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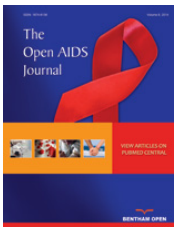

RESEARCH ARTICLE

Apolipoprotein B Gene Polymorphisms and Dyslipidemia in HIV Infected Adult Zimbabweans

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Abstract:

Background:

Dyslipidemia does not occur in all HIV-infected or antiretroviral therapy-experienced patients suggesting role of host genetic factors but there is paucity of data on association between dyslipidemia and gene polymorphisms in Zimbabwe.

Objective:

To determine association of lipoprotein levels and *apolipoprotein B* polymorphisms in HIV-infected adults.

Method:

Demographic data were collected from 103 consenting patients; lipoprotein levels were determined and blood samples were successfully genotyped for both *apolipoprotein B* 2488C>T Xba1 and *apolipoprotein B* 4154G>A p.Gln4154Lys EcoR1 polymorphisms by real time polymerase chain reaction.

Results:

Mean age of genotyped patients was 40.3 ± 10.1 years, 68% were female; prevalence of dyslipidemia was 67.4%. Of 103 samples genotyped for *apolipoprotein B* Xba1 polymorphism, 76 (74%) were homozygous C/C, 24 (23%) were heterozygous C/T and only three (3%) were homozygous T/T. *Apolipoprotein B* EcoR1 polymorphism showed little variability, one participant had rare genotype A/A, 68.3% had wild type genotype G/G.

Conclusion:

Observed frequencies of *apolipoprotein B* XbaI and EcoRI polymorphisms matched other African studies. In spite of low numbers of rare variants, there was positive association between both total cholestrol and high density lipoprotein with ECoR1 wild type G/G genotype, suggesting that ECoRI 4154 G allele could be more protective against coronary heart disease than EcoR1 4154 A allele. There is need for further research at population level to confirm whether *apolipoprotein B* ECoR1 genotyping is useful for predicting risk of dyslipidemia in HIV patients in our setting.

Keywords: *APOB*, ART, CHD, Dyslipidemia, EcoR1, HIV, Lipoproteins, Xba1.

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INTRODUCTION

Since the introduction of antiretroviral therapy (ART), quality of life of people living with human immunodeficiency virus (PLWHIV) has remarkably improved, not to mention the considerably reduced morbidity and mortality associated with HIV/AIDS infection [1]. Despite the clinical benefits, long term ART use is associated with a complex spectrum of unwanted metabolic effects such as lipodystrophy, insulin resistance, and dyslipidemia [2 - 4]. Dyslipidemia is a disorder of lipoprotein metabolism including lipoprotein overproduction and deficiency and is a major risk factor for coronary heart disease (CHD) [5, 6]. Lipoproteins contain triglycerides, phospholipids, cholesterol and amphipathic proteins called apolipoprotein and the four major types of lipoproteins are chylomicrons, very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) [7]. Earlier studies in Zimbabwe in the era before HIV and ART showed that ranges for lipoproteins, apolipoprotein B (ApoB), and triglycerides in healthy populations and diabetic patients, respectively were lower than those found in European populations [8, 9]. This suggested low prevalence of coronary heart disease (CHD) in Zimbabwean Africans. Similar findings were reported in Nigeria in the same pre-HIV era [10].

However, the current situation contradicts this perception across all groups of people in Africa as high prevalence comparable to Caucasians is being reported, due to urbanization and westernized lifestyle and the advent of HIV in most African countries [11]. Prevalence of dyslipidemia is reportedly higher in PLWHIV due to the effect of HIV and ART [12 - 15]. Concerns of increased incidence of CHD presenting as myocardial infarction in PLWHIV on antiretroviral (ARV) drugs have been confirmed in several studies [3, 16, 17]. For example, a study of young children in South Africa confirmed the adverse effect of HIV infection and ART on lipoprotein metabolism [12]. The severity of the dyslipidemia and the typical pattern of the lipid profile differs between and within the classes of antiretroviral (ARV) agents [3, 18, 19]. Several other studies in Sub-Saharan Africa suggest that the duration on ART also contributes to the severity of dyslipidemia [14, 17, 20].

