Cholesteryl Ester Transfer Protein (CETP) I405V (rs5882) Polymorphism affects plasma lipid profiles and lipoprotein ratio in hyperlipidemic ischemic stroke patients that visited Lagos University Teaching Hospital.

ABSTRACT

Background

Cholesteryl ester transport protein (CETP) plays a key role in the metabolism of high-density lipoprotein (HDL) and other lipoproteins. Polymorphism in the CETP gene is associated with cardiovascular risk and the development ischemic stroke.

Method**ology**

100 patients with ischemic stroke and 100 controls matched for sex and aged 46-87 were selected for the study. Lipid profiles were measured using Randox kits and lipoprotein ratios were calculated using Excel software. The genotyping of the of the CETP genewere performed by Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR-RFLP) combined with 2% gel electrophoresis.

Results

There were significant difference ($P<0.05$) in the genotypic and allelic frequencies of CETP SNP between the healthy and patients with ischemic stroke. The frequencies of I/I, I/V and VV genotypes of the CETP gene were 48%, 37% and 15% for the control and 17%, 33% and 50%, for the stroke subjects, respectively.The frequencies of I and V alleles were 67% and 33% for the control and 37.5% and 62.5% for the stroke subjects respectively. The V allele carriers of CETP gene had higher plasma TC, HDL-C, HDL_2-C, HDL_3-C and other lipid parameters when compared to the I allele carriers for both subjects. The V allele carriers were responsible for the increase in dyslipidemia for both subjects.

Conclusion

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The results of this study show that the mutation of CETP I405V (rs5882)polymorphism causes an increased in defective HDL-C, HDL_2-C and HDL_3-C concentration and is associated with an increased risk of ischemic stroke.

Keywords: Cholesteryl ester transfer protein, ischemic stroke, lipid parameters, SNP.

1.0 INTRODUCTION

Cardiovascular diseases (CVD) are the first leading cause of death in the world [1]. Its belong to a class of diseases that involve the blood vessels (veins, capillaries and arteries), heart,vascular diseases of the brain and kidney, cardiac disease,peripheral arterial disease and other diseases that affects cardiovascular system [1]. Stroke is a subclass of cardiovascular disease and is the second-leading causes of mortality in most countries in the wide and is the second most common causes of death and disability in developed countries [2]**.** Study has shown that 80% of all types of strokes are ischemic. They result from occlusion of a major cerebral artery by a thrombus or embolism which results in reduced blood flow and a major decrease in the supply of oxygen and glucose, all other nutrients as well as disrupting the nutrient and waste exchange process required to support brain metabolism [2]. If cerebral arterial blood flow is not restored within a short period, cerebral ischemia will result, and will subsequently lead to neuron death within the perfusion territory of the affected blood vessels [2].

Studies have shown that dyslipidemia, including high levels of serum or plasma triglycerides (TG), total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), and apolipoprotein B (ApoB) and low levels of high density lipoproteincholesterol (HDL-C) and apolipoprotein A (ApoA) are risk factors for the progression of atherosclerosis and the development of stroke and other cardiovascular disease [1, 2]. Researchworks have shown that plasma lipid parameters are modulated by genetic factors [1,2, Momoh et al, 2021 and Momoh, 2021] as well as environmental factors such as hypertension [3], demographics [4], diet [5], exercise [6], cigarette smoking [7,8], alcohol consumption [7],and obesity [9].

Lipid and lipoprotein ratios have been recently used to predict the risk of stroke and other cardiovascular diseases. Examples of some of these lipoprotein ratios are: HDLC/LDL-C,HDL-C/TC (Coronary disease risk ratio), TC/HDL-C (Castelli risk index-I), LDL-

C/HDL-C (Castelli risk index-II), TG/HDL-C, Logarithmic transformation of TG/HDL-C (Atherogenic index of plasma i.e AIP),and Atherogenic coefficient (AC) = (TCHDL-C)/HDL-C [1,2,10]. Jeppesen et al. [11] study shows that the better ability of these lipoprotein ratios to predict cardiovascular disease compared to single lipid marker is of particular clinical relevance and can be possibly explained by association of lipid ratios with a clusterof cardiovascular risk factors that are at least in part unrelated to cholesterol metabolism.

