RESPONSE OF SERUM LIPIDS TO A FAT MEAL IN BLACK SOUTH AFRICAN SUBJECTS WITH DIFFERENT APOE GENOTYPES

by

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DECLARATION

I declare that the dissertation hereby submitted to the University of Limpopo for the degree of Master of Science in Medical Sciences (Chemical Pathology) has not been submitted by me for any degree at this or any other University; that it is my own work in design and in execution, and that all material contained herein has been duly acknowledged.

S.A. DIKOTOPE

SIGNATURE: ______________________ Date: __________________________

DEDICATION

This study is dedicated to the following:

1. My late parents, Mr. Kgoloko Stephen and Mrs Maapetle Dikotope for raising me up and believing in me. I am what I am because of their efforts.
2. My family Tebogo, Witness and Goodness. Your presence motivated me to work harder.
3. My sister Stephina and two brothers, Moses and Johannes for your relentless moral support and encouragement.

Above all my Lord and Savior Jesus Christ for giving me life, wisdom, good health, physical and spiritual strength throughout the whole study and for taking a good care of me throughout my life.

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ABBREVIATIONS

A	Adenine
ABCA1	ATP-binding cassette A1
ACAT	Acyl CoA:cholesterol acyl transferase
ANOVA	Analysis of variance
Apo A	Apolipoprotein A
Apo B	Apolipoprotein B
ApoE	Apolipoprotein E protein
AUC	Area under the curve
BMI	Body mass index
CAD	Coronary artery disease
C	Cytosine
CETP	Cholesteryl-ester transfer protein
CHD	Coronary heart disease
CM	Chylomicron
CREAT	Creatinine
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetracetic acid
g	grams
G	Guanosine
HDL	High density lipoprotein

HDL-C	High-density lipoprotein cholesterol
HMG-CoA	Hydroxymethylglutaryl Coenzyme A reductase
HTL	Hepatic triglyceride lipase
IDL	Intermediate-density lipoproteins
kDA	Kilo Daltons
Kg/m^2	Kilograms per square meter
LCAT	Lysolecithin cholesterol acyl transferase
LDL	Low density lipoprotein
LDL-C	Low-density lipoprotein cholesterol
LPL	Lipoprotein lipase
LRP	LDL-receptor related lipoprotein
mmol/l	Millimoles per liter
MUFA	Monounsaturated fatty acids
ng	Nanograms
nm	Nanomoles
N-saline	Normal saline
OFTT	Oral fat-tolerance test
PCR	Polymerase chain reaction
PUFA	Polyunsaturated fatty acid
P-value	Probability value
RFLP	Restriction Fragment Length Polymorphism
SD	Standard deviation
SFA	Saturated fatty acid
SR-BI	Scavenger receptor BI
TBE	Tris-borate-EDTA

TRL	Triglyceride-rich lipoprotein
VLDL	Very low-density lipoprotein

DEFINITION OF TERMS

Apolipoprotein:	the protein component of lipoprotein complexes that is a normal constituent of plasma chylomicrons, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and very low-density lipoprotein cholesterol in man.
Genotype:	the genetic constitution of an individual.
Phenotype:	manifestation of a genotype
Polymorphism:	occurrence in more than one form
Postprandial:	following a meal

ABSTRACT

Objectives

The present study investigated how the serum lipids responded to a high-fat meal in black South African subjects with different *APOE* genotypes, a population that until recently was reported to be consuming a traditional diet of low fat and high carbohydrates.

Methods

Sixty students (males and females) of the University of Limpopo, Turfloop Campus were successfully genotyped using Restriction Fragment Length Polymorphism (RFLP) and grouped into four *APOE* genotype groups; 2, 2/ 4, 3 and 4. Only thirty-three subjects volunteered to participate in the oral fat-tolerance test (OFTT), but two were excluded for having abnormal total cholesterol (6.05 mmol/l) and LDL cholesterol (3.12 mmol/l) so only 31 subjects were left. The numbers per group were 2=5, 2/ 4=8, 3=9 and 4=9. After an overnight fast blood was drawn for measurements of baseline serum parameters. Subjects were administered a high fat meal 30 minutes after the baseline blood sample was drawn. Blood was drawn at intervals of 20, 40, 60, 120, 180, 240, 300 and 360 minutes for measurements of postprandial serum parameter levels. Serum parameters measured were triglyceride, total cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol, glucose and insulin.

Results

Mean levels of serum lipids at baseline in mmol/l were as follows; group 1[TG=0.69(0.55-0.81), TCHOL=3.10±0.29, HDL-C=1.12±0.32, LDL-C=1.67±0.28]; group 2 [TG=0.61(0.53-1.00), TCHOL=2.98±0.53, HDL-C=1.20±0.37, LDL-C=1.43±0.37]; group 3 [TG=0.67(0.28-0.86), TCHOL=2.96±0.54, HDL-C=1.22±0.30, LDL-C=1.46±0.47]; group 4 [TG=0.76(0.51-1.16), TCHOL=3.27±0.51, HDL-C=1.12±0.17, LDL-C=1.79±0.47]. There was no significant difference in the mean levels of baseline triglyceride, total cholesterol, low density lipoprotein cholesterol, and

high density lipoprotein cholesterol between the APOE groups hence no significant difference in the response to a fatty meal.

Conclusions

There was no significant change in serum lipid concentrations after a fatty meal in individuals with different *APOE* genotypes in a population that consume a traditional diet of low fat and high carbohydrates. Due to the small sample size, the results should be interpreted with caution. A larger study is recommended to ascertain the role of APOE genotypes on serum lipid response to a fatty meal in Black South African population.

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1 INTRODUCTION

Postprandial lipaemia is the term given to the metabolic events that take place after digestion and absorption of a fat-containing meal [1]. The common feature of postprandial lipaemia is the elevated concentrations of triglycerides after a fat-containing meal [2]. The magnitude and duration of the triglyceride response is influenced by a number of metabolic processes that include the rate of secretion of triglycerides from the intestine and the liver, enzyme activities involved in the processing of triglyceride-rich lipoproteins (lipoprotein lipase (LPL) and hepatic lipase (HL)) and the rate of receptor-mediated clearance of triglyceride-rich lipoproteins (TRLøs) [1]. At present there is no widely agreed definition of postprandial lipaemia [3].

Studies have reported that there is an inter-individual variation in postprandial response of serum lipids to a fat meal and that it is mainly due to the interaction of some single-nucleotide gene polymorphism and environmental factors such as diet and lifestyle [4, 5]. Among the genetic factors, apolipoprotein E (*APOE*) gene polymorphism has been extensively studied [4, 5]. Apolipoprotein E is a structural component of TRLøs (very low-density lipoproteins and chylomicrons) and HDL and plays a key role in the metabolism of TRLøs. Apolipoprotein E serves as a high affinity ligand for the receptor-mediated uptake of TRL remnant particles by the hepatic and extra-hepatic tissues [6, 7].

The gene that codes for apoE is located on the long arm of chromosome 19 and is polymorphic [8]. Three common alleles designated *ε2, ε3* and *ε4* give rise to six genotypes; Three homozygous (*ε2/ε2, ε3/ε3* and *ε4/ε4)* and three heterozygous (*ε2/ε3, ε2/ε4* and *ε3/ε4)* [9, 10]. The *APOE ε2* and *APOE ε4* alleles have been reported to exert dorminant characteristics over the *ε3* allele in their allelic combination [11].

The three alleles code for the three major isoforms: apoE2, apoE3 and apoE4 which differ in the binding activity to the low-density lipoprotein (LDL-) receptors also known as B/E receptors [7]. Relative to apoE3, apoE2 binds poorly to the hepatic LDL receptors resulting in the accumulation of chylomicrons and VLDL remnant particles [12]. The apoE4 isoform binds with the same affinity as the apoE3 isoform

to the LDL receptor *in vitro*, but its influence on the clearance of triglyceride-rich lipoproteins *in vivo* remains unclear [13, 14, 15]. It has been reported that *in vivo* lipoproteins that carry the apoE4 isoform is catabolized at a rate twice those carrying the apoE3 isoform [15]. The resultant down-regulation of LDL receptors by increased cholesterol influx is believed to explain the higher levels of LDL-C in healthy subjects with the *ε4* allele [15].

Population studies have demonstrated that individuals with the *ε4* allele have higher plasma total (TC) and LDL- C concentrations compared to individuals with *ε3/3* genotype [16, 17, 18]. Subjects with the *ε2* allele have been reported to have lower concentrations of TC and LDL-C compared to subjects with the *ε3/3* genotype [16, 17, 18]. Furthermore there is an increased susceptibility to coronary heart disease (CHD) among individuals with *ε4* allele [19, 20, 21, 22] while the *ε2* allele has been shown to have a protective effect against CHD [19, 20, 21, 22]. The association between the APOE genotypes and serum triglyceride remains unclear.

The *APOE* polymorphism may explain some of the inter-individual differences in the response of serum lipids to a fat meal [4, 5, 23]. In response to a fat meal, significantly higher and prolonged postprandial triglyceride levels were reported in subjects with the *ε4* allele in some studies [24, 25] while other studies observed higher and prolonged postprandial triglyceride levels in individuals with the *ε2* allele compared to individuals with *ε3/3* genotype [26]

In the Black South African populations, the frequency of the *ε4* allele has been reported to be higher than in the Caucasian population [27]. The *ε2/ε2* genotype is very rare in the South African Black populations which suggest that the population is at an increased cardiovascular risk [27, 28, 29].

The association between *APOE* genotypes and serum lipids has been suggested to be modulated by dietary and lifestyle factors. It has been suggested that the presence of the *ε4* allele does not negatively affect serum lipid parameters in physically active subjects consuming a low fat, carbohydrate-rich diet, but that the effect will be expressed when those indiviodulas become sedentary, obese or diabetic [30].

Black South Africans have been reported to have lower lipid profiles than whites, which makes them less-prone to coronary heart disease (CHD). However the situation is beginning to change with the adoption of western lifestyle that accompanies urbanization [27, 31, 32].The unfavorable pattern of the apoE allele distribution may lead to a rapid growth of CHD incidence among Blacks [27,31,32].

1.1 Rationale of the study

Although there is data about the effect of *APOE* genotypes on the postprandial serum lipids in many parts of the world, the studies that investigated the effect have produced conflicting results. Although the association between the *APOE* polymorphism and baseline serum lipid concentrations among the Black South African populations has been explored, no data is available on the effect of *APOE* genotypes on postprandial serum lipid response to a fat meal. The present study was aimed at establishing the response of serum lipids to a fat meal among Black South African subjects with different *APOE* genotypes.

1.2 Aim

To determine the response of serum lipids to a high fat-load in young and apparently healthy individuals with different *APOE* genotypes

1.3 Objectives

1. To determine the *APOE* genotypes of participants
2. To determine baseline serum triglyceride, total cholesterol, low density lipoprotein cholesterol and high density lipoprotein cholesterol for each subject.
3. To determine baseline concentrations of glucose and insulin for each subject
4. To determine the serum lipid, glucose and insulin response of subjects with different *APOE* genotypes to a high fat meal

1.4 Hypothesis

Subjects with the *ε3/ε4* and *ε4/ε4* genotypes will present with higher fasting and postprandial serum triglyceride concentrations compared to subjects with the *ε3/ε3* genotype after ingestion of a fat meal

2 LITERATURE REVIEW

2.1 LIPOPROTEIN METABOLISM

2.1.1 Structure, functions and classification of lipoproteins

a. Structure

Plasma lipoproteins are water-soluble particles consisting of hydrophobic lipids in their cores, surrounded by a shell of polar lipids and apolipoproteins. The inner core lipids consist of triacylglycerol (TAG) and cholesteryl-esters (CE) while the shell contains un-esterified cholesterol, proteins and phospholipids [4].

The protein moieties of lipoproteins are called apolipoproteins and there are 16 apolipoproteins to date that have been isolated and characterized. They are apo A-I, apoA-II, apoA-IV, apo (a), apoB, apoC-I, apoC-II, apoC-III, apoC-IV, apoD, apoE, apoF, apoG, apoH, apoI, and apoJ [4].

The apolipoproteins make the highly hydrophobic lipids soluble, permitting their transport in blood. Several apolipoproteins serve as ligands for recognition, uptake and removal of lipoprotein particles or for cellular mobilization of particular lipids [4].

b. Classification

Lipoproteins are classified according to their densities into chylomicrons (CM), very low-density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) [33, 34].

c. Chylomicrons

Chylomicrons are the largest lipoproteins (>100 nm in diameter) and are synthesized in mucosal cells from dietary fats and secreted into the lymphatic system where they enter circulation via the thoracic duct [33, 34]. The secreted chylomicrons contain

apoB-48 and apoA-I. Their function is to transport dietary triglycerides and cholesterol from the site of absorption to various tissues of the body and the fat-soluble vitamins to the liver [33, 34].

The secreted chylomicrons contain apoB-48 and apoA-I. In the blood chylomicrons acquire ApoE and Apo C-II from HDL. ApoC-II activates capillary endothelium-bound lipoprotein lipase that catalyzes removal of free fatty acids from the lipoproteinøs triglyceride pool, thereby progressively reducing its size. Cholesterol, phospholipids, apoA and apoC-II are released from the surface of the chylomicrons and are taken up by HDL. The triglyceride-depleted chylomicron remnant particles are enriched with cholesterol esters from HDL and are rapidly cleared from the circulation by the hepatic parenchymal cells. The clearance depends on recognition of apoE by the LDL-related receptor protein and LDL receptors [34]. In the liver cells the chylomicron remnant releases its remaining triglycerides, cholesteryl esters, phospholipids and apoproteins [33]. The remnant particles, together with endogenous triglycerides and cholesteryl esters are either re-assembled into very low-density lipoproteins (VLDLøs) and secreted into the circulation for delivery of lipids to the peripheral tissues or metabolized in the liver [33].

- **Very low-density lipoproteins (VLDL)**

Very low-density lipoproteins are 30 to 90 nm particles of a density less than 1.006 g/ml and responsible for transport of triglycerides and cholesterol from the liver for redistribution to various tissues [34]. They are formed from triglycerides synthesized in the liver through either de novo synthesis or by re-esterification of free fatty acids [34]. Very low-density lipoproteins contain fewer triglycerides than chylomicrons and contain apoC-II, apoE and apoB100, a physiological ligand for the LDL receptor [33, 34]. In the plasma triglycerides of VLDLs are hydrolyzed by lipoprotein lipase thus progressively depleting VLDLs of triglyceride. In the process phospholipids, apoC-II and apoE are transferred to HDL [34]. Very low-density lipoproteins are converted to denser particles (densities are 1.006-1.019 g/ml), intermediate density lipoproteins (IDL) [33, 34].

Cholesterol taken up by HDL becomes esterified and the cholesteryl ester is transferred back to IDL by cholesteryl ester transfer protein (CETP) in exchange for

triglycerides [3]. Hepatic triglyceride lipase (HTL) located on the hepatic endothelial cells removes more triglycerides, thus converting IDL to LDL [34].

- **Low-density lipoproteins (LDL)**

Low-density lipoproteins are particles of density of 1.019-1.063 g/m and are the principal carriers of cholesterol mainly in the form of cholesteryl esters to the peripheral tissues. They are formed from VLDL via IDL and contain ApoB100. Low-density lipoprotein particles can pass through the junctions between capillary endothelial cells and attach to LDL receptors on cell membranes that recognize apoB100. The LDL particle is then internalized and degraded in the lysosome releasing free cholesterol which is subsequently esterified [34].

Cholesterol synthesis can also take place in the peripheral tissues under regulation of the rate-limiting enzyme hydroxymethylglutaryl CoA reductase (HMG-CoA) which is inhibited by cholesterol. Free cholesterol stimulates its own esterification and is esterified to cholesterol ester by an activity of the enzyme acyl CoA: cholesterol acyl transferase (ACAT) [33]. When LDL concentration increases, macrophages derived from circulating monocytes take up LDL in the arterial wall via scavenger receptors, resulting in the formation of foam cells. This process contributes to pathogenesis of atherosclerosis [3].