In fact, there are variations in dyslipidemia even in individuals with comparable ART, demographic, immunologic, and virological characteristics [21]. This suggests that host genetic factors have a significant influence on the incidence of dyslipidemia [21, 22]. Genetic variation has been shown to account for about 43-83% of the variability of plasma lipoprotein levels in a normal healthy population [23]. Several single nucleotide polymorphisms (SNPs) associated with blood lipoprotein concentrations have been identified through genome wide association studies (GWAS) [21, 24]. Since levels of LDL predict risk of atherosclerotic CHD while ApoB is a major ligand of LDL for uptake by cell-surface receptors, the *apolipoprotein B* (*APOB)* gene was one of the candidate genes of interest in GWAS of dyslipidemia [22, 25 - 28]. Over ten polymorphisms within or flanking the *APOB* gene have been detected and two of the most extensively studied polymorphisms of the *APOB* gene are Xba1 (Pubmed ID: rs693) and EcoR1 (Pubmed ID: rs1042031) [29].

The Xba1 polymorphism arises due to a single base variation in exon 26 (at 2488th position ACC→ACT) of the *APOB* gene that does not lead to change in amino acid sequence [30, 31]. This base change creates a cutting site for the restriction endonuclease Xba1 and the polymorphism is known as APOB 2488C>T Xba1 polymorphism (-7673C>T) [31]. The XBa1 polymorphism has been associated with inter-individual variability of lipoprotein levels in several populations [7, 28, 32 - 34]. However, the results are conflicting in different ethnic groups.

On the other hand, the EcoR1 restriction site exists due to a change in guanidine to adenine at exon 29 (GAA→AAA), leading to the substitution of glutamine for lysine at position 4154 in the ApoB polypeptide product (p.Gln4154Lys) [32]. This nucleotide change leads to loss of the EcoR1 restriction site and the polymorphism is known as *APOB* 4154G>A polymorphism (-2669G>A) [32, 33]. The *APOB* EcoR1 polymorphism has been reported to have a positive association with raised serum lipoprotein levels, while others reported no such association [34, 35].

There have been reports of significant differences between Senegalese and Caucasians, and between Senegalese and Mongoloids for the EcoR1 allele of the *APOB* gene [33], but very few studies have been conducted in Africa, and to our knowledge, none in Zimbabwe. This study was conducted to determine frequency of *APOB* gene EcoR1 and Xba1 SNPs followed by tests for association with dyslipidemia in HIV infected Zimbabweans.

MATERIAL AND METHODS

Study Design

This was a nested cross sectional study of a cohort of 215 HIV infected, unrelated patients attending an

opportunistic infections clinic in Harare, Zimbabwe, between March and August 2013, who were previously described [36]. Two non-fasting blood samples were collected, one in 4ml EDTA tubes and the other in 4ml plain tubes using the vacutainer method. EDTA samples were used for DNA extraction whilst serum from the plain tubes was used for lipoprotein profiling. A sub-set of 103 samples was genotyped due to limited resources in our setting.

Ethical Clearance

The study was ethically cleared in Zimbabwe by the Joint Research Ethics Committee (JREC) and Medical Research Council of Zimbabwe (MRCZ) and in Norway by the Research Ethics Committee (REK).

Biochemical Measurements

Total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) serum levels were measured by enzymatic methods using the Mindray BS 120 analyser (Nanshan, People's Republic of China) according to the manufacturer's protocol, as previously described [36]. Dyslipidemia was defined according to the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) guidelines, 2004 as the occurrence of any one of the following conditions: TC >5.17 mmol/l (>200 mg/dl), LDL-C >3.16 mmol/l (>130 mg/dl), and HDL-C <1.03 mmol/l (<40 mg/dl) for males, <1.30 mmol/l (<50 mg/dl) for females [36 - 39].