Cholesteryl Ester Transfer Protein (CETP) is a plasma glycoprotein that contains 476 amino acids with molecular weight of 74 kDa. CETP is involved in the transfer of triglycerides, cholesteryl esters, phospholipids and retinal ester. CETP facilitates the transport of triglycerides and cholesteryl esters between lipoproteins. This enzyme promotes the transfer of cholesteryl esters from high density lipoprotein (HDL) to apolipoprotein B containing lipoproteins, such as low density lipoprotein (LDL) and VLDL in exchange for triacylglycerols[12, 13].Thus, CETP plays an important role in the reverse cholesterol transport pathway, as changes in CETP activity are inversely correlated with plasma HDL-C concentrations [14]. CETP inhibitors significantly raise plasma HDL-C concentration.High-density lipoprotein cholesterol (HDL-C) hasatheroprotectiveproperties and low HDL-C concentrations are the most common lipid abnormality in patients with premature CVD [15].

Cholesteryl ester transfer protein gene (Gene ID: 1071) is a single gene consisting of a 25 kb genomic DNA and is located on the long arm of chromosome 16 adjacent to the lecithin cholesterolacyltransferase (LCAT) (16q12–16q21). It consists of 15 introns and 16 exons[16, 17].Studies have shown that genetic changes in CETP gene may lead to change in the concentration of plasma and function of CETP and therefore affect the level of HDL-C and LDL-C in the plasma or serum [15, 18-20]. These CETP gene polymorphisms include -629C/A, I405V, D442G and Taq1B and can cause diseasesdue to their influence on serum lipids parameters [21-25].The CETP I405V (rs5882) polymorphism is located in a known binding domain and it causes an isoleucine/valineshift, leading to decreased protein levels. Takataet al. [26] study showed that CETP promoter -1337C >T polymorphism can affect plasma CETP concentration and lipid profile, in patients with familial hypercholesterolaemia.

Two CETP polymorphisms (R451Q and A373P) located in the coding region of the gene have been studied. The minor alleles of these two polymorphisms, Q and P, respectively, appear at a low frequency in the general population, each having a minor allele frequency of 2–7% in Western European cohorts [27, 28]. The minor alleles of these polymorphisms have been associated with lower HDL-C concentrations [27] and higher CETP activity [29].The study evaluatesthe effect of Cholesteryl Ester Transfer ProteinI405V Polymorphismon lipid profiles and lipoprotein ratios in patients with ischemic stroke.

2.0 MATERIALS AND METHODS

2.1. Study subjects

Clinical and laboratory data of 100 adult Ischemic stroke subjects who visited Lagos University Teaching Hospital (LUTH) in South-west, Nigeria were obtained for the analysis. 100 ischemic stroke patients' blood samples were collected and assayed. All the ischemic stroke patients had cerebral computerized tomography taken which showed cerebral infarction and they were confirmed by neurologists in LUTH to have ischemic stroke. The control subjects consist of 100 individuals within the same age range 46-87 and socio-economic status as the stroke patients. Blood samples were obtained in heparin vacutainer and ethylenediaminetetraacetic acid (EDTA)bottles from healthy individuals and stroke patients who have been fasting for 12 to 16 hours. All the healthy individuals and stroke patients were given consent forms and questionnaire. Ethical approval was also obtained from the Research and Ethical Committee of the Institution. The control and stroke subjects not willing to participate in the study were excluded from the study.

2.2. Determination of plasma lipid profiles and lipoprotein ratios

Blood was collected in tubes containing EDTA to give a final concentration of 0.1%. Plasma was separated from red blood cells by centrifugation at 1500 X g for 15 minutes at 4^{o}C. The Total Cholesterol (TC), Triglyceride (TG) and HDL-Cholesterol were assayed using Randox kits (RANDOX Laboratories Ltd., Ardmore, Diamond Road, Crumlin Co. Antrim, United Kingdom).

VLDL-C =TG/5, Non- HDL-Cholesterol = Total Cholesterol — HDL-Cholesterol

LDL-C=TC - HDL-C –TG/5

The atherogenic ratios were calculated as follows:

Castellis risk index I (CRI-1) = TC/HDL-C

Castellis risk index II (CRI-11) = LDL-C/HDL-C

HDL-C/LDL-C and TG/HDL-C values were also calculated

Atherogenic index of plasma (AIP) = log TG/HDL-C

Atherogenic coefficient (AC) = (TC - HDL-C)/HDL-C

Lipid and lipoprotein ratio were calculated by the procedure described by Momoh et al. [2].