- **High-density lipoprotein (HDL)**

High-density lipoprotein is synthesized in the intestinal mucosal cells and in the liver. The HDL particles consist of a number of discrete subpopulations of entities differing widely in density, size, shape, composition and surface charge. The two main sub-fractions are HDL2 and HDL3, with densities of 1.063-1.125 g/ml and 1.125-1.21 g/ml respectively. High-density lipoprotein is secreted as a nascent HDL particle which is disc-shaped and contains only a small amount of phospholipid and ApoA-I. Apolipoprotein A-I rapidly picks up phospholipid in the plasma and the nascent HDL particle is converted almost immediately to a pre 1 HDL. The process is catalyzed by the ATP-binding Cassette A1 (ABCA1) transporter, which is expressed in the peripheral tissues, intestine and liver. The nascent pre 1 HDL cholesterol is then esterified by lysolecithin cholesterol acyl-transferase (LCAT) to give rise to mature spherical HDL. Another transporter, ATP-binding Cassette loads more cholesterol

onto the mature HDL from peripheral tissues and along with ABCA1 enables removal of artery wall cholesterol by macrophages. HDL-cholesterol particle is taken up by scavenger receptor BI (SR-BI) in the liver and is hydrolyzed. The resulting free cholesterol is then metabolized to bile acids and is eventually excreted [33, 35, 36].

2.1.2 ENZYMES INVOLVED IN LIPOPROTEIN METABOLISM

a. Lipoprotein Lipase

Lipoprotein lipase (LPL) is a 57 kilo-Dalton glycoprotein synthesized and secreted by many tissues [37]. Lipoprotein lipase is expressed on the luminal surface of the capillary endothelial cells of various tissues in the body after synthesis [38].

Lipoprotein lipase is a key enzyme in lipoprotein metabolism and transport [39]. The function of LPL is to hydrolyze the triglycerides of the circulating chylomicrons and VLDL [40, 41, 42].

b. Cholesteryl ester transfer protein

Cholesteryl-ester transfer protein (CETP) is responsible for transfer of cholesteryl esters from HDL particles to low-density lipoprotein cholesterol (LDL) particles. The CETP-mediated transfer of cholesteryl-ester from HDL to apoB-containing particles (VLDL and LDL) and the subsequent uptake by hepatic LDL receptors is a major route for delivery of cholesterol to the liver [3, 43, 44, 45].

Cholesteryl-ester transfer protein is secreted primarily by the liver and adipose tissue and circulates in plasma in association with HDL [46]. Cholesteryl-ester transfer protein may be both pro-atherogenic and anti-atherogenic [47]. Cholesteryl-ester transfer protein plays a major role in the reverse cholesterol transport [44]. Cholesteryl-ester transfer protein is anti-atherogenic in that it optimizes LDL particle structure and apoB100 conformation for high binding affinity to LDL receptors [46, 48]. High CETP activity has been reported to have a lowering effect on the HDL/total cholesterol ratio thus predisposing to an increased risk of coronary artery disease (CAD) [49, 50].

c. Lecithin cholesterol acyl-transferase

Lecithin cholesterol acyl-transferase plays a role in lipoprotein metabolism especially in the reverse cholesterol transport. The enzyme is synthesized in the liver and circulates in the blood plasma complexed to components of HDL. Cholesterol from peripheral tissues is transferred to HDL particles, and esterified before it is transported into the liver [4].

2.2 APOLIPOPROTEIN E

2.2.1 Apolipoprotein E structure and function

a. Structure

The human apoE has a molecular mass of 34kDa and contains 299 amino acid residues. Apolipoprotein E contains two regions; the amino terminal (22kDa) and the carboxyl-terminal (10kDa) regions. The amino terminal region comprises residues 1-191 that has lysine- and arginine-rich-receptor-binding region situated between amino acids 136 and 150. The carboxyl-terminal comprises residues 225-299 and contains the major lipid-binding determinants necessary for apoE-lipoprotein complex formation [51].

Apolipoprotein E is synthesized mainly in the liver while 20% to 40% of the total apoE protein has been estimated to be produced by the extra-hepatic tissues with the brain, and monocyte-derived macrophages reported to produce the highest amount of apoE. Apolipoprotein E is also produced by other tissues like adrenal glands, testes, ovary, lungs, adipose tissues and the retinal pigment epithelial cells [52, 53].

b. Function

Apolipoprotein E (apoE) serves as a high affinity ligand for the receptor-mediated uptake of TRL remnant particles by the hepatic and extra-hepatic tissues [6, 7]. In addition, apoE has several other functions. Macrophage-derived apoE acts in both an autocrine and paracrine manner by by causing the reverse efflux of cholesterol from macrophages [54]. Furthermore ApoE helps in the maintenance of structural integrity of lipoproteins [25, 55].

Apolipoprotein E has several functions which are not related to lipoprotein metabolism. They include inhibition of platelet aggregation by interacting with apoE receptor 2, resulting in an increase in cellular nitric oxide (NO) concentrations by simulating the NO synthase signaling cascade [56, 57, 58]. In the vascular smooth muscle cell, apoE inhibits smooth muscle cell migration directed by platelet-derived growth-factors by binding to LDL receptor-related protein, which activates the cyclic AMP protein kinase cascade [8]. Furthermore apoE inhibits cell proliferation by binding to cell surface proteoglycans [59]. Apolipoprotein E was also found to modulate the cell inflammatory response through pro-inflammatory cytokine production [60, 61]

2.2.2 Apolipoprotein E polymorphism

The *APOE* polymorphism results from mutations in the coding sequence of the *APOE* gene that results in amino acid substitutions (Arg to Cys) at amino acid positions 112 and 158 of the apoE protein [62, 63]. The substitution affects the metabolic activity of the apoE protein since the amino acid terminal domain approximately between residue 140 and 160 is a sequence enriched with basic amino acids which interact with acidic amino acids of the ligand-binding domain of LDL receptor and hepatic LDL receptors [64, 65]

Three common alleles of the *APOE* gene; *ε2*, *ε3* and *ε4* result from this polymorphism and give rise to six different genotypes (homozygous *ε2/ε2*, *ε3/ε3*, *ε4/ε4* and heterozygous *ε2/ε3*, *ε2/ε4*, *ε3/ε4* (9). The proteins coded by the three alleles in their order are apoE2, apoE3, and apoE4 respectively [66, 67, 68, 69].

The apoE3 isoform contains cysteine and arginine at position 112 and 158 respectively. The cysteine-arginine position of the apoE3 isoform enhances a normal binding activity to LDL receptor which enhances normal lipid metabolism [70]

The apoE2 isoform has cysteine residues at both positions 112 and 158 [70]. The substitution dramatically changes salt bridges within helix 4 and between helices 3 and 4, lowering the positive ion potential of the receptor-binding regions of residues 140-150 to LDL receptors [64, 65].

The apoE4 isoform has arginine at both amino acid positions 112 and 158 [70]. The substitution of arginine for cysteine at amino acid residue 112 in the apoE4 isoform causes noticeable shifts in side chain orientation of specific residues and a few salt bridge re-arrangements. The presence of arginine at amino acid residue 112 leads to formation of a new salt bridge between glutamic acid 109 and residue 112 in helix 3. Furthermore, the side chain of arginine 61 is caused to adopt a different, exposed position in helix 6 [51]. The apoE4 has a high affinity to the LDL receptors which induces a faster uptake of triglyceride-rich lipoprotein particles by the liver [51, 64, 65].

2.2.3 Apolipoprotein E allele and genotype distribution

The *ε3* allele is the most common of the three alleles globally with frequencies ranging from 60 to 90%. In populations with a long-established agricultural economy such as those of Mediterranean basin the frequency of the *ε3* allele ranges from 85 to 90% [66, 67, 68, 69, 71, 72, 73, 74].

The second most prevalent allele is the *ε4* allele which has been referred to as the ancestral allele (75, 76). The frequency of the *ε4* allele is higher in populations such as Ugandans (25%), Khoi-Sans (37%), Pygmies (41%), aborigines of Malysia (24%), Australians (26%) and Papuans (37%) and in African Americans (28%) [66,67, 68, 69, 70, 71, 72]. The frequency of the *ε4* allele is reported to be low in Asian and Chinese populations where a frequency of 9% and 5% respectively has been reported [73, 77]. In European populations a North to South decrease of the frequency of the *ε4* allele has been reported [55, 67, 74, 78, 79].
The *ε4* allele has been reported to be associated with increased risk of cardiovascular disease and Alzheimerøs disease [80]. The association between the *ε4* allele with Alzheimerøs disease among African Americans still remains unclear [81] and no study could be found on the association between the *ε4* allele with Alzheimerøs disease among Black South Africans.

The *ε2* allele is common among Caucasians, low or absent among African Americans [82] , Mexicans [83, 84], and most African populations including Black South African

populations [27,29, 71, 72]. However a high frequency was reported in the Ugandan populations (15.71%) [73]

2.3 APOLIPOPROTEIN E GENOTYPES AND SERUM LIPID CONCENTRATIONS

2.3.1 Apolipoprotein E genotypes and serum triglycerides, total cholesterol, low-density lipoprotein cholesterol and high-density lipoprotein cholesterol concentrations

The association between the *APOE* genotypes and serum lipids has been extensively studied worldwide but has produced inconsistent results.

Several studies reported that *APOE* genotypes influence serum triglyceride levels [15, 85, 86] while other studies did not find any association between APOE genotypes and serum triglycerides [87, 88]. Regarding the effect of *APOE* genotypes on HDL-C, inconsistent results have been observed across populations [19].

A meta-analysis by Bennet et al. (2007) was carried out on the association between *APOE* genotypes and serum lipid levels. Individuals with the *ε2* allele had significantly lower levels of LDL-C and TC than individuals with the *ε4* allele. The authors reported significantly higher triglyceride levels in individuals with the homozygous *ε2/ε2* genotype than in individuals with the homozygous *ε3/ε3* genotype [22]. Similar results were reported by other studies [15, 18, 88, 89, 90, 91, 92].

In Black South African populations serum TC and LDL-C concentrations were found to be higher in subjects with the *ε4* allele compared to those with the *ε3* allele [27, 29]. Triglyceride concentrations were also significantly higher in the *ε2* allele group compared to *ε3* allele group. A study by Corella et al. (2001), in a Mediterranean Spanish population indicated that environmental factors are important modulators of the effect of the *APOE* allele on serum lipid concentrations. Their results suggested that higher LDL-C levels in subjects with the *ε4* allele are only present in populations exposed to Western diets that are high in saturated fat and dietary cholesterol and are

leading a sedentary type of lifestyle [93]. Other studies have also indicated that environmental factors modulate the effect of *APOE* alleles on serum lipid levels [62, 77, 91, 94, 95, 96, 97].

Aguilar et al. (1999) investigated the association between the *ε4* allele and serum lipid profiles in 142 middle-aged healthy individuals from a Native American population following their traditional lifestyle. The *ε4* allele was not associated with an abnormal lipid profile [30]. Other studies have also reported the absence of an association between *APOE* genotypes and serum lipids [30, 98, 99, 100].

Contrary to these reports, a study in a Korean population exposed to a low-fat, high carbohydrate diet reported that the *ε4* allele was significantly related to higher concentration of LDL-C and small, dense LDL particles compared to the *ε3/3* genotype [101]. Several other studies have reported similar results [12, 16, 102, 103, 104, 105].

2.3.2 Apolipoprotein E genotypes and glucose and insulin

The mechanism by which apoE genotypes affect serum glucose and insulin levels have not been elucidated and the reports have not been consistent. Some studies have reported higher levels of baseline glucose and insulin in diabetic subjects with *ε2/ε4* and *ε3/ε4* genotypes than in diabetic subjects with the *ε3/3* genotype [106], while other studies have found no difference on either glucose or insulin [24, 25, 107].

2.4 Effect of gender on association between APOE genotypes and serum lipid concentrations

Gender has been suggested as a potential effect modifier of the association between *APOE* polymorphism and serum lipid concentrations. The effect of the *ε4* allele on serum lipids was reported to be higher and more detectable in men than in women independent of cholesterol concentration [108]. Mahley et al. (2000) reported higher HDL-C concentrations in women with the 2 allele than in those with the *ε3* allele [20]. However Ward et al. (2009) did not find any modifying effect of age and gender on the association between APOE polymorphism and serum lipid concentrations [97].

2.5 APOLIPOPROTEIN E POLYMORPHISM AND POSTPRANDIAL SERUM LIPID, GLUCOSE AND INSULIN CONCENTRATIONS

2.5.1 Association between apolipoprotein E genotypes and concentrations of triglyceride, total cholesterol, low-density lipoprotein cholesterol and high-density lipoprotein cholesterol levels after a fat meal

a. The response of serum triglycerides to a fat meal

Studies have shown that the capacity of individuals to regulate circulating triglyceride concentrations and to clear triglyceride-rich lipoproteins (TRLøs) after a fat-containing meal is modulated by *APOE* polymorphism [4, 5, 109].

Dart et al. (1997) assessed postprandial lipaemia among patients with recently diagnosed CHD and their age-matched controls. The mean age of subjects was over 60 years of age. The test meal yielded 66% of daily anticipated energy and contained 45% fat, 35% simple carbohydrate and 25% proteins. Postprandial triglyceride concentrations were found to be higher in CHD cases with the *ε4* allele than in those with *ε3* allele [104]. Similar results have been reported by several studies [13, 82, 110]

Dallongeville et al. (1999) did a study on male students aged between 18 and 28 years with paternal history of myocardial infarction and those without myocardial infarction. The standard test meal yielded 1.493kcal of energy and contained 65,5g fat (41.6g saturated), 21.6g protein, 56.2g carbohydrate and 416.6 mg of cholesterol. Serum triglyceride levels were higher in subjects with the *ε2* allele and to a lesser extent in those with *ε4* allele than those with the *ε3* allele independently of fasting triglyceride concentrations in both the cases and controls. The peak triglyceride concentrations were reached at 6 hours in all the three allele groups with and without paternal history of myocardial infarction [7].

Kobayashi et al. (2001) investigated how the apoE4 isoform affects postprandial lipid metabolism by comparing individuals with the *ε3/ε4* genotypes to those with the *ε3/ε3* genotypes matched for abdominal visceral fat among a Japanese population. The test meal was a 40g/m^2 cream with 45% emulsified fat with polyunsaturated: saturated ratio of 0.06 and contained 0.001% cholesterol and 2.8% carbohydrates.The cream

contained 50 000U/m^2 of vitamin A as well. Plasma triglyceride concentrations were significantly increased in both groups after a fat meal and concentrations were significantly higher in the *ε3/ε4* genotype individuals than in the *ε3/ε3* genotype individuals [24]. The gender effect on the association between apoE polymorphism and postprandial serum lipid levels was also assessed. Apolipoprotein E polymorphism was associated with higher postprandial serum lipid concentrations in men than in women [24].

Carvalho-Wells et al. (2010) in their study on the impact of *APOE* genotype on postprandial lipaemia reported exaggerated postprandial triglyceride response in subjects with the *ε4* allele in healthy United Kingdom adults. Subjects homozygous for the *ε2* allele were also reported to have an exaggerated postprandial triglyceride response while subjects heterozygous for the *ε2* allele presented with a non-significant tendency towards higher postprandial triglyceride response compared to those with the *ε3/3* genotype. The authors concluded that the *ε4* allele is associated with elevated postprandial triglyceride levels particularly in subjects older than 50 years [25].

A study by Couch et al. (2000) conducted in New York, U.S.A investigated the postprandial response after a fat-containing meal in children. In contrast to the findings by Carvalho-Wells et al. (2010), Couch et al. found no difference in postprandial triglyceride concentrations between individuals with *ε3* and *ε4* alleles [111]. Different experimental conditions and different age groups may explain the conflicting results obtained in the two studies.

b. Response of serum cholesterol to a fat meal

The majority of postprandial studies focused mainly on the plasma triglyceride response and only few data is available on the response of serum cholesterol levels to a fatty meal. The presence of the *ε2* allele has been associated with impaired cholesterol uptake by the hepatic cells mainly due to up-regulation of LDL receptors associated with the results of its isoform. Conversely the uptake of apoE4 isoform-containing TRL particles enhances the uptake of cholesterol in the liver, down-regulating the LDL receptors and resulting in increase in plasma cholesterol levels after a fatty meal [12]. Furthermore, Hallman et al. (1991) suggested that the magnitude of the *ε4* allele on LDL-C concentrations after a fatty meal is directly

related to the habitual dietary cholesterol and fat consumption [112]. Other studies have reported a marked increase in LDL-C levels in subjects with the *ε4* allele compared to subjects with the *ε3* allele when a high fat diet was administered and a substantial decrease in LDL-C levels when a low fat, low cholesterol diet was administered [110, 113, 114].

Studies on the association between HDL-C and *APOE* alleles have also produced conflicting results. In response to high cholesterol diet, HDL-C concentrations were increased in subjects with *ε3/ε4* genotype while subjects with *ε2/ε3* genotype showed a decrease in HDL-C concentrations compared to those with *ε3/ε3* genotypes [115]. However the study by Tso et al. (1998) reported an increase in HDL-C among subjects with the *ε3* allele compared to subjects with either the *ε2* or the *ε4* allele after a fatty meal [113].