Genotype Detection

DNA was extracted from whole blood cells using the Qiagen Mini Extraction Kit (QIAmp®, Valencia, California, USA) as per manufacturer's protocol. A final volume of 20µl pure DNA was obtained. Quality and purity of the extracted DNA was checked using ultra-violet visible spectrophotometry at absorbance 260nM, 280nM and 360nM. Genotyping was performed by Real Time PCR (qPCR) on the Applied Biosystems 7500 platform (Life Technologies® Corporation, Foster City, USA) using Taqman® Chemistry, (Cheshire, United Kingdom). The assay kit consists of a single, ready-to-use tube containing two sequence-specific primers for amplifying the *APOB* polymorphism and two allele-specific TaqMan MGB probes for detecting the alleles of the specific polymorphism of interest. Vic dye is linked to the 5'end of allele 1 probe (reporter) whilst Fam dye is linked to allele 2 probe. A non-fluorescent quencher (NFQ) at the 3'end of the probe allows the detection of the reporter dye fluorescence with greater sensitivity. Reactions were performed in MicroAmp™ 96 well optical tubes in a final volume of 25µl.The reaction conditions were pre PCR read at 60^{o}C for 1 minute, a holding stage for 10 minutes at 95^{o}C and cycling stage of 40 cycles (denaturation 100^{o}C, annealing 60^{o}C and elongation at 72^{o}C). Allelic discrimination was performed by the Sequence Detection Software (Applied Biosystems, Foster City, California, USA).

Statistical Analysis

Results were expressed as mean ± standard deviation (SD) or median (interquartile range-IQR) for continuous variables and as proportions for categorical variables, respectively. Allele and genotype frequencies were determined by direct counting of alleles, whilst Fisher's exact tests were used to determine association of *APOB* SNPs with dyslipidemia status.

Comparison of demographic and biochemical data was carried out using student's t-tests for means, Kruskal-Wallis equality-of-populations rank tests for medians and Pearson's or Fisher's exact chi-square tests for proportions. In order to adjust for sex, age, CD4 count and viral load for significant tests, post-hoc testing was carried out using multivariate regression analysis.

Chi-square analysis was used to test for deviations in genotype frequencies from the Hardy-Weinberg equilibrium (HWE) using web tool [40]. Linkage disequilibrium between rs693 and rs1042030 were evaluated with the correlation of alleles test (r2) and were considered in disequilibrium when r2≥0.33 using SHesis [41].

RESULTS

Demographic and Biochemical Characteristics

Demographic characteristics of participants by dyslipidemia status are shown in Table **1**. Twenty-seven (26.1%) of participants were males, frequency of dyslipidemia, as defined in the Methods section, was 70% and there was no difference in any independent variables including age, sex, ART experience, CD4 count and viral load when patients were compared by dyslipidemia status. The most prevalent type of dyslipidemia was HDL-C deficiency followed by

excess LDL-C and excess TC respectively. There was no difference in frequency of HDL-C deficiency, excess LDL-C and excess TC for those aged >35 and <35 years, respectively and when patients were stratified by sex (Table **2**).

Table 1. Demographics of genotyped patients (N=103).

Characteristics	Non-dyslipidemic (n=31) 30%	Dyslipidemic (n=72) 70%	P value
Age in years (mean ± SD)	41.8 (10.0)	43.0 (9.4)	0.5708[e]
Male n (%)	7 (25.9)	20 (74.1)	0.3650*
Female n (%)	24 (31.6)	52 (68.4)	
ART naïve n (%)	2 (18.2)	9 (81.8)	0.3750*
ART experienced n (%)	29 (31.5)	63 (68.5)	
Years Since Diagnosis/Median (IQR)	4 (3-8)	5 (2-8)	0.5772[§]
Years Since ART started/Median (IQR)	3 (2-7)	3 (1-6)	0.6810[§]
CD4 count (cells per mm^3) after nine months/median (IQR)	491 (299-590)	470 (303-661)	0.7247[§]
Viral Load (copies per mm^3) after nine moths/Median (IQR) [Range]	37 (20-37) [20-27184]	37 (20-58) [20-350000]	0.8760[§]
Log_{10}Viral Load/Median (IQR) [Range]	1.6 (1.3-1.8) [1.3-5.5]	1.6 (1.3-1.7) [1.3-4.4]	0.7380[§]

SD=standard deviation, IQR=interquartile range, ART=antiretroviral therapy, CD4=cluster of differentiation 4, P[e] from student's t-tests, P* from Pearson chi-squared tests, P[§] from Kruskal-Wallis equality-of-populations rank median tests

Table 2. Frequencies of dyslipidemia in genotyped patients.

