones (HIV negative vs HIV positive)

|                      | Control      | HIV          | P-value     |
|----------------------|--------------|--------------|-------------|
|                      | n = 5        | n = 5        |             |
| Age                  | 29.60 ± 5.41 | 39.60 ± 6.19 | 0.0267      |
| BMI                  | 34.06 ± 5.98 | 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 \(LDLr\) (Fig. 1A). Quantitative PCR results for miR-148a-3p showed significantly lower hepatic expression in HIV positive individuals [0.396-fold (RQ min: 0.297; RQ max: 0.527); Fig. 1B]. Analysis of target gene \(ABCA1\) showed higher mRNA levels [1.541-fold (RQ min: 1.266; RQ max: 1.875); Fig. 1C] while \(LDLr\) mRNA levels were significantly reduced in HIV positive patients [0.181-fold (RQ min: 0.07; RQ max: 0.467); Fig. 1D].

**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 negative patients [0.127-fold (RQ min: 0.088; RQ max: 0.184); Fig. 2].

**Cholesterol metabolism**

HIV positive patients showed higher levels of total cholesterol, therefore the gene responsible for cholesterol metabolism was quantified. \(HMGCR\) mRNA levels were lower in the HIV positive group, albeit
insignificantly \([0.595\text{-fold RQ min: 112; RQ max: } 1.172]; \text{Fig. 3}\). 

**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 positive females \((n = 18)\) showed that these patients do not conform to these risk factors, with this population being significantly younger with lower BMI relative 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 positive 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 negative female patients, but rises dependant on the use of PI-ART (34). In male patients 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 males being relatively protective against gallstones as indicative of the low incidence of gallstones in HIV positive males (35).

These findings are not dissimilar to that in our study group where all patients were female 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 group compared to the control (Table 1). Altered circulating LDL-c would prompt cellular homeostatic responses. As HIV positive 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 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 positive patients with lipodystrophy compared to HIV negative controls and positive 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 positive patients as these mimic our findings in this study. Further to this we demonstrated that the downregulation of LDLr (Fig. 1D) was due to a decrease in SREBP2 mRNA (Fig. 2) with overall significantly raised LDL-c levels in the HIV group.

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 inverse relationship between ABCA1 by miR-148a is evident in HIV positive patients with gallstones compared to their negative counterparts as demonstrated in our study (Fig. 1B, C). The effect on plasma HDL-c however was negligible in our study (Table 1).

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 down regulates 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 positive patients 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 group having lower HMGCR mRNA levels (Fig. 3) which may be due to cholesterol levels being higher in this group.

**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 positive 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 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.

**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) and all subsequent procedures were followed in accordance with the Declaration of Helsinki. 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**

All participants consented for the data to be published subject to anonymity.

- **Availability of data and material**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

- **Competing interests**

N/A

- **Funding**

N/A

- **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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