2.5.2 Apolipoprotein E genotypes and glucose and insulin concentrations after a high fat meal

Data suggests that if mixed meals are used, glucose provided by digestible carbohydrates will transiently increase postprandial glucose and insulin levels [117]. The authors reported that glucose and insulin responses to a meal are very rapid, which arises a need to measure these variables with 30 minutes interval within the first 2 to 3 hours and then hourly thereafter.

In a study by Dart et al. (1997) glucose response to high-fat meal was significantly greater in subjects with *ε4/ε4* genotype than in subjects with *ε3/ε3* genotype. Postprandial insulin response did not differ significantly between the groups, suggesting that there is no association between *APOE* genotypes and postprandial insulin response [106]

2.6 POSTPRANDIAL LIPAEMIA

Postprandial lipaemia is characterized by elevation of triglyceride-rich lipoproteins in the plasma [116]. The first and main change elicited in the circulation after a fatty meal is the transient elevation of triglyceride levels resulting from accumulation of triglyceride-rich lipoproteins (TRLøs) [116]. The accumulation of these lipoproteins

during the postprandial period is explained in part by the competition between the intestinal and hepatic TRLøs for the same lipolytic and receptor-mediated uptake pathays [117].

Postprandial lipaemic response is therefore a dynamic and non-steady state condition accompanied by rapid remodeling of lipoproteins compared to the relatively stable fasting condition [24]. Human beings spend the majority of their time in a postprandial state when eating regular meals because plasma triglyceride levels from the previous meal have not returned to baseline before another meal is consumed [118]

Studies have suggested that elevated postprandial TG levels predispose to increased risk of atherogenesis, and a correlation has been observed between postprandial triglycerides and presence or progression of coronary heart disease (CHD) and carotid intima- media thickness (CIMT) [119, 120, 121, 122].

2.6.1 Postprandial lipaemia and atherosclerosis

Many individuals develop atherosclerosis despite normal fasting lipid concentrations [119]. Studies have reported an impaired postprandial lipid metabolism, particularly a prolonged postprandial triglyceride concentration, as an important risk factor in the onset of coronary heart disease (CAD) [124, 125].

A suggestion that postprandial dyslipidaemia contributes to atherosclerosis was proposed over 30 years ago [126]. Recent studies have confirmed the role of postprandial lipaemia in the development of atherosclerosis. It has been reported that chylomicron remnants can enter the arterial wall by penetrating into the sub-endothelial space by endocytotic mechanism at a rate equal to or greater than that of LDL which predisposes to formation of atherosclerotic plaques [127].

It has also been reported that acute postprandial hypertriglyceridaemia provokes endothelial dysfunction by inducing oxidative stress on the endothelial layer causing an inflammatory response and production of tumor necrosis factor- (TNF- , interleukin-6 (IL-6) and C-reactive protein (CRP). The mechanism behind formation

of atherosclerotic lesion is that postprandial hypertriglyceridaemia induces secretion of toxic substances such as oxidizing agents and cytokines that may harm the arterial walls and also induce an inflammatory response [128, 129, 128]

Stimulation of smooth muscle cells and their migration into the subendothelial space by macrophage-secreted cytokines may also cause development of plaques that may occlude luminal blood flow [130, 131] resulting in an increased pro-thrombosis [132]. Accumulation of chylomicron remnants in the sub-endothelial space also causes differentiation of circulating monocytes into macrophages, which continually take up lipids and become foam cells, an initial step to atherogenesis [130].

2.6.2 Factors affecting postprandial response

A variety of factors affect the duration and magnitude of the elevated triglyceride response in the postprandial period. Fasting concentrations of triglycerides have been reported to be important determinants of postprandial lipaemia [13] with elevated baseline concentrations being the main determinant of prolonged postprandial response [2, 133, 134]. A decrease in activity of lipoprotein lipase (LPL) has been reported to account for four-fold prolonged residence-time of TRL⌀s, particularly those of intestinal origin [119].

The degree of insulin sensitivity has been suggested as a determinant of postprandial lipaemia among healthy middle-aged men [135]. The mechanism behind this is unclear but might be due to the impaired insulin-mediated suppression of hepatic VLDL synthesis and fatty acid release from adipose tissue [136]. The resultant increase in VLDL secretion is associated with a prolonged circulatory residence-time of TRL⌀s due to increased competition with intestinal chylomicron for the common removal pathways [136].

Obesity is associated with several metabolic disoders which include hypertriglyceridaemia and hyperinsulinaemia, which affects postprandial lipaemia [137].

Protocols of postprandial studies involve administration of a test meal or a fat challenge to subjects following an overnight fast and interval-based collection of blood samples for a given period of time. The amount and type of dietary fat, as well as other dietary components including fibre, glucose, starch and alcohol influence magnitude and duration of postprandial lipidaemia [116, 119, 138, 139, 140, 141].

Increase in fat content of a meal increases the area under the curve of the postprandial triglyceride response [116]. Polyunsaturated fatty acids (PUFA) increase the postprandial triglyceride peak while dietary cholesterol exacerbates postprandial increase in triglyceride concentration and delays its clearance [116]. The concentration of TG fails to return to baseline after a postprandial period when dietary cholesterol is ingested [116, 119, 139].

The amount or nature of carbohydrates in a meal alters postprandial lipid metabolism, with fiber addition reported to generally decrease the triglyceride response. It is higher after a load of saturated fatty acid (SFA) than polyunsaturated fatty acids [116, 119, 139].

Apart from diet, other lifestyle factors may also affect postprandial lipaemia. Exercise has been shown to be associated with not only lower fasting triglyceride but also a decreased postprandial triglyceride response. This reduction in postprandial lipaemia after exercise has been attributed to energy expenditure of the exercise and the enhanced associated insulin sensitivity [142].

Gender has also been considered as a significant factor in postprandial lipaemia. Pre-menopausal women were reported to have lower fasting and postprandial lipaemia than men and menopause itself is associated with altered lipid parameters (increased fasting and exaggerated postprandial lipaemia) [143]. Hormone replacement therapy (HRT) has been reported to be associated with an increase in triglyceride levels in parallel with a decrease in remnant cholesterol levels after a fatty meal [145].

Age has also been reported to affect postprandial lipaemia with an increase in age being associated with an increase in the postprandial triglyceride response [117].

Genetic factors also influence postprandial response. Several apolipoprotein genes have been associated with postprandial lipaemia [1]. Variants in the majority of apoprotein genes (A1, A4, A5, C3, and apolipoprotein E), fatty acid-binding protein 2 (FABP2), LPL, HL, microsomal transfer protein and scavenger receptor-B1 have been associated with the extent of postprandial lipaemia [120]. ApoA-I and apoA-IV are also important factors in postprandial clearance [145, 147].

2.7 ENVIRONMENTAL FACTORS (DIET, TOBACCO USE AND ALCOHOL CONSUMPTION) AND APOE GENOTYPES ON POSTPRANDIAL SERUM LIPID RESPONSE

2.7.1 Diet

The effect of apoE genotypes on serum lipids is modified by environmental factors such as dietary intake. Subjects with the *ε4* allele have been reported to have a greater lipid response to a fatty meal than individuals without the allele (145). Replacement of carbohydrate-rich diet by a monounsaturated fatty acid (MUFA) diet resulted in a noticeable decrease in LDL-C in subjects with *ε3/ε4* genotypes whereas the opposite or no effect was observed for *ε2/ε3*and *ε3/ε3* subjects respectively (148, 149). The MUFA intake has also been associated with increased LDL clearance (150).

2.7.2 Tobacco use

Because of the association of the *ε4* genotype with elevated concentrations of LDL-C, Talmud et al. (2005) proposed that individuals who smoke cigarettes and who carry the *ε4* genotype are prone to smoking-related LDL oxidation [149]. Oxidation of LDL results in foam cell formation, a common feature in the pathogenesis of atherosclerosis. Some studies have reported that cigarette smoking predisposes to increased risk of CHD among individuals with the *ε4* allele (151, 152, 153) while others did not find any association (154, 155, 97).

2.7.3 Alcohol consumption

Alcohol has also been suggested to have an effect on the association between *APOE* genotypes and serum lipids. Correla et al. (2000) investigated the association between alcohol intake and plasma lipid concentrations in individuals with different ApoE genotypes. Low density lipoprotein cholesterol was higher in men with *ε4* and lower

in men with the *ε2* allelethan in men with *ε3/ε3* genotype among alcohol drinkers while in women LDL-C was higher in those with *ε4* allele than in those with *ε3* allele in both drinkers and non-drinkers [156].

3 METHODOLOGY

3.1 Study design

The study was experimental in design. Serum parameters were measured at baseline and at intervals after consumption of a fatty meal.

3.2 Study area

The study was conducted at the University of Limpopo, Turfloop campus which lies about 23 kilometers east of Polokwane, the capital city of Limpopo Province.

3.3 Population sampling

Subjects were recruited among the student population of the University of Limpopo, Turfloop Campus aged between 18 and 25 years through personal communication and in response to public notices on the main entrances and prominent places in the campus.

A total of one hundred and sixty-two students responded positively and consented to participate in the study but only sixty students were willing to donate blood and were successfully screened for *APOE* genotypes (genotyping method explained in section 3.9). Of the sixty students, forty-two were females and eighteen were males aged between 18 and 25 years.

Subjects were grouped into four groups according to their genotypes. Since the *ε2* allele has been reported to act in a dominant manner in its combination [11] the *ε2/ε2* and the *ε2/ε3* genotype subjects were grouped together as group 1, *ε2/ε4* genotype subjects as group 2, the *ε3/ε3* genotype subjects as group 3 and the *ε3/ε4* and the *ε4/ε4* genotype subjects as group 4 since the *ε4* allele is dominant over the *ε3* allele [156]. The *ε2/ε4* genotype group was analyzed as a separate group because it has been referred to as a conflicting genotype since the apoE2 isoform has been reported to have opposite effect to the apoE4 isoform on the metabolism of triglyceride-rich lipoproteins.The initial intention was to have ten subjects per group, but as there were only nine subjects with the *ε2/ε4* genotype, automatically limiting the number of

subjects to thirty-nine. Out of the 39 eligible subjects only thirty-three completed the OFTT.

3.4 Exclusion criteria

Subjects with abnormal concentrations of total cholesterol (×5.00 mmol/l), low-density lipoprotein cholesterol (×3.0 mmol/l), fasting serum glucose (×7.00 mmol/l), creatinine (×130 umol/l), and triglyceride (×1.7 mmol/l) concentrations were excluded from the study.

3.5 Ethical consideration

The study was approved by the Polokwane-Mankweng Hospital Complex Ethics Committee (project number 003/2008). All participants signed a written consent form prior to commencement of the study.

3.6 Data collection

Questionnaires about alcohol consumption, smoking and current medication were administered to the participants prior to participation (Appendix V-VII).

Anthropometric measurements (weight, height) were taken. Body weight was measured using a digital balance according to standard procedures to the nearest 0.1 kg with subjects in light clothing.

Height was measured with subjects standing upright with the head up straight and barefoot to the nearest 0.1 cm using a stadiometer.
Body mass index was calculated using the formula weight/ $(\text{height})^2$.

3.7 Blood Sampling procedure

The study consisted of two sets of blood collections: the first for apoE genotype screening and the second set during the oral fat-tolerance test. All blood samples were collected by a registered nurse.

For extraction of DNA, venous blood was collected into two six milliliter purple-top tubes containing EDTA (anti-coagulant). The DNA was extracted from fresh blood samples and stored at four degrees Celsius.

For determination of glucose concentrations, blood was collected into a four milliliter grey-top tube which contained sodium-fluoride/potassium-oxalate. Blood was centrifuged at 1510 x g for ten minutes using a centrifuge from Beckman Coulter and analyzed immediately.

For determination of serum concentrations of creatinine, total cholesterol, HDL-cholesterol, triglycerides and insulin, a five milliliter silicon-coated yellow-top tube was used. The blood was centrifuged at 771 x g for twenty minutes using a centrifuge from Beckman Coulter. Serum was then separated using sterile Pasteur pipettes and stored at minus seventy degrees Celsius for further analysis.

3.8 DNA extraction

DNA was extracted using a commercially available DNA isolation kit (Illustra Nucleon Genomic DNA Extraction Kit, GE Healthcare UK limited, and Buckinghamshire, United Kingdom) according to the manufacturerøs protocol. Approximately ten milliliters of whole blood from subjects were collected into the EDTA tubes and were were transferred to fifty milliliter Falcon tubes and centrifuged at 1510 x g for twenty minutes. Plasma was removed using sterile Pasteur pipettes and stored at minus seventy degrees Celsius for future use. Reagent A (lysing reagent) was added into the Falcon tubes up to four times the initial volume of the content of the Falcon tubes. The Falcon tubes were inverted several times for four minutes and centrifuged at 1734 x g for ten minutes.

Carefully and without disturbing the pellet, the supernatant was removed using a Pasteur pipette and discarded. Two milliliters of reagent B (supplied with the kit) was added and the solution was vortex-mixed until the pellet was completely dissolved. Five hundred milliliters of Sodium Perchlorate were added and the tube inverted twenty times. Two milliliters of chloroform were added into the tube. The tube was inverted seven times. Three hundred micro-liters Nucleon Resin (supplied with the

kit) were added into the tube and without shaking or mixing, centrifuged at 1734 x g for ten minutes. The supernatant was transferred into a screw capped tube using a Pasteur pipette and ice-cold ethanol was added. The screw-capped tube was shaken until the DNA strand could be seen. The DNA was spooled out using spooling rods and suspended in one milliliter of TE buffer in Eppendorf tubes. The Eppendorf tubes were placed on rotator mixer until thoroughly mixed and then stored at four degrees Celsius until the day of analysis.

Preparation of reagents/solutions are presented in Appendix I

3.8.1 Determination of DNA concentration

Twenty times dilution of the DNA sample was made and absorbance read at 260 nm on a spectrophotometer. Protein contamination was checked by reading absorbance at 280 nm. A ratio of A_{260}/A_{280} was acceptable when it was greater than 1.75. When the ratio was too low the samples were treated with proteinase K to remove protein contamination.

The concentration of DNA was calculated as follows:

dsDNA concentration = 50µg/ml X A_{260} X 20 (dilution factor)

3.8.2 Amplification of DNA

Genomic DNA was amplified by Polymerase Chain Reaction (PCR) using the DNA thermal cycler (AEC-AMERSHAM, KELVIN, SOUTH AFRICA). PCR was carried out in a 50µl reaction volume in sterile Eppendorf tubes containing 375 ng DNA, 100 pmol of each primer that is 5ø-AGAATTCGCCCCGGCCTGGTACAC-3-ø reverse primer (Roche Diagnostics) and 5ø-TAAGCTTGGCACGGCTGTCCAAGGA-3ø-forward primer (Roche Diagnostics) as described in th restriction isotyping method by Hixon and Vernier, (1990) [153], dNTP-mix each at 0.1 mmol/l (Roche Diagnostics), 5U/ µl Taq polymerase (Roche diagnostics), 100ml/l dimethylsulfoxide (DMSO), and sterile distilled water. As negative control, a no-template control (NTC) was used where no sample was added in one tube but only distilled water.

The PCR Cocktail was set on ice. After adding all the required PCR cocktail reagents to the Eppendorf tubes and the mixtures were gently vortex-mixed. The tubes were

then loaded on a block of thermo-cycler, which had been warmed to 50 degrees Celsius for 8 minutes.

The PCR was set up as follows;

Denature	95°C for 5 minutes
Cycles 30 cycles of	95°C for 1 minute (denature)
	60°C for 1 minute (anneal)
	70°C for 2 minutes (extend)
Extension	70°C for 7 minutes

After running the PCR, the samples were removed from the block and cooled at 4°C

Preparation of PCR cocktail and principles are presented in Appendix I

3.8.3 Detection of PCR products

Ten microliters of PCR products were mixed with three microliters of loading buffer and run on a 2% agarose gel with Tris-Borate-EDTA (TBE) buffer at 60V. DNA molecular marker consisting of a 50 base-pair ladder (Whitehead) was run along with the PCR products. After electrophoresis the gel was stained with 0.5 µg/l ethidium bromide, then visualized with by UV trans-illuminator and photographed on Polaroid type 667 film using Polaroid camera.

The staining procedure involved carefully placing the gel in a 20cm X 30cm plastic trough and 200 milliliters of the ethidium bromide solution were added. The gel was stained for ten minutes while shaking using a Roto Mix type 50800.

Preparations of buffers and ethidium bromide reagent are presented in Appendix I.