Characteristic	All (N=103)	Males (n=27)	Females (n=76)	P*	>35years (n=20)	≤35years (n=83)	P**
Non-dyslipidemic n(%)	31 (30)	6 (23)	25 (33)	0.344	4 (22)	27 (32)	0.458
Dyslipidemic n(%)	72 (70)	21 (77)	51 (67)		16 (78)	56 (68)	
Normal HDL-C n(%)	43 (42)	11 (40)	32 (42)	0.776	7 (35)	36 (43)	0.452
HDL-C deficient n(%)	60 (58)	16 (60)	44 (58)		13 (65)	47 (57)	
Normal LDL-C n (%)	76 (74)	19 (70)	57 (75)	0.507	16 (78)	60 (72)	0.763
LDL-C excess n (%)	27 (26)	8 (30)	19 (25)		4 (22)	23 (28)	
Normal TC n (%)	95 (92)	27 (100)	68 (89)	0.083	20 (100)	75 (90)	0.249
TC excess n (%)	8 (8)	0 (0)	8 (11)		0 (0)	8 (10)	

P-values from Pearson's chi-squared tests after stratifying by gender (P*) and age (P**), TC=total cholesterol, HDL-C=high density lipoprotein cholesterol, LDL-C=low density lipoprotein cholesterol, Dyslipidemic denotes presence of at least one NCEP ATP III characteristics of dyslipidemia (elevated TC> 5.2mmol/L, depressed HDL-C< 1.1mmol/L, elevated LDL-C>3.2mmol/L, elevated TC/HDL-C ratio>4.5)

Genotypic Characteristics

The genotype distributions of the two SNPs conformed to the Hardy-Weinberg equilibrium (HWE) (genotype frequencies appear in Table **3**) and the two loci are in linkage disequilibrium. Seventy-six (74%) were homozygous 2488 C/C, 24 (23%) were heterozygous 2488 C/T and only three (3%) were homozygous 2488 T/T (Table **3**). Seventy (68%) were homozygous wild type: 4154 G/G, 32 (31%) were heterozygous: 4154 G/A and only one patient (1%) had the homozygous mutant genotype: 4154 A/A (Table **3**). No significant association was observed when genotypes were stratified by gender (Table **3**).

Frequency of the 2488 C/C genotype was slightly lower (72.2%) in dyslipidemic patients compared to non-dyslipidemic patients (77.4%), but there was no statistically significant association between *APOB* 2488 XbaI and dyslipidemia status (Table **3**). *APOB* minor (2488 T) allele had a frequency of 21% in the population studied and no significant association was observed between dyslipidemia and the *APOB* minor allele, P>0.05 (Table **3**).

APOB EcoR1 polymorphism had only one patient with minor genotype 4154 A/A, while frequency of the heterozygous genotype 4154 G/A was slightly higher in non dyslipidemic (38.7%) than in dyslipidemic group (28.0%), though no significant association was observed, P=0.675. Table **3** shows the allelic frequency and distribution amongst patients. Frequency of the A allele was 15% and there was no association between the *APOB* EcoR1 minor allele (4154 A) and dyslipidemia status (P=0.675).

Table **4** shows median values for metabolic variables according to different genotypes; values were compared using multivariate analysis and Kruskal-Wallis equality-of-populations rank median tests. HDL-C levels were marginally

higher (P=0.056) in patients with the 4154 G/G genotype than the genotypes of 4154 A/A and 4154 G/A, respectively. One patient had the rare variant 4154 A/A and was subsequently dropped from statistical analysis, whilst three patients had the rare variant 2488 T/T and were also subsequently dropped. Hence, results for comparisons of median lipoprotein levels reported in Table **4** were based on comparison between two groups consisting wild type 4154 G/G and heterozygotes (4154 G/A) or wild type 2488 C/C against 2488 C/T. The difference in median levels of TC and HDL-C was more apparent for 4154 G/G *versus* 4154 G/A (P=0.0049 and P=0.0106, respectively), but there was still no difference in levels of LDL-C and TC/HDL-C ratio, (Table **4**). The association of *APOB* EcoR1 SNPs with TC (P=0.012) and HDL-C (P=0.011) remained when adjusted by age, gender, CD4 count and viral load (Table **5**). There was no statistically significant association between all lipoprotein levels and Xba1 genotype variants (2488 C/C against 2488 C/T) p<0.05, (Table **4**).