Dyslipidemia was defined according to Momoh et al.[2] and National Cholesterol Education Program 2001[30].Dyslipidemia was defined by the presence of one or more abnormal plasma lipid indexes.

2.3. Isolation of DNA from blood sample

Genomic DNA was isolated from peripheral blood leukocytes using DNA Qiagen kits according to manufacturer instructions. The extracted DNA was stored at 4℃ until analysis. The quality and quantity of extracted DNA were determined using the spectrophotometric method with NANODROP *1000*^{R} (Thermo Fisher Scientific, United States of America), which quantified the amount of extracted DNA in nanogramme per microlitre (ng/µL) and assessed the quality (purity) based on the ratio of absorbance at 260nm:280nm for all the samples [31].

2.4. Amplification of CETP gene

The DNA samples were amplified by using the Polymerase Chain Reaction (PCR) method. The *CETP* genes were amplified using PCR machine (TECHNE TC-4000) with the necessary PCR reagents and primers. The amplification of the CETP gene was done by polymerase chain reaction with the use of primer pairs. The substitution of valine for isoleucine is caused by an A→G mutation at codon 405 in exon 14 of the *CETP* gene on chromosome 16. Exon 14 was amplified by PCR with primers located in intron 13(F: 5'-AATGCTTGTCCAGGCCGTGCAGCAT-3')and in intron 14(R: 5'-CAGTTTCCCCTCCAGCCCACACTTA-3') using methods

described by Agerholm-Larsen et al., [28]. The PCR cycling conditions for the CETP SNPs were as follows: initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 2 minutes. All PCR reactions were carried out in a total volume of 23 µl containing 2.5 µl dNTPs, 2 nM $MgCl_2$, 1µl of each primer, and 1 unit of AmpliTaq polymerase (Perkin Elmer England), 2.5 µl PCR buffer and 13.8µl grade water. Negative controls were included in each set of reaction.

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2.5. Amplification and genotyping of *CETP*gene

The DNA samples were genotyped by using the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method. The genotype of the CETP gene was done by polymerase chain reaction with the use of the primer pairs. Exon 14 was amplified by PCR with primers located in intron 13 (F:5'-AATGCTTGTCCAGGCCGTGCAGCAT-3') and intron 14 (R: 5'-CAGTTTCCCCTCCAGCCCACACTTA-3'). The PCR Cycling conditions for the CETP SNPs were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 2 minutes. The PCR products were then digested with 1 µL restriction enzyme *Fok1* at 37°C.Briefly, 1 µL of restriction enzyme (*Fok1*) was added to 1 µL of PCR amplicon and 10x NEBuffer (5 µL) in a reaction volume of 50 µL. The solution was mixed properly and incubated for 37^{o}C for maximum of 15 minutes. The digested PCR products were followed by electrophoresis on 2% agarose gel containing ethidium bromide. To validate the results, genotyping experiments were repeated for all samples. The genotyping of these samples were completely consistent. The genotypes identified were named according to the absence or presence of the enzyme restriction sites, when an I to V transversion at nucleotide position of the *CETP*. The I/I genotype is homozygote for the absence of the site (band at 120 and 55 bp), I/V genotype is heterozygote for the presence and absence of the site (bands at 120, 85 and 55bp), and V/V genotype is homozygote for the presence of the site (bands at 85 bp and 55bp).A common band of 55 bp was found in all the DNA samples.A negative control was included in each set of the reaction.

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2.6. Agarose gel electrophoresis of amplified CETP gene

Amplification was confirmed by electrophoresing of PCR amplicons on 2% w/v agarose gel stained with ethidium bromide, 0.5 mg/mL in Tris-borate EDTA. Electrophoresis was carried out at 120 V, 50 W, and 300 mA for 40 minutes. On completion of electrophoresis, bands were visualized with the gel documentation system (Infinity 3026, France). The sizes of the fragments obtained were estimated by comparison with the 50 bp DNA ladder (Jena Bioscience GmbH, Germany) run alongside with the negative control. The genomic DNA of all the subjects after amplification with PCR and imaged by 2% agarose gel electrophoresis, the purpose gene of 120 bp, 85 bp and 55 bp were foundand a common band of 55 bp were found in the DNA samples.