3.9 Apolipoprotein E genotyping by restriction enzyme isotyping

Amplified DNA was digested with 5U of HhAI for at least 3 hours at 37°C. The process did not require purification of the PCR products or addition of specific buffer components for HhAI digestion. The reaction was then stopped by adding 1/10 volume of loading buffer after removal from the water bath. Deoxyribonucleic acid fragments were run on a 6% Meta-Phore agarose gel in TBE buffer at 4°C for 240

minutes at 6V/cm. After electrophoresis, the gel was stained with ethidium-bromide for ten minutes and visualized by UV Trans-illuminator and photographed on Polaroid type 667 film using a Polaroid camera (Appendix VIII)

The numbers and sizes of produced HhAI fragments were used to determine the apoE genotypes according to the method described by Hixon and Vernier [157]. The *ε2/ε2* genotype was characterized by 91 and 83 base pair HhAI fragments reflecting the absence of sites 112cysteine and 158cysteine. The *ε3/ε3* genotype contained the 91 base pair fragment (112cysteine) as well as the 48 and 35 base pair fragments, while the *ε4/ε4* genotype also contained the 48 and 35 base air fragments as well as the unique 72 base pair fragment. The ε3/ε3 genotype was used as reference genotype (normal type) as mentioned in the literature [67].

Protocols of gel preparation are presented in Appendix I.

3.10 Oral fat-tolerance test (OFTT)

Subjects who participated in the oral fat-tolerance test were asked to fast overnight and abstain from any smoking, alcohol use and physical exercise for at least twelve hours prior to the morning of the oral fat-tolerance test. Subjects were also advised to abstain from eating breakfast in the morning of the oral fat-tolerance test.

Subjects reported to the Universityøs Chemical Pathology laboratory by 08h00 in the morning after an overnight fast. Only clear water was allowed during the oral fat-tolerance test. An in-dwelling venous catheter was inserted in the ante-cubital vein. A fasting blood sample was then drawn and a slow infusion of N-saline, without heparin, was used to maintain catheter patency. Subjects were then administered a high-fat test meal which had to be consumed within 30 minutes after the fasting blood sample was collected.

The standard test meal administered consisted of 40g corn flakes, 100g macadamia nuts (ground), 200ml low-fat milk (2% fat) and 1 big banana which provided 56% of energy as fat, 36% as carbohydrate and 8% as protein. At first a food homogenizer was used to mix the meal components but after complaints by subjects that

homogenization makes the meal insipid the usage of homogenizer was discontinued. Subjects were then given the unhomogenized meal that they consumed within the stipulated time. The test meal yielded 1300 calories of energy [158].

When blood was collected the first few milliliters of blood was discarded. Blood samples were taken at 20, 40, 60, 120, 180, 240, 300 and 360 minutes after the fat meal for measurement of postprandial plasma lipids, glucose and insulin.

3.11 Biochemical analysis

Total cholesterol (%CV= 6.2), high-density lipoprotein (%CV=3.0) cholesterol, triglycerides (%CV=12.6), glucose (%CV=4.23) and creatinine (%CV=5.6) were determined using ILAB300 PLUS Chemistry System with kits from Instrumentation Laboratory, Milano, Italy.Creatinine was measured for exclusion of individuals with renal disease (Creatinine >130 umol/l).

Insulin (%CV=7.15) was determined on Beckman ACCESS Immunoassay System using Ultra-sensitive insulin reagent from Beckman Coulter (USA).

Low-density lipoprotein (LDL-C) cholesterol was calculated using the Friedewald equation: LDL-C (mmol/l) = total cholesterol-(HDL-C+ TRIG/2.2) [159].

Principles of the biochemical methods used are presented in Appendix II.

3.12 STATISTICAL ANALYSIS

Data was analyzed using the program SPSS version 17 (SPSS UK Ltd, Chertsey, UK). Serum triglyceride and insulin data (baseline and postprandial) are presented as median and interquartile ranges and were logarithmically transformed to achieve a normal distribution before analysis.

Glucose and serum lipids (total cholesterol, low-density lipoprotein cholesterol and high-density lipoprotein cholesterol) were presented as mean± standard deviation.

Analysis of variance (ANOVA) and Bonferronni test were used to assess differences in parameters between the four apoE genotype groups.

General linear model for repeated measures was used to assess whether there was changes in the concentrations of the biochemical parameters with time within each apoE genotype group after a fatty meal.

The lipid response was measured as a change in concentration from baseline at any given interval and was calculated at each time-point as V_x-$V_{baseline}$ where V_x and $V_{baseline}$ are the values of the variable at time x and at baseline respectively [160]. The area under the curve was measured using Trapezoid rule.

Independent Student-t-test was used to compare means of variables between the *ε4* (*ε3/ε4, ε4/ε4*) and non- *ε4* (*ε2/ε2*, *ε2/ε3*, *ε3/ε3*) allele groups. A p-value of less than 0.05 was regarded as statistically significant.

Postprandial curves were plotted using GraphPad Prism 5 Windows Software (GraphPad Software, Inc, U.S.A)

4 RESULTS

Sixty students (40 females and 20 males) were successfully genotyped and the distribution was as follows; *ε2/ε2* (n=2), *ε2/ε3* (n=9), *ε2/ε4* (n=9), *ε3/ε3* (n=18), *ε3/ε4* (n=18) and *ε4/ε4* (n=4). After grouping the subjects into four *APOE* genotype groups (group 1= *ε2/ε2 and ε2/ε3*; group 2= *ε2/ε4*; group 3= *ε3/ε3* and group 4= *ε3/ε4 and ε4/ε4)* the totals were 11 in group 1, 9 in group 2, 18 in group 3 and 22 in group 4.

Although our initial plan was to have forty subjects (ten from each genotype group) for oral fat-tolerance tests, thirty-nine subjects were available for participation. Only nine (9) subjects were available in group 2. During the oral fat-tolerance test two subjects had intolerance to the fat meal and four had blood clotting and vein collapse and were withdrawn from participation. An effort to replace the withdrawn subjects was unsuccessful which could possibly be the fear of needles, vein collapse and the long duration of the OFTT.

As a result thirty-three subjects (twenty-two females and eleven males) completed the OFTT (six in group 1 (*ε2/ε2, ε2/ε3*), eight in group 2 (*ε2/ε4*), ten in group 3 (*ε3/ε3*) and nine in group 4 (*ε3/ε4, ε4/ε4*) and had all results available. Since in our study blood samples for both baseline and postprandial serum parameter measurements were collected during the day of OFTT, two more subjects were excluded from the study after analysis due to abnormal baseline serum total cholesterol and LDL cholesterol. Finally only 31 subjects were considered for analysis. Since none of the subjects were smokers and only two subjects (6%) used alcohol (3 days a month) the results have not been included.

4.1 Anthropometric measurements and baseline concentrations of serum lipids, glucose and insulin of subjects by APOE genotype group

There was no significant difference in the parameters between males and females among the four *APOE* genotype groups and thus data were combined.

Descriptive statistics for anthropometric measurements (weight, height, BMI,) and baseline concentrations of glucose, insulin and serum lipids (triglyceride, total

cholesterol, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol are presented in Table 4.1.

Concentrations are presented as means ± standard deviation (S.D) for anthropometric measurements (weight, height, BMI, waist circumference and hip), total cholesterol, low-density lipoprotein cholesterol, high density lipoprotein cholesterol and glucose which were normally distributed. Serum triglyceride and insulin levels are presented as medians (interquartile ranges) since they were not normally distributed.

Table 4.1. Anthropometric measures and biochemical parameters at baseline by *APOE* genotype group

Variables	Group 1 n=5	Group 2 n=8	Group 3 n=9	Group 4 n=9	P-Value
Age (years)	20.20 ± 1.64$^{\$}$	20.5 ± 1.07$^{\#}$	22.78±1.56	20.78±1.09$^{@}$	0.002
Weight (kg)	59.98±6.18	60.35±5.94	67.23±7.73	67.59±9.96	0.122
Height (m)	1.59±0.01	1.65±0.08	1.69±0.06	1.66±0.10	0.187
BMI (kg/m^2)	23.59±2.19	22.22±2.22	23.44±1.99	24.47±3.88	0.430
TRIG (mmol/l)	0.69(0.55-0.81)	0.61(0.53-1.00)	0.67(0.28-0.86)	0.76(0.51-1.16)	0.533
TCHOL(mmol/l)	3.10±0.29	2.98±0.53	2.96±0.54	3.27±0.51	0.264
HDLC (mmol/l)	1.12±0.32	1.20±0.37	1.22±0.30	1.12±0.17	0.854
LDLC (mmol/l)	1.67±0.28	1.43±0.37	1.46±0.47	1.79±0.47	0.264
GLUC (mmol/l)	4.61±0.53	4.68±0.27	4.47±0.44	4.47±0.67	0.784
INS (mIU/l)	9.20(3.64-16.69)	6.18(4.30-12.48)	6.67(4.15-10.00)	4.31(3.31-6.82)	0.390

Reference values: triglyceride <1.7 mmol/l, total cholesterol <5 mmol/l, high-density lipoprotein cholesterol >1 for males; >1.3 for females, low-density lipoprotein cholesterol <3.0 mmol/l, glucose <6.00 mmol/l and insulin <26 mIU/l.

P-value <0.05 considered statistically significant

P-value =$^{\$}$Difference between group 1 (*ε2/ε2*, *ε2/ε3*) and group 3 (*ε3/3*) (p=0.011), # between group 2 (*ε2/ε4*) and group 3 (*ε3/3*) (p=0.009) and @ between group 4 (*ε3/ε4*, *ε4/ε4*) and group 3 (*ε3/3*) (p=0.022). Group 1= (*ε2/ε2*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

Age was significantly different among the groups. Subjects in group 3 were significantly older than those in group 1 (p = 0.011), group 2 (p = 0.009) and group 4

(p = 0.022). Weight and height measures did not differ significantly between the four *APOE* genotype groups (p>0.05). There was no significant difference in the baseline concentrations of triglyceride, total cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol, glucose and insulin between the four *APOE* genotype groups.

4.2 Response of serum triglyceride, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, glucose and insulin concentrations to a fatty meal by APOE genotype group

4.2.1 Changes in triglyceride concentrations during a fat meal by *APOE* genotype groups

The postprandial triglyceride concentrations by *APOE* genotype group are presented in Table 4.2. The values are presented as medians (interquartile ranges).

Table 4.2. Fasting and postprandial triglyceride concentrations of subjects by *APOE* genotype group

Variables	intervals	Group 1 n=5	Group 2 n=8	Group 3 n=9	Group 4 n=9	P-value
TRIG (mmol/l)	0	0.69(0.56-0.81)	0.61(0.53-1.00)	0.67(0.28-0.87)	0.76(0.51-1.16)	0.553
	20	0.67(0.44-0.82)	0.56(0.43-.85)	0.79(0.53-.91)	0.91(0.68-1.22)	0.176
	40	0.66(0.41-1.19)	0.66(0.39-1.03)	0.94(0.74-1.04)	0.87(0.54-1.32)	0.522
	60	0.65(0.46-0.95)	0.61(0.42-.96)	0.94(0.70-.18)	1.07(0.78-1.72)	0.056
	120	0.81(0.58-1.16)	0.72(0.43-1.13)	0.95(0.82-1.08)	1.12(0.54-1.72)	0.654
	180	0.86(0.70-1.42)	0.82(0.59-1.07)	1.21(0.75-1.53)	1.09(0.92-1.76)	0.176
	240	0.99(0.66-2.08)	0.81(0.48-1.34)	0.99(0.60-1.45)	1.45(0.92-1.56)	0.262
	300	0.81(0.56-1.47)	0.84(0.62-1.06)	0.89(0.61-1.17)	0.92(0.78-1.48)	0.758
	360	0.58(0.50-1.04)	0.82(0.68-1.18)	0.82(0.55-1.02)	0.82(0.70-1.20)	0.433
AUC		288.10(216.75-468.00)	301.10(196.55-388.95)	374.50(246.35-423.10)	339.2(323.10-577.60)	0.319

P-value < 0.05 considered statistically significant

Group 1= (*ε2/ε2*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

There was no significant difference in the triglyceride concentrations and in the areas under the postprandial curve between the four apoE genotype groups at any of the time intervals ($p > 0.05$) after the fat load.

The general linear model of repeated measures was used to assess the within-the-group changes in concentrations of triglyceride after a fat-rich meal. No significant difference between the concentrations at baseline and at different time intervals within each group was observed. The results show that the *APOE* genotype does not have any effect on the triglyceride response to a fatty meal meal during the OFTT.

A graphic representation of the postprandial response of serum triglyceride to a fatty meal is presented in Figure 4.1. To plot the graph of triglyceride concentration versus time, means and standard deviations were used as required by Graph Pad software.

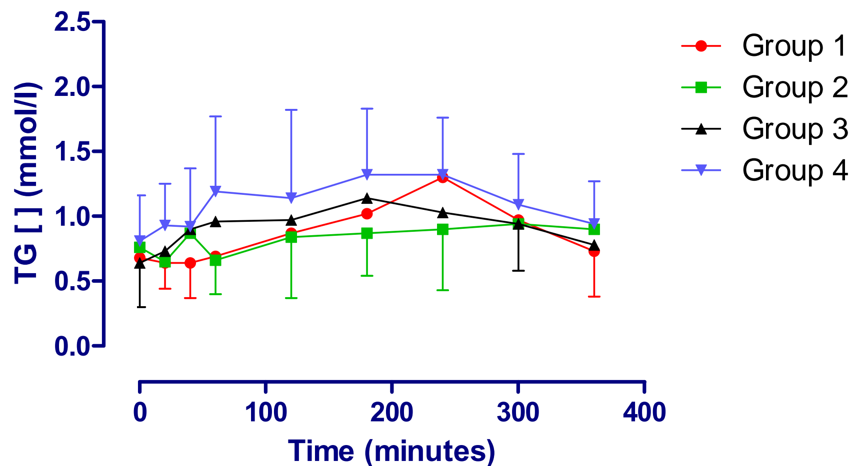

Figure 4.1. Postprandial triglyceride concentrations versus time by *APOE* genotype group. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group 1= (*ε2/ε2*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

4.2.2 Changes in serum total cholesterol, HDL cholesterol and LDL cholesterol concentrations during oral fat-tolerance test between the *APOE* genotype groups

There was no significant difference in the postprandial response of serum TC, HDL-C and LDL-C to the fatty meal. The tables of their respective results are presented in the Appendix 3. (Tables A1-A3).

Total cholesterol and LDL cholesterol showed a significant difference among the four *APOE* genotype groups ($p<0.05$) at the 300th minute but the difference was not confirmed by the Post-hoc analysis of Bonferroni test.

The general linear model of repeated measures was used to assess the within-the-group changes in concentrations of total cholesterol, HDL cholesterol and LDL cholesterol after a fat-rich meal (Results not shown). No significant difference between the concentrations at baseline and at different time intervals within each group was observed, indicating that the *APOE* genotypes had no effect on the response of total cholesterol, HDL cholesterol and LDL cholesterol to a fat meal during the OFTT.

Graphic representations of the postprandial response of serum total cholesterol, HDL cholesterol and LDL cholesterol to a fat meal is presented in Figures 4.2-4.4. Means and standard deviations were used to plot the graphs.