Table 3. Distribution of *APOB* polymorphisms by gender and dyslipidemia status.

Genotypes	**Male (N=27)**	**Female (N=76)**	**P value**
Distribution of 2488 XbaI genotype and allele frequencies by gender			
Genotypic frequency			
C/C (n=76)	23 (86.2)	53 (69.5)	0.232
C/T (n=24)	3 (10.3)	21 (28.1)	
T/T(n=3)	1 (3.5)	2 (2.4)	
Allelic frequency			
C (0.86)	0.911	0.83	0.0926
T (0.14)	0.09	0.17	
Genotypes	**Male**	**Female**	**P value**
Distribution of 4154 EcoRI genotype and allele frequencies by gender			
Genotypic frequency			
G/G (n=70)	18 (67.0)	52 (68.4)	0.937
G/A (n=32)	9 (33.0)	23 (30.2)	
A/A (n=1)	0	1 (1.4)	
Allelic frequency			
G (0.84)	0.84	0.84	0.910
A (0.16)	0.16	0.16	
Distribution of 2488 XbaI genotype and allele frequencies by dyslipidemia status			
Genotypic frequency			
Genotype	**Dyslipidemic n(%)**	**Non-dyslipidemic n(%)**	**P value**
C/C (n=76)	52 (72.2)	24 (77.4)	0.653
C/T (n=24)	19 (26.4)	5 (16.1)	
T/T (n=3)	1(1.4)	2 (6.5)	
Allelic frequency			
C	0.85	0.86	*0.845
T	0.15	0.14	
Distribution of 4154 EcoRI genotypes and allele frequencies by dyslipidemia status			
Genotypic frequency			
Genotype	**Dyslipidemic n (%)**	**Non-dyslipidemic n(%)**	**P value**
G/G (n=70)	51 (70.8)	19 (61.3)	0.675
G/A (n=32)	20 (28.0)	12 (38.7)	
A/A (n=1)	1(1.2)	0	
Allelic frequencies			
G	0.85	0.81	*0.699
A	0.15	0.19	

Genotype frequencies presented as numbers (%), G and A are alleles at the *APOB* EcoR1 locus, C and T are alleles at *APOB* XBaI locus, *Genotype frequencies conformed to the Hardy-Weinberg equilibrium (q=1-p)

DISCUSSION

Many studies have been performed to date to investigate association between *APOB* gene polymorphisms and lipoprotein levels in other parts of the world [42, 43]. However, this is the first research showing both allelic frequencies of *APOB* XBAI and EcoRI SNPs in a Zimbabwean HIV cohort and association between *APOB* EcoR1 gene

variants with TC and HDL-C levels; with higher levels of TC and HDL-C in 4154 G/G carriers after adjusting for age, gender, CD4 count and viral load (P<0.05). However no association was found between *APOB* XBa1 gene variants and lipoprotein levels, which confirm previous observations, that 2488T allele is not a risk factor for dyslipidemia.

Table 4. Metabolic variables according to polymorphisms.

Lipid Profiles	**TC/mmol/L**	**HDL/mmol/L**	**LDL/mmol/L**	**TC/HDL ratio**
Single nucleotide Polymorphisms				
APOB EcoR1				
GG n=70	4.7 (3.7-5.2)	1.3 (1.0-1.6)	2.6 (2.1-3.5)	3.6 (2.8-4.7)
GA n=32	4.3 (4.1-5.2)	1.1 (0.9-1.3)	2.4 (0.9-2.8)	3.6 (3.0-4.4)
P	*0.0049*	*0.0106*	0.1041	0.6625
APOB XBa1				
CC n=76	4.4 (3.7-5.2)	1.2 (1.0-1.5)	2.6 (2.1 – 3.4)	3.6 (3.0-4.5)
CT n=24	4.3 (4.1-5.2)	1.2 (1.0-1.5)	2.4 (2.1-3.3)	4.2 (3.0-4.7)
P	0.5248	0.8405	0.4210	0.3172

P values calculated using Kruskal-Wallis equality-of-populations rank median tests in Stata, Rare variants dropped from analysis, Genotypes: EcoR1 (GG, GA, AA); XBa1 (CC, CT, TT)

Table 5. Metabolic variables according to APOB EcoR1 SNPs.