2.7. Statistical Analyses

Data are presented as Mean ± SD. GraphPad prism computer software version 5.01 was used to compare lipid profiles and lipoprotein ratios levels between genotypes for control and stroke subjects respectively. Allele frequency was determined via direct counting and the standard-goodness-of-fit test was used to test the Hardy-Weinberg equilibrium. One-way ANOVA Postdoc Turkey's test was used for comparing significantdifference between wild type and mutant genotypes for both separate subjects. One-way ANOVA Bonferroni's multiple comparisontest was also used for comparing the significant difference between genotypes for both subjects.A P-value < 0.05 was considered statistically significant.

3.0. RESULTS

3.1. CETPGenotype Frequencies

This is the first time the research work will be carried out in Lagos, Nigeria. Two hundred subjects were genotyped. The CETP gene polymorphism was highly prevalent in the stroke subjects compared to the controls. For the control subjects: 48 were homozygous for the wild-type (Ile/Ile) and 52 were carriers of the V allele (37 I/V and 15V/V). The stroke subjects had 17 homozygous for the Ile/Ile genotype and 83were carriers of the V allele (33 I/V and 50V/V). The frequencies of I/I, I/V and V/V genotypes for the CETPwere 48%, 37% and 15% for the control while 17%, 33% and 50% were for the stroke subjects (P<0.0001) respectively. The frequencies of

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I and V alleles were 67% and 33% for the control subjects while 37.5% and 62.50% were for the ischemic stroke subject´s respectively. These frequencies did not differ from those predicted from the Hardy-Weinberg equilibrium. The allele frequencies were consistent with Hardy–Weinberg equilibrium for both control and stroke subjects (P = 0.8050 for control and 0.6650 for stroke subjects). The prevalence of the V allele was significantly higher in the ischemicstroke subjects compared to the control (62.50% Vs 33.00%).

Table 1. . The number of observed and expected genotype of examined CETP SNP for the control and the ischemic stroke subjects according to Hardy-Weinberg equilibrium.

SNP	Genotype/ Allele Frequency	**Control subject**		**Stroke subject**		**P value; OR (95%CI)**
CETP		Observed Frequency	Expected H-W Frequency	Observed Frequency	Expected H-W Frequency	
	I/I	48 (48%)	44.2225	17 (17%)	11.2225	
	I/V	37 (37%)	44.5550	33 (33%)	44.5550	
	VV	15 (15%)	11.2225	50 (50%)	44.2225	

P Value		0.6650		0.3350		0.33-0.67
X^2 Value		2.8753		6.7258		
	I	133 (66.50%)		67 (33.50%)		0.0001
	V	67 (33.50%)		133 (66.50%)		0.0001