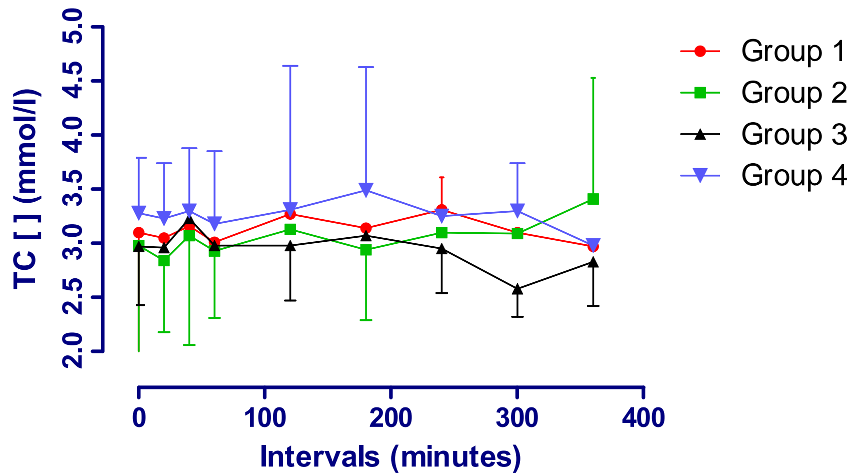

Figure 4.2. Postprandial total cholesterol concentration versus time by *APOE* genotype group. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group 1= (*ε2/ε2*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

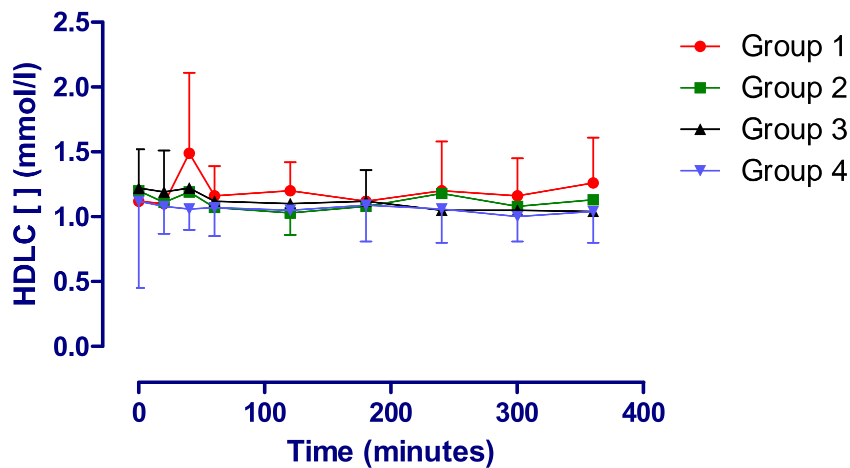

Figure 4.3. Postprandial high-density lipoprotein cholesterol versus time by *APOE* genotype group. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group 1= (*ε2/ε2*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

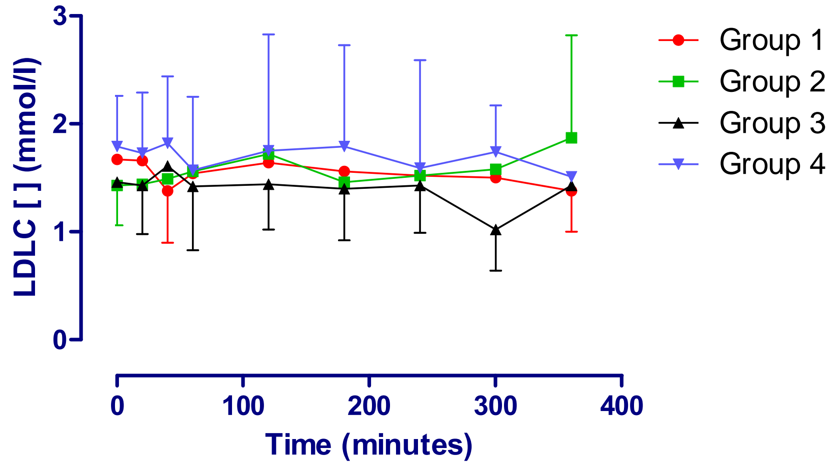

Figure 4.4. Postprandial low-density lipoprotein cholesterol concentration versus time by *APOE* genotype group. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group 1= (*ε2/ε2*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

4.2.3 Changes in glucose and insulin concentrations during a fat-tolerance test between the *APOE* genotype groups

The postprandial concentrations of glucose and insulin are presented in Tables 4.3 and 4.4 respectively. Glucose concentrations were presented as mean ± standard deviations while insulin concentrations were presented as medians and interquartile ranges.

Table 4.3. Fasting and postprandial glucose concentrations of subjects by *APOE* genotype group

Variables	intervals	Group 1 n=5	Group 2 n=8	Group 3 n=9	Group 4 n=9	P-value
GLUC (mmol/l)	0	4.61±0.53	4.68±0.27	4.47±0.44	4.47±0.67	0.784
	20	4.91±1.18	5.54±1.16	5.00±0.94	4.84±1.87	0.728
	40	4.91±1.17	5.24±0.87	4.71±0.86	4.88±0.94	0.712
	60	4.50±1.52	4.88±0.67	5.02±1.16	4.64±0.86	0.782
	120	4.29±1.09	4.76±0.42	4.67±0.81	4.70±0.99	0.782
	180	4.06±0.89	4.39±0.59	4.54±0.54	4.33±0.46	0.565
	240	4.39±0.51	4.63±0.36	4.34±0.57	4.43±0.42	0.629
	300	4.27±0.66	4.61±0.49	4.27±0.40	4.46±0.64	0.563
	360	4.35±0.31	4.51±0.45	4.39±0.26	4.30±0.47	0.726
AUC		1573.5±182.45	1696.56±73.63	1640.22±136.60	1628.19±190.64	0.548

P-value <0.05 considered statistically significant.

Group 1= (*ε2/ε2*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

Table 4.4. Fasting and postprandial insulin concentrations of subjects by *APOE* genotype group

Variables	interv	Group 1 n=5	Group 2 n=8	Group 3 n=9	Group 4 n=9	P-value
INS (mu/l)	0	9.20(3.64-16.69)	6.18(4.30-12.48)	6.67(4.15-10.00)	4.31(3.31-6.82)	0.390
	20	57.28(43.31-140.72)	54.02(30.22-87.48)	49.95(32.64-65.46)	48.10(34.34-62.84)	0.526
	40	28.71(18.54-51.17)	50.31(40.08-63.76)	30.57(19.45-38.78)	36.24(17.22-51.11)	0.119
	60	31.63(7.92-47.88)	48.06(27.23-72.40)	39.90(29.81-68.22)	37.95(12.26-42.23)	0.176
	120	22.04(4.42-27.10)	26.47(12.60-36.09)	24.37(8.54-32.74)	8.75(5.92-27.38)	0.463
	180	10.80(4.68-20.23)	11.82(8.34-14.41)	12.16 (6.60-26.84)	11.14(6.07-16.44)	0.921
	240	10.03(4.60-13.24)	7.52(6.95-10.17)	9.57 (4.91-12.12)	6.29(5.18-8.81)	0.550
	300	5.88(4.89-6.86)	5.78(4.40-7.19)	8.38 (5.76-10.59)	7.78(5.00-8.51)	0.431
	360	6.15(3.26-7.19)	6.42(3.86-8.21)	8.02 (5.70-11.42)	5.02(3.00-7.20)	0.541
AUC		6589.80 (3462.40-9321.25)	6878.40(5400.22-10415.82)	7111.00 (5323.45-9064.70)	5185.10(2995.60-8091.30)	0.534

P-value <0.05 considered statistically significant.

Group 1= (*ε2/ε2*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

There was no significant difference in the concentrations and areas under the postprandial curve of both the glucose and insulin among the four *APOε* genotype groups after a fat-rich meal (p>0.05).

The general linear model of repeated measures was used to assess the within-the-group change in concentrations of glucose and insulin after a fat-rich meal (Results not shown). No significant difference between the concentrations at baseline and at different intervals within each group was observed.

Graphic representations of the postprandial response of glucose and insulin after a fat meal are presented in Figures 4.5 and 4.6 respectively. Means and standard deviations were used to plot the graph of postprandial concentrations of insulin as required by the GraphPad Software.

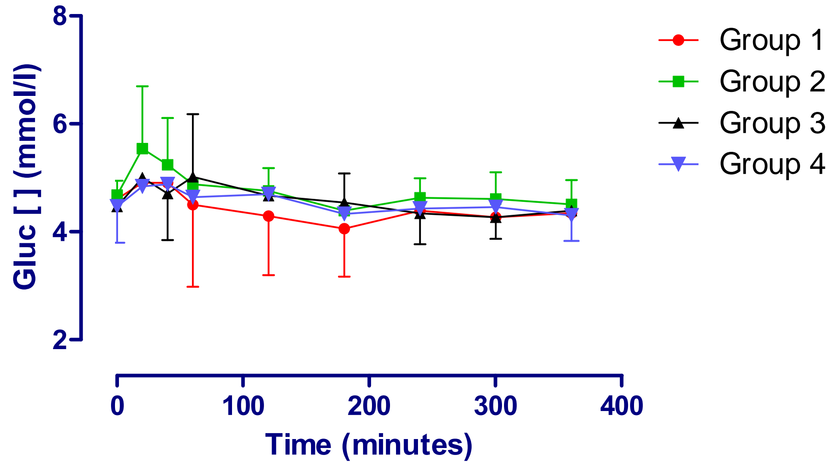

Figure 4.5. Postprandial glucose concentration versus time by *APOE* genotype group. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group 1= (*ε2/ε2*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

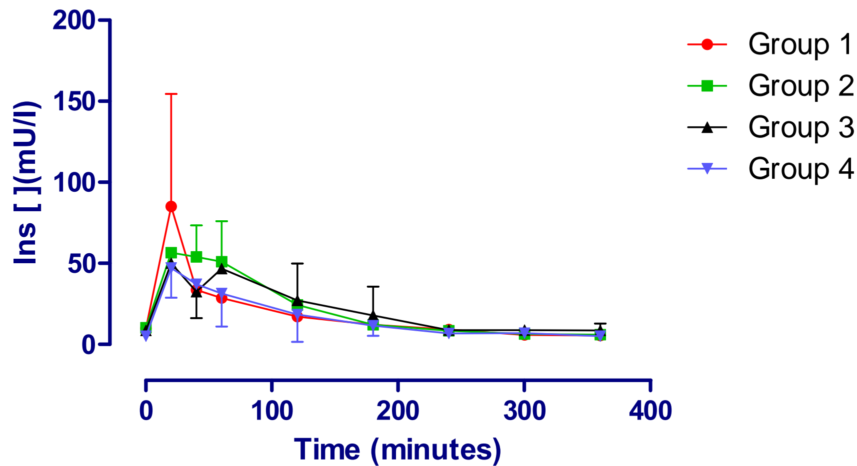

Figure 4.6. Postprandial insulin concentration vesus time by *APOE* genotype group. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group 1= (*ε2/ε2*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

4.3 Changes in serum lipids, glucose and insulin concentrations after subtraction of baseline concentrations among the four APOE genotype groups

Because fasting triglyceride concentration is a major determinant of postprandial lipid and lipoprotein levels [13], changes in concentrations of serum lipids, glucose and insulin between the four *APOE* genotype groups after a fat load were determined by subtracting baseline concentrations from concentrations at every time interval. The changes in concentrations did not differ significantly between the groups. The graphic representations of the change in concentrations of the biochemical parameters are presented in figures 4.7 - 4.12.

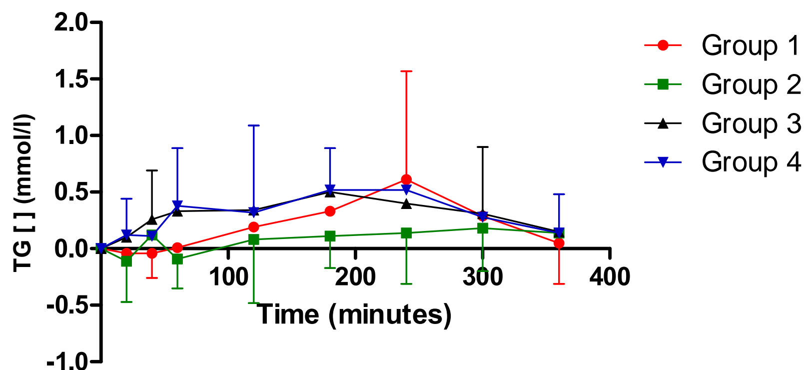

Figure 4.7 Change in triglyceride concentrations after subtraction of baseline concentrations by *APOE* genotype group. Error-bars indicate the standard error of mean (S.E.M). Overlapping error bars have been omitted for clarity. Group 1= (*ε2/ε2*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

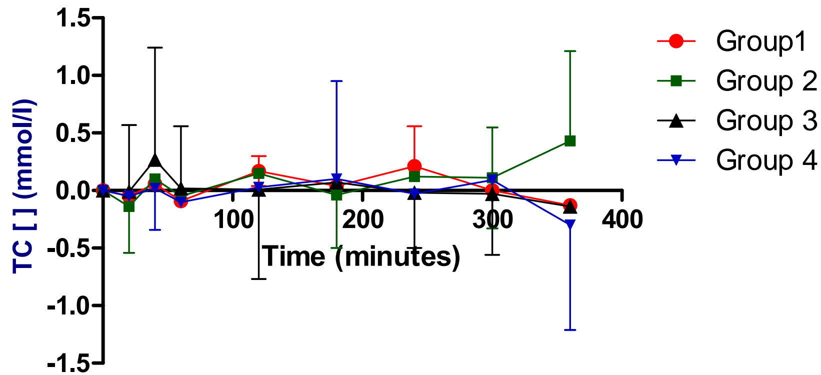

Figure 4.8. Change in total cholesterol concentrations after subtraction of baseline concentrations by *APOE* genotype group. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group 1= (*ε2/ε2*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

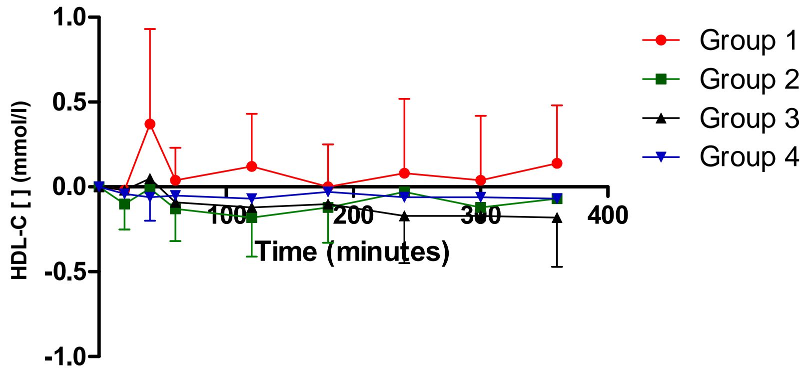

Figure 4.9. Change in high-density lipoprotein cholesterol concentrations after subtraction of baseline concentrations by *APOE* genotype group. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group 1= (*ε2/ε2*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

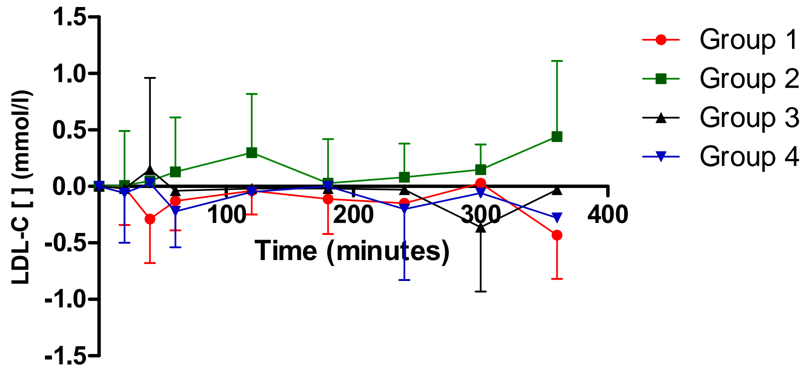

Figure 4.10. Change in low-density lipoprotein cholesterol concentrations after subtraction of baseline concentrations by *APOE* genotype group. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group 1= (*ε2/ε2*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

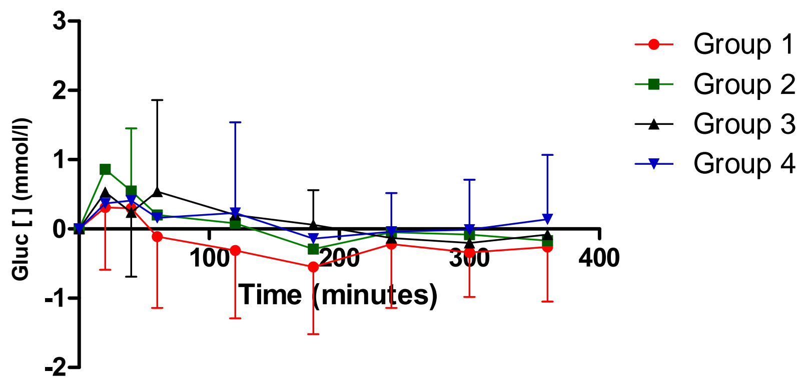

Figure 4.11. Change in glucose concentrations after subtraction of baseline concentrations by *APOE* genotype group. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group 1= (*ε2/ε2*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

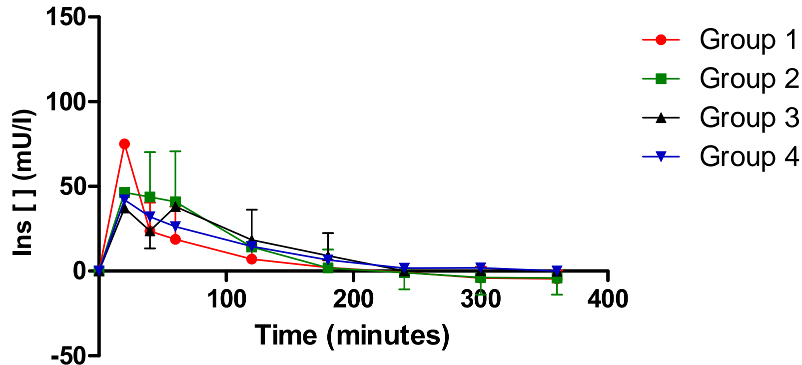

Figure 4.12. Change in insulin concentrations after subtraction of baseline concentrations by *APOE* genotype group. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group 1= (*ε2/ε2*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

4.4 Postprandial response of serum lipids, glucose and insulin to a fatty meal in subjects with and without the ε4 allele.