Lipid Profiles	**TC/mmol/L**	**HDL/mmol/L**
GG n=70	4.7 (3.7-5.2)	1.3 (1.0-1.6)
GA n=32	4.3 (4.1-5.2)	1.1 (0.9-1.3)
*P (adjusted)	*0.012*	*0.011*

*P values calculated using multivariate regression analysis, to adjust for age, sex, CD4 count and viral load

Frequency of the EcoR1 4154 G allele is high in our population compared to other populations. In agreement with our study, a study conducted in India [30] reported only the presence of 4154 G/G and 4154 G/A genotypes in their population while a study in Senegalese had slightly higher frequency of the 4154 A/A genotype *i.e.* 0.08 [33]. Likewise, the frequency of the EcoR1 rare genotype 4154 A/A reported in Caucasians (0.24) [33] is higher than that reported in Zimbabweans, as well as in other African studies. The XBa1 2488 T/T genotype polymorphism is rare in our population. Its frequency (0.03) is slightly lower than that reported from a study in Senegalese HIV patients (0.08-0.16) whilst a study in Nigeria reported a similar frequency which is comparable to results from Mongoloids where frequencies ranged from 0.01 to 0.07 [28, 33].

In studies that involved both Africans and Mongoloids, no association of the Xba1 2488 T allele and plasma lipoproteins were observed [7, 28, 33]. However, association of the 4154 A allele to plasma lipoproteins has been reported in many studies involving HIV positive Caucasians, though lack of association was reported in HIV positive Senegalese and Chinese populations [21]. Unlike Zimbabwean individuals, Caucasian individuals have higher frequencies of the 2488 T allele, reported allele frequencies range between 0.33 and 0.61 in Caucasians [34].

Patients with the homozygous 4154 G/G genotype showed trend toward more favorable lipoprotein values (higher HDL-C) and could be more protected against CHD. Of note, a high fraction of the patients carry this genotype in the population studied. An earlier study conducted to examine the role of *APOB* gene polymorphisms in responsiveness of plasma lipoproteins to diet in 44 healthy participants in Finland [44] observed that a high-fat diet induced a larger increase in plasma LDL-C in subjects with the 4154 A/A genotype than in those with the 4154 G/A or 4154 G/G genotype. Hence 4154 G/G carriers are protected against CHD for slightly different reasons in Finns compared to the Zimbabwean cohort reported here.

Finally, it is very difficult to explain the association between APOB gene variation and HDL-C levels reported here because genetic variations of enzymes, receptors, and apoB a protein essential to LDL-C metabolism, are partially involved in the regulation of serum LDL-C and TC but not HDL-C [45]. It is however important to remember that serum lipids have a multi-factorial etiology that is determined by a large number of environmental and genetic factors and detailed mechanisms of their interactions are not well known. HIV infection in patients has prior been associated

with a highly atherogenic lipid profile with increased levels of TC and LDL-C accompanied by decreased levels of HDL-C [45]. Thus results of this study should be received with caution and may need further enquiry.

LIMITATIONS OF STUDY

Failure to get sufficient numbers of the mutant alleles, inadequate choice of genetic variant, phenotype being affected by many variants at several genes, use of single markers instead of haplotypes and gene-environment interactions might have contributed to lack of associations observed in our study for Xba1 2488 variants [46]. Study results and conclusions for the EcoR1 variants in the current study were also severely weakened by the small sample size, scarcity of investigated variants and cross-sectional nature of the study. Future population-wide longitudinal studies are warranted in this setting to strengthen the power of study and reduce type II errors. Furthermore, the inclusion of patients on different ART regimens is a difficulty inherent to many studies involving patients already receiving ART [21], hence larger population-based studies can improve the number of patients in sub-groups, a necessary step for enhancing power in any subsequent comparative analysis.

CONCLUSION

Dyslipidemia is highly prevalent in HIV patients in Zimbabwe both ART experienced and ART naives. Frequencies for TC, LDL-C excess and HDL-C deficiency were similar across gender and age lines. Observed frequencies of *APOB* Xba1 and EcoR1 polymorphisms matched other African studies. There was positive association between EcoR1 wild type genotype 4154 G/G with HDL-C levels which could indicate that homozygous 4154 G/G genotype carriers are more protected from CHD via increase in HDL-C.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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