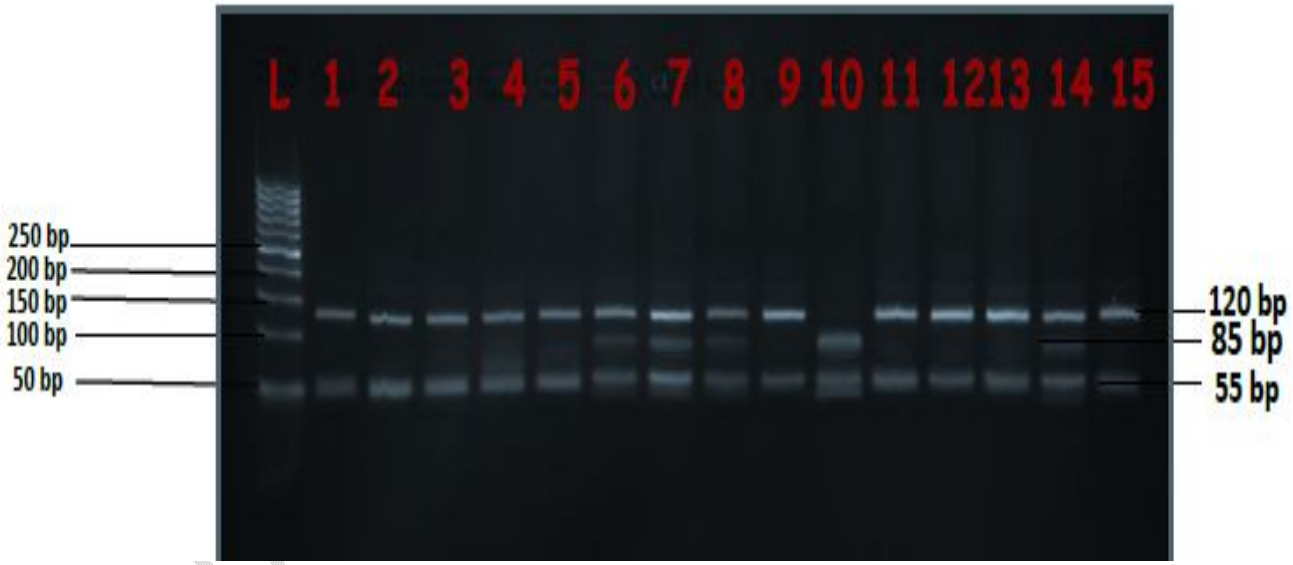

Figure 1. L is DNA Ladder (50 – 500 bp), Lanes 1-5, 9, 11 – 13, and 15 are Ile/Ile genotypes (bands at 120 bp and 55 bp)

Lanes 6-8, and 14 are Ile/Val genotypes with 120 bp, 85 bp and 55 bp respectively. Lane 10 is Val/Val with 85 bp and 55 bp

Table 2: The effect of CETPgene genotypes on plasma lipid parameters for both control and ischemic stroke subjects.

Parameters	Control subjects			Ischemic stroke subjects		
	Genotype			Genotype		
	I/I (48)	I/V (37)	V/V (15)	I/I (17)	I/V(33)	V/V(50)
TC (mg/dl) OK	140.80 ±12.88[c]	165.60 ±10.38[b]	173.10 ±9.32[a]	[c]205.42 ±8.38	[b]209.83 ±11.47	[a]222.13±11.30
TG (mg/dl) OK	105.50 ±11.45[c]	133.20 ±12.84[a]	108.80 ±11.04[b]	[c]152.33 ±9.24	[b] 159.60 ±10.02	[a] 174.01±9.94
HDL-C (mg/dl)	100.90 ±5.128[c]	118.50 ±9.66[b]	124.90 ±9.58[a]	[b]47.47 ±3.83	[b] 49.82 ±3.79	[a] 58.61±4.37
HDL_2-C (mg/dl)	27.29 ±0.93[c]	40.67±0.74[b]	42.98±0.66[a]	16.08 ±0.16[c]	17.78 ±0.13[b]	19.12 ±0.10[a]
HDL_3-C (mg/dl)	73.61 ±2.21[c]	77.83 ±2.09[b]	81.92 ±2.01[a]	31.39 ±0.94[c]	32.04 ±1.03[b]	39.49±1.01[a]

VLDL-C (mg/dl)	21.10 ±2.29 [b]	26.64 ±2.57[a]	21.76 ±2.21[b]	30.47 ±1.85[b]	31.92 ±2.00[b]	34.80 ±1.99[b]
LDL-C (mg/dl)	18.80 ±1.67 [c]	20.46 ±1.87 [b]	26.44 ±1.86[a]	[a]127.48 ±7.42	128.09 ±8.27[a]	[a]128.72. ±8.37
Non-HDL-C (mg/dl)	39.90 ±3.02 [b]	47.10 ±3.46 [a]	48.2 ±2.99 [a]	157.95 ±7.32[c]	160.01±8.45[b]	163.52 ±8.59 [a]
TC/HDL-C	1.395±0.036 [b]	1.397±0.031 [b]	1.386±0.028[b]	4.327±0.121 [b]	4.212 ±0.182 [b]	3.790 ±0.106 [b]

Data are presented as Mean ± SD (n=100). TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; VLDL-C, very low-density lipoprotein-cholesterol; Non-HDL-C, Non-high-density lipoprotein cholesterol, AC, atherogenic index; TC/HDL-C, total cholesterol/high-density lipoprotein-cholesterol; LDL-C/HDL-C, low-density lipoprotein-cholesterol/high-density lipoprotein-cholesterol; TG/HDL-C, triglyceride/high-density lipoprotein-cholesterol; AIP, atherogenic index of plasma..One-way ANOVA Posthoc Turkey´s test was used for comparing significantdifference between wild type and mutant genotypes for both separate subjects. a=highest, b= medium, c=lowest. Those genotypes that have the same letters are not statistically significant (P>0.05) while those that have different letters are statistically significant (P<0.05).