It has been reported that the presence of the *ε4* allele is associated with an impaired triglyceride-rich lipoprotein metabolism [24, 26, 103, 108] hence the decision to assess the effect of the *ε4* allele on the postprandial serum lipid . Subjects were grouped into group A (*ε2/ε2*, *ε2/ε3*. *ε3/3*) and group B (*ε3/ε4*, *ε4/ε4*) which are non- *ε4* and *ε4* allele groups respectively.

4.4.1 Baseline characteristics of subjects between the two *APOE* genotype groups

Anthropometric measures and serum concentrations of triglyceride, total cholesterol, HDL cholesterol, LDL cholesterol, glucose and insulin in the two groups [A (*ε2/ε2*, *ε2/ε3*. *ε3/ε3*) and group B (*ε3/ε4*, *ε4/ε4*)] are presented in Table 4.5. Concentrations are presented as mean ± S.D for total cholesterol, HDL cholesterol, LDL cholesterol and glucose and as median (interquartile ranges) for insulin and triglyceride.

Table 4.5. Anthropometric measures and baseline biochemical parameters between the two *APOE* genotype groups

Variables	Group A	Group B	P-values
	N=14	N=9	
Age (years)	21.86±1.99	20.78±1.09	0.154
Weight (kg)	64.64±7.85	67.59±9.96	0.438
Height (m)	1.66±0.07	1.66±0.10	0.818
BMI (kg/m^2)	23.49±1.98	24.48±3.88	0.429
BTRIG (mmol/l)	0.69(0.40-0.86)	0.76(0.51-1.16)	0.294
BTCHOL (mmol/l)	3.01±0.46	3.28±0.51	0.207
BHDL-C (mmol/l)	1.18±0.30	1.12±0.17	0.560
BLDL-C (mmol/l)	1.53±0.42	1.79±0.47	0.181
BGLUC (mmol/l)	4.52±0.46	4.47±0.67	0.840
BINS (mmol/l)	7.94(4.10-11.66)	4.31(3.31-6.82)	0.092

P-value <0.05 considered statistically significant. Group A= (*ε2/ε2*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

There were 14 and 9 subjects in group A and group B respectively. The *ε2/ε4* subjects were excluded because it is regarded as a conflicting genotype, since both *ε2* and *ε4* genes are reported to display a dominant characteristic [11]. There was no significant difference in age, anthropometric measures (weight, height, BMI) and baseline concentrations of serum lipids (triglyceride, total cholesterol high-density lipoprotein cholesterol and low-density lipoprotein cholesterol), glucose and insulin between the two *APOE* genotype groups.

4.4.2 Changes in concentrations of serum lipids, glucose and insulin after a fat-meal between the two *APOE* genotype groups

The response of serum triglyceride, glucose and insulin to a fatty meal is presented in Tables 4.6-4.8. Concentrations are presented as mean ± standard deviations for triglyceride and glucose and as medians and interquartile ranges for insulin.

Table 4.6. Triglyceride response to a fatty meal in the two *APOE* genotype groups

		Group A n=14	Group B n=9	
Variables	Intervals			P-values
TRIG (mmol/l)	0	0.69(0.40-0.86)	0.76(0.51-1.16)	0.294
	20	0.71(0.50-0.86)	0.91(0.68-1.22)	0.088
	40	0.88(0.60-1.02)	0.87(0.54-1.32)	0.709
	60	0.91(0.64-1.08)	1.07(0.78-1.72)	0.204
	120	0.95(0.78-1.06)	1.12(0.54-1.72)	0.753
	180	0.99(0.71-1.52)	1.09(0.92-1.76)	0.224
	240	0.99(0.60-1.40)	1.45(0.92-1.56)	0.218
	300	0.86(0.57-1.14)	0.92(0.78-1.48)	0.316
	360	0.77(0.54-1.02)	0.82(0.70-1.20)	0.175
AUC		329.95(220.28-419.55)	339.20(323.10-577.60)	0.183

P-value <0.05 considered statistically significant. Group A= (*ε2/ε2*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

Table 4.7. The glucose response to a fatty meal in the two *APOE* genotype groups

		Group A n=14	Group B n=9	
Variables	Intervals			P-values
Gluc (mmol/l)	0	4.52±0.46	4.47±0.67	0.294
	20	4.97±0.99	4.84±1.87	0.088
	40	4.78±0.94	4.88±0.94	0.709
	60	4.83±1.27	4.64±0.86	0.204
	120	4.53±0.89	4.70±0.99	0.753
	180	4.36±0.69	4.33±0.46	0.224
	240	4.36±0.53	4.43±0.42	0.218
	300	4.27±0.48	4.46±0.64	0.316
	360	4.38±0.27	4.30±0.47	0.175
AUC		1616.40±151.08	1628.19±190.64	0.871

P-value <0.05 considered statistically significant. Group A= (*ε2/ε2*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

Table 4.8. The insulin response to a fatty meal in the two *APOE* genotype groups after a fat meal

		Group A n=14	Group B n=9	
Variables	Intervals			P-values
INS (mU/l)	0	7.94(4.10-11.66)	4.31(3.31-6.82)	0.092
	20	50.20(38.90-71.18)	48.10(34.34-62.84)	0.520
	40	29.64(19.80-37.97)	36.24(17.22-51.11)	0.870
	60	38.78(23.55-58.61)	37.95(12.26-42.23)	0.517
	120	22.76(7.31-58.61)	8.75(5.92-27.36)	0.495
	180	11.48(5.76-17.60)	11.14(6.07-16.44)	0.715
	240	9.66(4.74-11.99)	6.29(5.18-8.81)	0.256
	300	6.86(5.52-8.64)	7.78(5.00-8.51)	0.635
	360	7.54(3.96-10.56)	5.02(3.00-7.26)	0.318
AUC		6850.80(4473.48-8929.90)	5185.10(2995.60-8091.30)	0.871

P-value <0.05 considered statistically significant. Group A= (*ε2/ε2*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

There was no significant difference in the response of triglyceride, glucose and insulin to a fatty meal between the two *APOE* genotype groups.

There was also no significant difference in the postprandial concentrations and areas under postprandial curves (AUCøs) of TC, HDL-Cand LDL-C. The results are presented in Appendix 3 (Tables B1-B3). Total cholesterol and LDL cholesterol showed a significant difference between the two *APOE* genotype groups ($p<0.05$) at the 300th minute but the difference was not confirmed by the Post-hoc analysis of Bonferroni test ($p>0.05$).

Graphic representations of concentrations of triglyceride, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, glucose and insulin are presented by Figures 4.13-4.18. Means ± standard deviations were used to

plot the graphs representing postprandial concentrations of triglyceride and insulin as required by GraphPad Software.

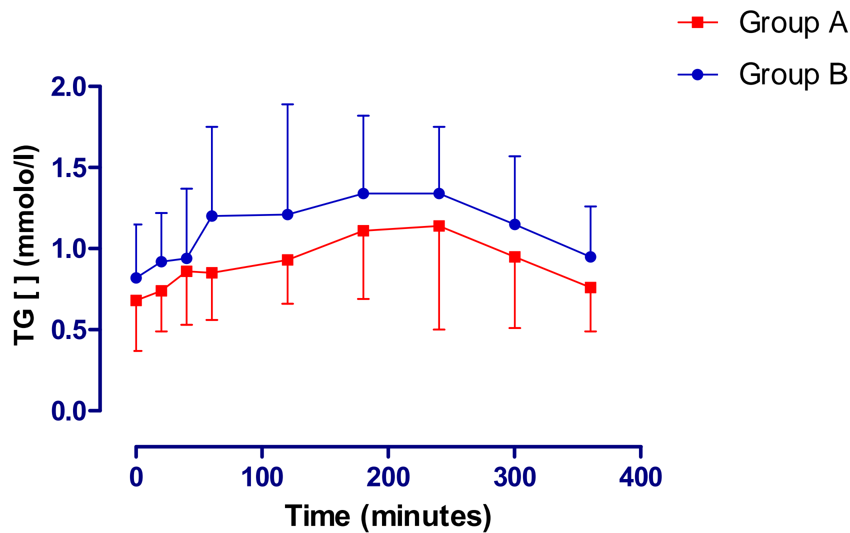

Figure 4.13. Postprandial TG concentration versus time between the two *APOE* genotype groups. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group A= (*ε2/ε2*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

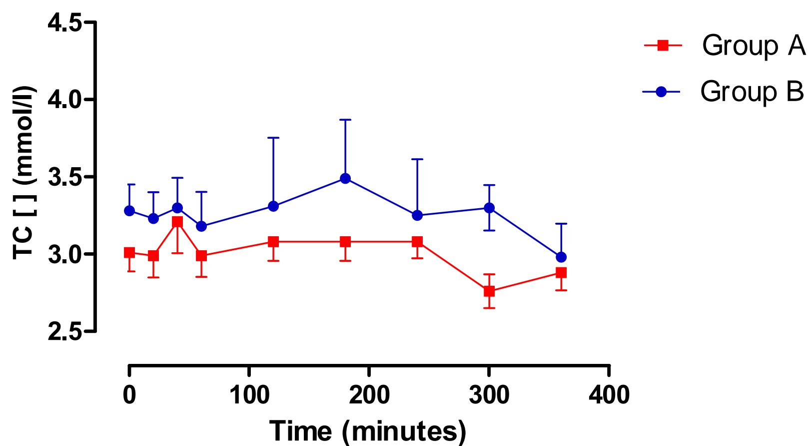

Figure 4.14. Postprandial TC concentration versus time between the two *APOE* genotype groups. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group A= (*ε2/ε2*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

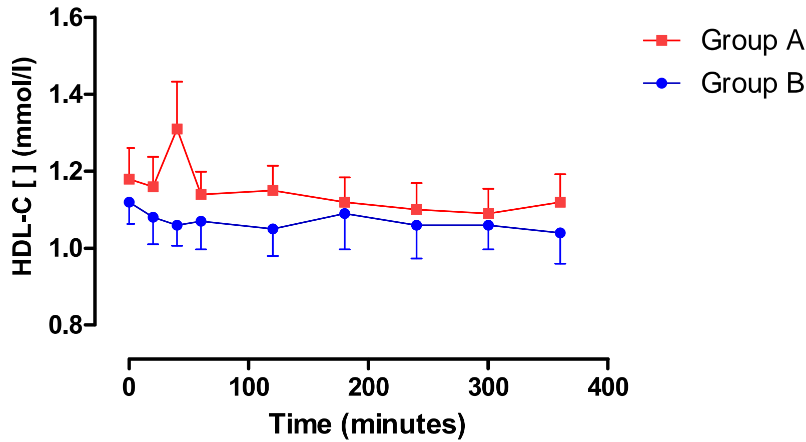

Figure 4.15. Postprandial HDL-C concentration versus time between the two *APOE* genotype groups. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group A= (*ε2/ε2*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

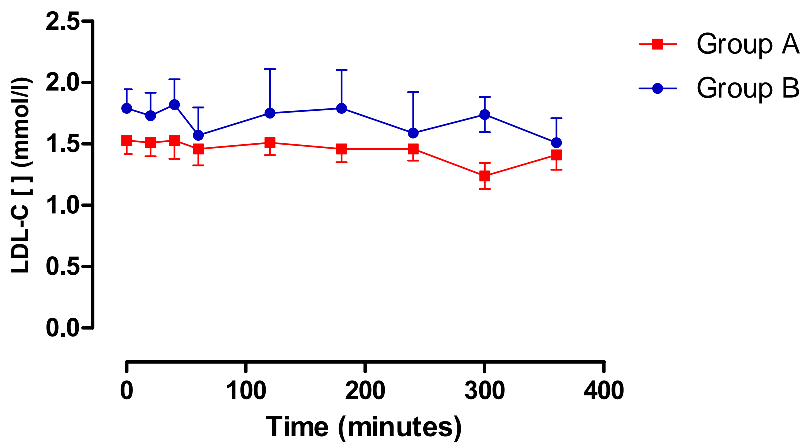

Figure 4.16. Postprandial LDL-C concentration versus time between the two *APOE* genotype groups. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group A= (*ε2/ε2*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

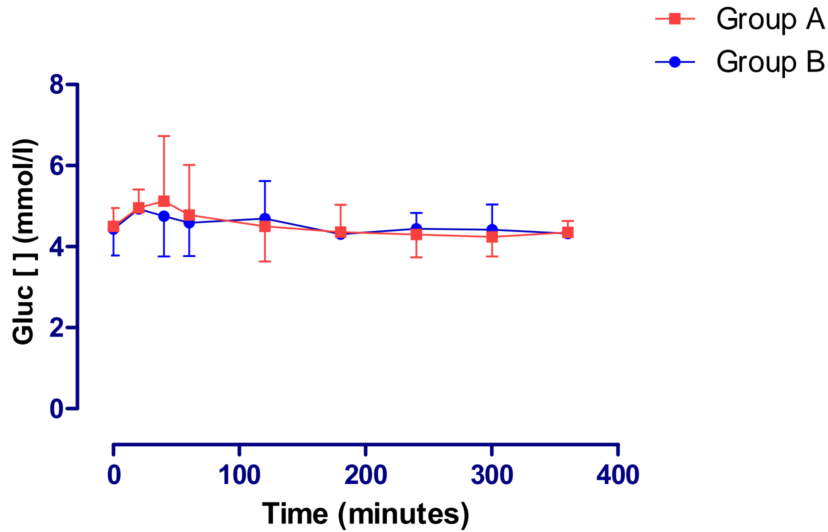

Figure 4.17. Postprandial GLUC concentration versus time between the two *APOE* genotype groups. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group A= (*ε2/ε2*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

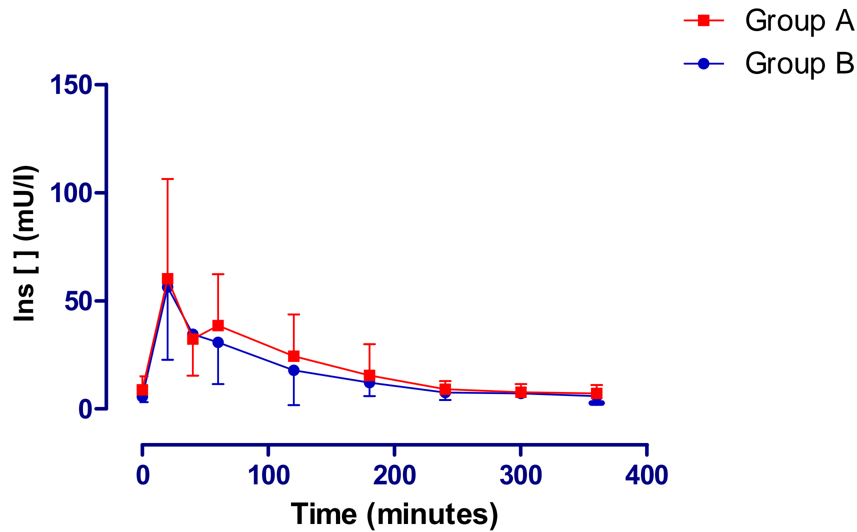

Figure 4.18. Postprandial insulin concentration versus time between the two *APOE* genotype groups. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group A= (*ε2/ε2*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

4.4.3 Changes in concentrations of serum lipids, glucose and insulin after subtraction of baseline concentrations by *APOE* genotype group

Changes in concentrations of serum lipids, glucose and insulin did not differ significantly between the two groups.

The graphic representation of changes in concentrations after subtraction of baseline values are presented in figures 4.19 - 4.24..

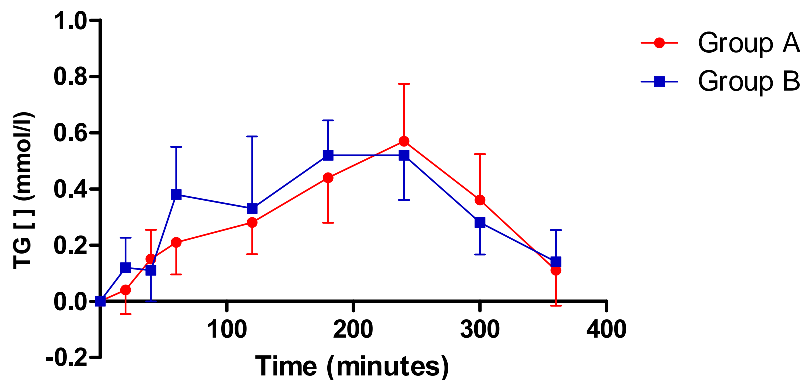

Figure 4.19. Change in triglyceride concentrations after subtraction of baseline concentrations in the two *APOE* groups. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group A= (*ε2/ε2*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

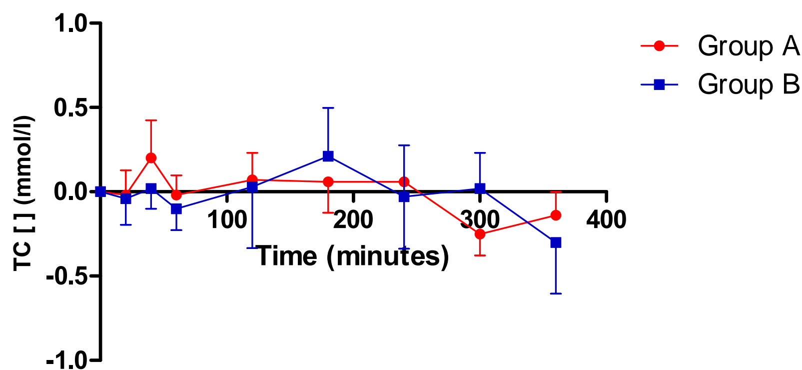

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Figure 4.20. Change in total cholesterol concentrations after subtraction of baseline concentrations in the two *APOE* groups. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group A= (*ε2/ε2*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

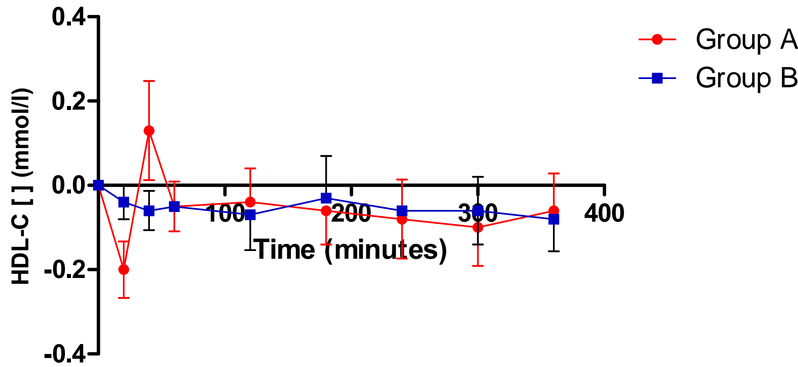

Figure 4.21. Change in high-density lipoprotein cholesterol concentrations after subtraction of baseline concentrations in the two *APOE* groups. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group A= (*ε2/ε2*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

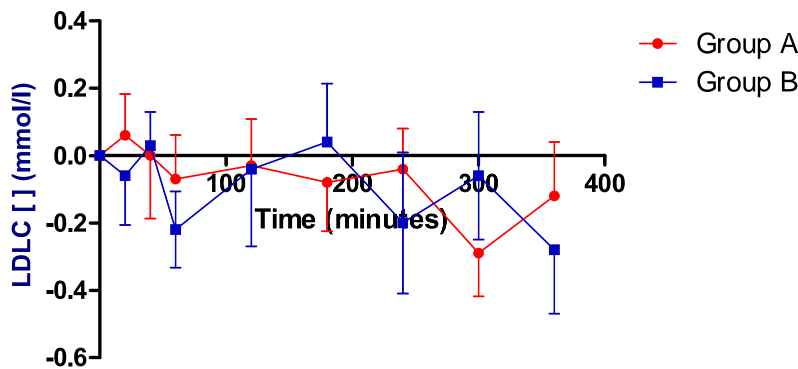

Figure 4.22. Change in low-density lipoprotein cholesterol concentrations after subtraction of baseline concentrations in the two *APOE* groups. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group A= (*ε2/ε2*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

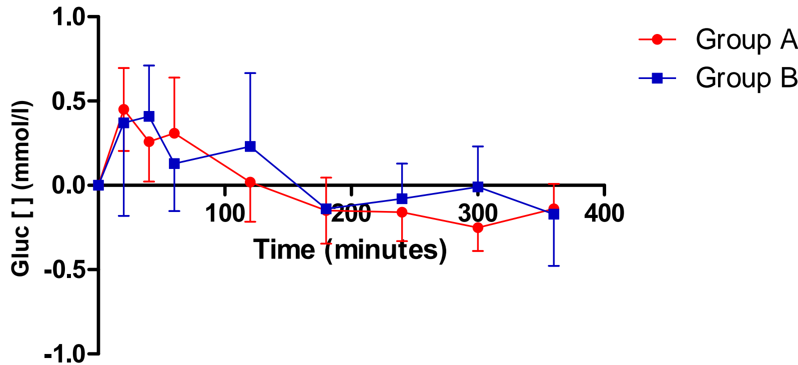

Figure 4.23. Change in glucose concentrations after subtraction of baseline concentrations in the two *APOE* groups. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group A= (*ε2/ε2*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

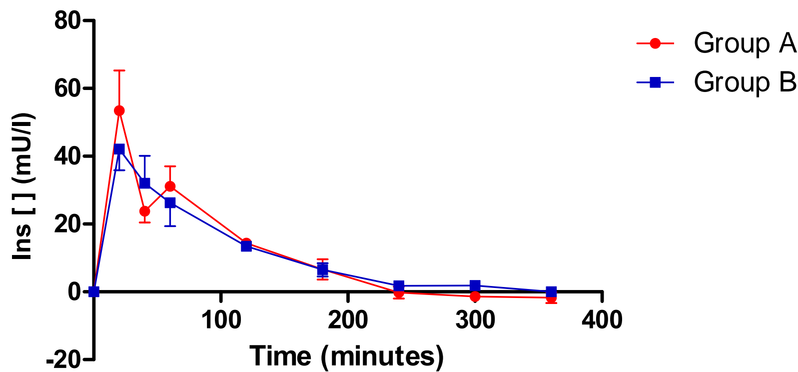

Figure 4.24. Change in insulin concentrations after subtraction of baseline concentrations in the two *APOE* groups. Error-bars indicate the standard error of mean (S.E.M). Overlapping error-bars have been omitted for clarity. Group A= (*ε2/ε2*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

According to the current results, the hypothesis that triglyceride levels will increase after a fat meal in black subjects with the *ε4* allele can be rejected.

5 DISCUSSION

In the present study concentrations of serum triglyceride, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, glucose and insulin were assessed both at baseline and after a fatty meal in individuals with different *APOE* genotypes. Subjects were grouped into four genotype groups: group 1 (*ε2/ε2*, *ε2/ε3*), group 2 (*ε2/ε4*), group 3 (*ε3/ε3*) and group 4 (*ε3/ε4, ε4/ε4*).

a. APOLIPOPROTEIN E GENOTYPES AND BASELINE SERUM LIPID LEVELS

Baseline serum lipid levels in the present study were similar to values reported by a study conducted in a Black South African population and lower than those reported in Caucasians [27]. The students at the University of Limpopo are mainly from rural areas and their lipid levels may be a reflection of their adherence to a traditional low fat diet [27] Baseline serum lipid levels in the present study did not differ significantly between the four *APOE* genotype groups. Environmental factors such as diet and lifestyle of participants might have contributed to the outcome of the present study. It has been established that gene-environment interactions have an effect on the association between serum lipids and *APOE* genotypes [15, 85, 86].

The results of the present study are in agreement with a study on healthy Native American subjects residing in a rural area following their traditional lifestyle [97]. No difference in LDL-C concentrations between the E3/E3 and E3/E4 subjects was observed, while subjects with the E2/E3 genotype had lower LDL-C levels. Similar results were obtained for cholesterol.The authors concluded that the effect of the *ε4* allele does not adversely affect plasma lipid parameters in physically active subjects consuming their traditional diet which is low in fat and high in carbohydrates, but that the effect may be expressed once the subjects resort to a sedentary lifestyle, become obese or are diabetic.

A lack of association between serum lipids and *APOE* genotypes have also been been reported in other population studies [90, 91, 100].

Environmental factors such as diet and lifestyle of participants in the present study may have contributed to the lack of an association between APOE genotypes and serum lipids [15, 84,86]

The results are however contrary to previous reports that have shown an effect of *APOE* genotypes on serum lipid parameters [16, 18, 88, 161, 162, 163]. In these studies serum total cholesterol and low-density lipoprotein cholesterol have been found to be higher in subjects with the ε4 allele and lower in subjects with the ε2 allele compared to subjects with the *ε3* allele. The same studies reported higher levels of high density lipoprotein cholesterol in subjects with the ε2 allele and lower levels in those with the *ε4* allele compared to subjects with the *ε3* allele.

b. APOLIPOPROTEIN E (*APOE)* GENOTYPES AND BASELINE GLUCOSE AND INSULIN LEVELS

Baseline glucose and insulin levels also did not differ significantly between the groups. Elosua et al. (2003) determined whether or not the association between *APOE* genotypes and fasting glucose and insulin levels is modulated by obesity in male and female subjects. Higher glucose and insulin levels were observed in obese men with the ε4 allele compared to those with the *ε3* allele. No association was found between *APOE* genotype and glucose and insulin levels in non-obese men. In obese women no association between *APOE* genotype and fasting glucose and insulin levels was found [164].

Lower levels of fasting glucose and insulin were reported in non-Hispanic whites and Mexican-Americans with the *ε2/*3 genotype compared to subjects with the ε3/4 genotype [165].

c. APOLIPOPROTEIN E GENOTYPES AND POSTPRANDIAL SERUM LIPID LEVELS

- **Apolipoprotein E genotypes and postprandial triglyceride concentrations**

Studies have reported a greater and prolonged postprandial hypertriglyceridaemia in patients with familial combined hyperlipidaemia with the *ε4* allele compared to those without the *ε4* allele [24, 164]. Several other population studies have also reported an increased and prolonged postprandial triglyceride response to a fatty meal in subjects with the *ε4* allele compared to those without the *ε4* allele [106, 110, 167]. Some studies reported higher levels of postprandial triglyceride levels in healthy individuals with the ε2 allele compared to those with the ε3 allele [1, 26, 168,]. The explanation given was that apoE2 isoform has lower affinity and thus a slower uptake of TRLøs while apoE4 isoform have higher affinity and thus a faster uptake of TRLøs by the hepatic LDL receptors.

The clearance of triglyceride-rich lipoproteins has also been reported to be delayed in subjects with *ε3/ε4* genotype compared to subjects with *ε3/ε3* genotype, which suggests that the ε4 allele is responsible for the impaired metabolism of lipoproteins [26, 106, 110]. Compared to subjects with the ε3/ε3 allele, subjects with the ε4 allele were found to have higher postprandial area under the curve [25].

No significant statistical difference in triglyceride levels after the fatty meal was observed between the apoE genotype groups in the present study. However there was a trend to higher triglyceride levels after the fatty meal in groups 3 and 4.The lack of significant difference may be because of the small sample size. There was also no significant difference in postprandial triglyceride concentrations between the ε4 and non- ε4 allele groups.

The absence of a significant increase in postprandial triglyceride concentrations in the present study could be due to increased lipoprotein lipase activity which enhances the hydrolysis of serum triglycerides. It has been reported that Black subjects have a higher lipoprotein lipase activity compared to white subjects [169]

Why no difference in the response of triglycerides to a fatty meal was observed between the *APOE* genotype groups in the present study is not known but could be due to several factors. Fasting serum triglyceride concentrations in the present study did not differ significantly between the groups. A linear relationship between baseline concentrations of serum triglyceride levels and the postprandial response has been observed, where high baseline concentrations have been associated with a pronounced and exaggerated postprandial lipaemia [2, 13, 134, 170]. The authors reported that the response of serum triglycerides to a fatty meal will differ significantly if the baseline concentrations of triglyceride differed significantly between the groups. Furthermore baseline serum triglyceride levels in the present study were low which might explain why no pronounced postprandial triglyceride levels were observed. Studies have shown that postprandial triglyceridaemia is positively associated with fasting triglyceride levels [2, 133, 170].

The inconsistency of reports regarding association between *APOE* genotypes and the postprandial triglyceride response may also be the lack of standardization of the test meal and postprandial lipaemia, making it difficult to compare studies [171, 172, 173, 172]. Study designs, composition of test meals and blood sampling frequencies differ from one study to another and might explain the difference in results reported [25]. Some studies measured TG concentrations every hour [173] and others after every two hours [175, 177].

A recent study suggested that for standardized postprandial testing a single OFTT meal should be given after an 8 hour fast and should consist of 75g of fat, 25g of carbohydrates and 10g of proteins [177]. The authors also shared a view that a single triglyceride measurement 4 hour after OFTT meal provides a good evaluation of the postprandial triglyceride response and that preferably subjects with fasting triglyceride levels of 1-2 mmol/l should be tested.

The results of the present study are in agreement with a study of young adults where no association was observed between *APOE* genotypes and postprandial serum triglyceride concentrations [162]. Other studies have also reported that *APOE* genotypes have no influence on the postprandial triglyceride resonse [14, 178].

- **Association of *APOE* genotypes with total cholesterol, low-density lipoprotein cholesterol and high density lipoprotein cholesterol concentrations after a fat load.**

Changes in concentrations of total cholesterol, low density lipoprotein cholesterol and high density lipoprotein cholesterol after a fatty meal were not significant in any of the groups. Similar results were observed even after dividing the subjects into the $\varepsilon4$ and non- $\varepsilon4$ allele groups (Appendix 3). The areas under postprandial curves did not differ significantly between the groups.

It has previously been reported that the effects of *APOE* gene polymorphism on the response of serum lipids to a fatty meal may be more pronounced when baseline concentrations of cholesterol and LDL-cholesterol differ significantly between subjects with different *APOE* genotypes [114], which was not the case in the present study. Again factors like difference in the methods or dietary protocols used, the number of subjects and their characteristics have also been reported to affect the response of serum cholesterol to a fatty meal hence the contradicting results [179].

Dietary fat quality and amount have been shown to be important factors in the determination of postprandial cholesterolaemia [114, 179]. In the study of individuals on their habitual diet who were given two test meals of different fat composition, serum cholesterol response was higher after a diet containing saturated fat compared to a meal with high cholesterol [114]. The authors found that normolipidaemic subjects with the $\varepsilon3/\varepsilon4$ or $\varepsilon4/\varepsilon4$ genotypes had a greater response of LDL cholesterol to saturated fat than those with the $\varepsilon3/\varepsilon3$ genotype. In relation to HDL-C, there was a gender-specific association between *APOE* genotypes and HDL-C where men with either $\varepsilon3/\varepsilon4$ or $\varepsilon4/\varepsilon4$ genotype had smaller postprandial concentrations of HDL-C than men with the $\varepsilon2/\varepsilon3$ genotype after ingestion of a meal containing dietary cholesterol [114].

Some studies have reported that subjects with the $\varepsilon4$ allele were more responsive to changes in the amount of dietary cholesterol than subjects without the $\varepsilon4$ allele [180, 181]. However, other studies did not find any difference in the respose of serum cholesterol to dietary cholesterol between subjects with and without the $\varepsilon4$ allele [24, 182, 183]. The explanation to these differences in results have been suggested to be

the sample size and gender, where men have been reported to have a greater response than women and to baseline serum concentrations of total cholesterol and LDL cholesterol which did not differ between the groups [114].

The difference between the results of the present study and studies that reported positive associations between serum lipid concentrations and *APOE* gene genotypes may be explained by several factors. Most studies but not all were conducted on Western populations consuming a high-fat diet. In some of the studies the subjects were having some metabolic abnormalities such as hyperlipidaemia [24], hypercholesterolaemia [179], diabetes, obesity [165], history or onset of myocardial infarction, CAD [186], CHD [106], metabolic syndrome [107], or over 40 years of age [24, 25, 29, 97].

- **Association of *APOE* genotypes with glucose and insulin concentrations after a fat load**

The change in glucose and insulin concentrations after a fatty meal in the present study was not significant in any of the APOE genotype groups. As the test meal was a mixed meal containing fat, carbohydrate (fibre) and protein no pronounced increase in glucose levels was observed. Insulin levels reached a peak at 20min in all groups and no difference in the concentrations was observed between the APOE genotype groups.