4.0. DISCUSSION

The plasma cholesteryl ester transfer protein (CETP) increases the transfer and substitution of cholesteryl ester and triglyceride between HDL-C and apo B-containing particles. Due to the main role of CETP in the metabolism of lipoproteins, it is assumed that genetic polymorphism in the CETP gene may be one of the risk factor for the development of ischemic stroke. In this study, a base variation of Ile405Val in the CETP gene was determined by PCR-RFLP. Thepolymorphism in this study was presented in 52% (IIe/Val=37% and Val/Val=15%) of the non-symptomatic control and 83% (IIe/Val=33% and Val/Val=50%) of the ischemic stroke patientssubjects. The present study showed that the frequencies of I and V alleles were 33.50% and 66.50% for the stroke and 66.50% and 33.50% for the control subjects respectively (P<0.000 1). The V allele was significantly (p<0.0001) more abundant in the ischemic stroke subjects compared with the control subjects. TheIle405Val was shown to be associated with higher concentration of TG, TC, LDL-C,VLDL-C and dyslipidemia for both the control and the stroke subjects.The Ile405Val genetic mutation of CETP gene is responsible for the elevation increase in the plasma cholesterol and triglyceride levels of the homozygous and heterozygous mutant genotypes for the control and ischemic stroke subject respectively. We found that the IIe/Val and Val/Val genotypes were responsible for higher HDL-C, HDL_2-C, and HDL_3-C (P<0.000 1) levels for both the control and the stroke subjects. The SNPs in the CETP gene

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is responsible for higher ($P<0.0001$) increase in the production of defective HDL-C, HDL_2-C, and HDL_3-C levels as a result of dysfunctional cholesteryl ester transfer protein activity. This mean that increased concentration of these lipoproteins may lead to an impaired reversetotal cholesterol transport and paradoxically increase the risk of ischemic stroke for both subjects. The study reported that the common Ile405Val mutation in the *CETP* gene, was associated with increased levels of HDL-cholesterol, and this mutation is one of an independent risk factor for ischemic stroke patience that visited LUTH. HDL particles in the plasma or serum accept cholesterol from non-liver cells; CETP facilitates the transfer of cholesteryl ester onto triglyceride rich lipoproteins as part of the reverse cholesterol transport pathway, ultimately leading to cholesterol excretion by the liver [32, 33]. When CETP is dysfunctional, cholesterol accumulates in HDL, and the transfer of cholesterol from peripheral cells to the liver is blocked and this may be responsible for the significant increase ($P<0.0001$) in plasma HDL-C, HDL_2-C and HDL_3-C as observed in our study. Different studies have shown that homozygosity for Val405 was associated with increased HDL-cholesterol levelsin 102 Japanese men with hypertriglyceridemia [34], in 234 Dutch men [21] and in 145 Icelandic men [35]. Complete CETP deficiency as seen in Japanese studies lead to massive elevated levels of HDL-cholesterol and apoA-I [36-39] and various studies have demonstrated that the Ile405Val mutation leads to reduced CETP activity [40] and mass [21, 34] in the plasma. In another study, the association of CETP with HDL levels was observed and the study suggests that CETP is an atherogenic protein increasing the risk of myocardial infarction (MI) [41].Agerholm-Larsen et al.[28] study shows thatin a large population sample,it was observed that HDL-cholesterol level increases in both heterozygotes and homozygotes of Val405 in premenopausal women and in postmenopausal women not treated with HRT, whereas inhypertriglyceridemic men, only Val/Val homozygosity is associated with increased in HDL-cholesterol. Their study also shows that increased in HDL-cholesterol levels caused by mutations in CETPgene are associated with an increased risk of ischemic heart disease in white women [28].ApoA-I is found only in HDL and chylomicrons [42], the effects we observed on apoA-I most likely reflect the changes in the levels of apoA-I in HDL.In the present study,there is a clear increased risk of dyslipidemia in the control andthe ischemic stroke subjects from Ile/Ile to Ile/Val to Val/Val respectively.

5.0 CONCLUSION

The results of this study show that the mutation of Cholesteryl ester transfer protein (CETP) Ile405Val (rs5882)polymorphism causes an increased in defective HDL-C, HDL_2-C and HDL_3-C concentration and is associated with an increased risk of ischemic stroke.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the author and producers of the products because I do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

As per international standard or university standard, patient's written consent was collected and preserved by the authors

ETHICAL APPROVAL

The research ethical approval was obtained from Lagos University Teaching Hospital Research and Ethical Committee with Healthy Research Committee assigned no: ADM/DCST/HREC/100. Control and stroke subjects who were not willing to participate in the research were excluded from the study.

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