There is a scarcity of data on the association between glucose and insulin responses to a fat meal and *APOE* genotypes. In the study on 51 CHD patients and 164 controls, Dart et al. (1997) reported that the presence of the $\varepsilon 4$ allele was associated increased postprandial response of glucose but not insulin. The significant peak was reached after 2 hours [106]. In another study in the United Kingdom where meals with different fatty acid composition were administered and compared to the habitual diet, the postprandial glucose and insulin concentrations changed significantly but the effect of *APOE* genotypes was not assessed [185].

The present study was however in agreement with a study on 17 healthy normolipidaemic men in Austria where no significant increases in either glucose or insulin were noticed during a 16 hour postprandial period [187].

The limitation of the present study is the small sample size, which may have lacked power to detect any significant difference in the measured variables between the groups. Several other studies were conducted with small sample sizes [13 (n=25), 14 (n=14), 110(n=16), 113 (n=36), 158 (n=25), 187 (n=20), 188 (n=34), 189 (n=28), 190 (n=24), 191 (n=15), and 192 (n=9)]. The other limitation is that female subjects were not screened for contraceptive use which has been reported to influence serum lipid metabolism. A further limitation may be that the present study had fewer male (n=10) than female (n=20) participants. It has been reported that the effect of the $\varepsilon 4$ allele was higher and more pronounced in men than in women [1068].

In conclusion, *APOE* genotypes did not have any effect on the baseline and postprandial serum lipid, glucose and insulin concentrations in our study of young and apparently healthy Black South African subjects. However the results of the present study must be interpreted with caution, considering the limitations. Larger studies on the association between *APOE* genotypes and serum lipid response to a fatty meal are needed. The results of the present study may however be used as a baseline for future larger scale studies.

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APPENDIX I. PREPARATION OF SOLUTIONS

a. Preparation of TE buffer

1) Prepare 1M Tris (PH 7.6)

Mix 30.28g Tris

200 ml distilled water

Adjust PH to 7.6

Fill up to 250 ml

Autoclave

2) Prepare 0.5M EDTA

Mix 18.61g EDTA-Na_2

800 ml distilled water

Adjust the PH to 8.0

Fill up to 1000 ml

3) TE buffer

Mix 500 µl 1M Tris

100 µl 0.5M EDTA

Fill up to 50ml with distilled water

b. Preparation of 10X TBE buffer

108g Tris

55g Boric Acid

9.3g Na_2EDTA

Add distilled water up to 1 litre (PH 8.0-8.3)

c. Preparation of 1X TBE buffer

Mix 100 ml of 10X TBE buffer with 900 ml distilled water

d. Preparation of loading buffer II

Mix 0.025g Bromophenol blue

0.025g xylene cyanol

40g sucrose

100ml distilled water

Aliquot in 5ml tubes and store at 4-8°C

e. Preparation of Ethidium Bromide dye

Add 0.1g EtBr (lyophilized) to 10 ml distilled water

Store in aliquots of tubes in the dark

Then mix 150 µl ethidium bromide solution with 200 ml 1X TBE buffer

f. Preparation of Reagent A

Add 400 milliliters of Reagent A into 1600 milliliters of distilled water to a final volume of 2 liters.

g. PCR Cocktail preparation

Master Mix was prepared by adding the following to the eppendorf tube in the order they appear with.

5ml PCR buffer

5ml DMSO

1 µl dNTP

1 µl Primer (forward)

1 µl Primer (reverse)

21 µl Distilled water

15 µl of DNA (into 7 of 8 tubes, distilled water in the 8th tube to check for further contamination)

1 µl Taq polymerase (just before running) into master mix

35 µl of master mix into each tube

h. Agarose gel preparation

2% (2g) agarose gel powder was weighed on a weighing scale and transferred into a 250ml conical flusk containing 98ml 1X TBE buffer solution. The solution was mixed using a magnetic stirrer on a hotplate until completely dissolved. The conical flask was then closed and put in a microwave oven until it bubbled up. As big bubbles

appeared, the conical flask was removed from microwave oven and put in a beaker of boiling water until all the bubbles disappeared. The flask was then cooled at room temperature for approximately 2 minutes and then transferred into cellotaped gel trays. The combs were loaded on the gel and the gel was cooled was cooled for approximately 30 minutes and then put into a gel chamber filled with 1X TBE buffer solution.

i. MetaPhore gel preparation

6% (6g) Meta-Phoreagarose gel powder was weighed using a weighing scale and was transferred into a 250ml conical flask containing 100ml 1X TBE buffer solution. The solution was mixed using a magnetic stirrer on a hotplate until completely dissolved (no lumps formed). The conical flask was then closed and put in a microwave oven until the gel solution bubbled up. As big bubbles appeared, the conical flask was removed from microwave oven and put in a beaker of boiling water until all the bubbles disappeared. The flask was then cooled at room temperature for approximately 2 minutes and then transferred into cello-taped gel trays. The combs were loaded on the gel and the gel was cooled was cooled for approximately 30 minutes and then put into a gel chamber filled with 1X TBE buffer solution.

APPENDIX II: METHODS AND PRINCIPLES

a. Determination of serum creatinine levels

Principle

Creatinine + Picrate → Red complex

Fixed time analysis, colorimetric methodology based on the reaction of creatinine with picric acid under alkaline conditions.

The increase in absorbance due to the red complex is proportional to the creatinine concentration in the sample. Absorbance measurements were taken at 510nm.

Composition:

Creatinine R1 contains: Sodium hydroxide
Creatinine R2 contains: Picric acid

b. Determination of blood Glucose levels

Principle:

B-D-Glucose + O_2 glucose oxidase → Gluconic acid + H_2O_2

$2H_2O_2$ + Phenol + 4- Aminoantipyrine peroxidase → red quinoneimine + $4H_2O$

The increase in absorbance generated by the red dye is proportional to the glucose concentration in the sample.

The absorbance was measured at 510nm.

Composition:
Glucose reagent contains: Glucose oxidase, Peroxidase, Phenol, 4- Aminoantipyrine, Phosphate buffer and less than 0.1% of sodium azide.

c. Determination of serum HDL-Cholesterol levels

Principle

The HDL-cholesterol assay is a homogeneous, direct method for measuring levels of HDL-C without the need for sample pre-treatment.

The Anti-human -lipoprotein antibody in R1 binds to lipoproteins (LDL, VLDL AND chylomicrons) other than HDL. The antigen-antibody complexes formed block enzyme reaction with all lipoproteins except HDL-C when R2 is added.

Cholesterol esterase and cholesterol oxidase react only with HDL-C.

Hydrogen peroxide produced by enzyme reactions with HDL-C yields a blue colored complex upon oxidative condensation of the chromogen. The concentration of the blue colored complex is proportional to the concentration of HDL-C.

The absorbance was taken at 620 nm.

Composition:

HDL-R1 contains: Good`s buffer pH 7.0, 4-aminoantipyrine, Peroxidase, Ascorbate oxidase, Anti human -lipoprotein antibody and preservatives.

HDL-R2 contains: Good`s buffer pH 7.0, Cholesterol esterase, Cholesterol oxidase, N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3, 5-dimethoxy-4-fluoroaniline.

d. Determination of Insulin

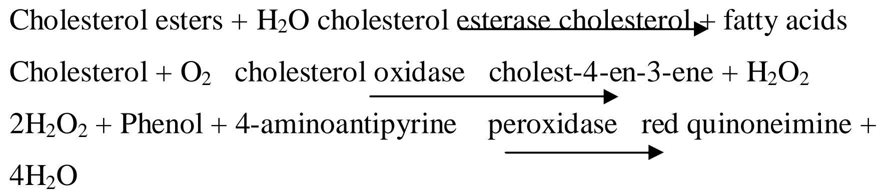
Principle

This is a one step immune-enzymatic (sandwich) assay. A sample containing insulin was added to a reaction vessel along with mouse monoclonal anti-insulin alkaline phosphatase conjugate and paramagnetic particles with coated mouse monoclonal anti-insulin antibody. The serum or plasma insulin bound to the antibody on a solid phase, while conjugate reacts with a different antigenic site on the insulin molecule. Separation in a magnetic field and washing removed materials not bound to the solid phase. A chemiluminiscent substrate, lumi-phosa 530, was added to the reaction vessels and light generated by the reaction was measured with a luminometer. The photon production was found to be proportional to the amount of conjugate bound to

the solid support. The amount of analyte in the sample was determined by means of stored, multi-point calibration curve. This method measured samples within the range of 0.003-300µU/ml. Three control sera (level 1, level 2, and level 3) were included in each run.

e. Determination of serum total cholesterol levels

Principle



Cholesterol esters + H_2O cholesterol esterase → cholesterol + fatty acids

Cholesterol + O_2 cholesterol oxidase → cholest-4-en-3-ene + H_2O_2

$2H_2O_2$ + Phenol + 4-aminoantipyrine peroxidase → red quinoneimine + $4H_2O$

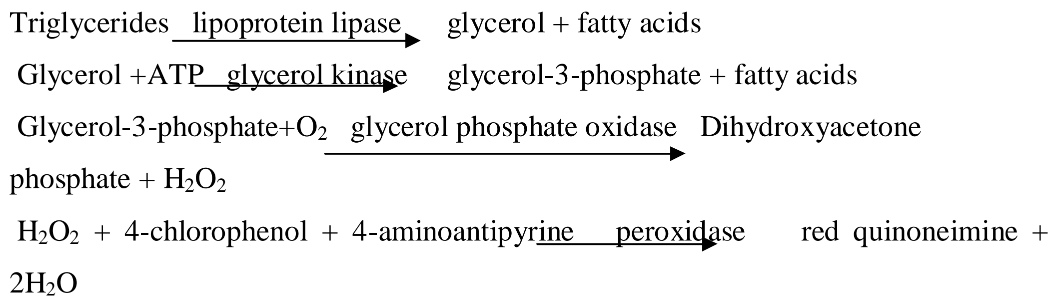
The increase in absorbance as generated by the red dye is proportional to the cholesterol concentration in the sample. Absorbance measurement was taken at 510nm.

Composition:

Total cholesterol reagent contains: Cholesterol esterase, Cholesterol oxidase, Peroxidase, Phenol, 4-aminoantipyrine, Tris buffer.

f. Determination of serum triglyceride levels

Principle



Triglycerides lipoprotein lipase → glycerol + fatty acids

Glycerol +ATP glycerol kinase → glycerol-3-phosphate + fatty acids

Glycerol-3-phosphate+O_2 glycerol phosphate oxidase → Dihydroxyacetone phosphate + H_2O_2

H_2O_2 + 4-chlorophenol + 4-aminoantipyrine peroxidase → red quinoneimine + $2H_2O$

The concentration of the red colored complex is proportional to the concentration of triglyceride.

The absorbance was taken at 510 nm.

Composition:

Triglyceride reagent contains: Lipoprotein lipase, Glycerol kinase, Glycerophosphate oxidase, Peroxidase, 4-chloro-phenol, 4-aminoantipyrine, ATP, Mg++, Good`s buffer.

g. Principle of Polymerase Chain Reaction

The PCR is used to amplify DNA fragments in-vitro without first separating the target sequence from other DNA molecules. Two primers, which are complementary to the DNA sequence flanking the sequence to be amplified, are added to denature DNA. The primers bind to each of the separated DNA strands, and in the presence of DNA polymerase and deoxyribonucleotides, DNA synthesis proceeds to give two new DNA strands. In the second cycle, four single strands of DNA are available to act as template to give eight strands of DNA containing the target sequence. In the third cycle the short form with fixed ends begin to predominate. After 30 continuous cycles, nearly 1 million copies of target sequence are produced.

APPENDIX III. TABLES FOR POSTPRANDIAL RESPONSE OF SERUM LIPIDS, GLUCOSE AND INSULIN BY APOLIPOPROTEIN E GENOTYPE GROUP

Table A1-A3 and Table B1-B3 = data is presented as mean ± SD

Table A1. Fasting and postprandial total cholesterol concentrations of subjects by *APOE* genotype group

Variables	intervals	Group 1 n=5	Group 2 n=8	Group 3 n=9	Group 4 n=9	P-value
TCHOL(mmol/l)	0	3.10±0.29	2.98±0.53	2.97±0.54	3.28±0.51	0.533
	20	3.05±0.44	2.84±0.66	2.96±0.60	3.23±0.51	0.550
	40	3.16±0.61	3.07±1.01	3.23±0.87	3.30±0.58	0.945
	60	3.01±0.33	2.93±0.62	2.98±0.60	3.18±0.67	0.837
	120	3.27±0.29	3.13±0.52	2.98±0.51	3.31±1.33	0.837
	180	3.14±0.28	2.94±0.65	3.07±0.55	3.49±1.14	0.489
	240	3.31±0.30	3.10±0.66	2.95±0.41	3.25±1.09	0.770
	300	3.10±0.44	3.09±0.52	2.58±0.26	3.30±0.44	0.010
	360	2.97±0.50	3.41±1.12	2.83±0.41	2.98±0.65	0.417
AUC		1224.4±1134.08	1102.94±184.89	2.83±0.41	2.98±0.65	0.503

P-value <0.05 considered statistically significant.

Group 1= (*ε2/ε3*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

Table A2. Fasting and postprandial high-density lipoprotein cholesterol concentrations of subjects by *APOE* genotype group

Variables	intervals	Group1 n=5	Group 2 n=8	Group 3 n=9	Group 4 n=9	P-value
HDL-C mmol/l)	0	1.12±0.32	1.20±0.37	1.22±0.30	1.12±0.67	0.854
	20	1.10±0.25	1.11±0.31	1.19±0.32	1.08±0.21	0.844
	40	1.49±0.62	1.19±0.36	1.22±0.35	1.06±0.16	0.258
	60	1.16±0.23	1.07±0.38	1.12±0.22	1.07±0.22	0.914
	120	1.24±0.22	1.03±0.17	1.10±0.25	1.05±0.21	0.340
	180	1.12±0.28	1.08±0.40	1.12±0.24	1.09±0.28	0.992
	240	1.20±0.38	1.18±0.55	1.05±0.17	1.06±0.26	0.805
	300	1.16±0.29	1.08±0.42	1.05±0.22	1.00±0.19	0.923
	360	1.26±0.35	1.13±0.39	1.04±0.19	1.04±0.24	0.518
AUC		429.92±95.27	396.42±126.57	395.49±72.78	383.64±63.46	0.837

P-value <0.05 considered statistically significant.

Group 1= (*ε2/ε3*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

Table A3. Fasting and postprandial low-density lipoprotein cholesterol concentrations of subjects by *APOE*genotype group

Variables	intervals	Group 1 n=5	Group 2 n=8	Group 3 n=9	Group 4 n=9	P-value
LDL-C (mmol/l)	0	1.67±0.28	1.43±0.37	1.46±0.47	1.79±0.47	0.264
	20	1.66±0.31	1.44±0.64	1.43±0.45	1.73±0.56	0.558
	40	1.38±0.48	1.49±0.75	1.61±0.63	1.82±062	0.604
	60	1.54±0.31	1.56±0.64	1.42±0.59	1.57±0.68	0.951
	120	1.64±0.31	1.72±0.54	1.44±0.42	1.75±1.08	0.779
	180	1.56±0.26	1.46±0.58	1.40±0.48	1.79±0.94	0.615
	240	1.52±0.17	1.52±0.45	1.43±0.44	1.59±1.00	0.962
	300	1.50±0.32	1.58±0.40	1.02±0.38	1.74±0.43	0.015
	360	1.38±0.38	1.87±0.95	1.43±0.51	1.51±0.60	0.474
AUC		553.47±67.89	568.31±170.88	497.32±151.71	609.08±219.72	0.588

P-value <0.05 considered statistically significant.

Group 1= (*ε2/ε3*, *ε2/ε3*), group 2= (*ε2/ε4*), group 3= (*ε3/3*) and group 4= (*ε3/ε4*, *ε4/ε4*).

Table B1: Total cholesterol concentrations between the two *APOE* genotype groups after a fat meal

		Group A n=14	Group B n=9	
Variables	Intervals			P-values
TCHOL (mmol/l)	0	3.01±0.46	3.28±0.51	0.207
	20	2.99±0.53	3.23±0.51	0.289
	40	3.21±0.76	3.30±0.58	0.758
	60	2.99±0.51	3.18±0.67	0.460
	120	3.08±0.46	3.31±1.33	0.555
	180	3.08±0.46	3.49±1.14	0.239
	240	3.08±0.40	3.25±1.09	0.584
	300	2.76±0.41	3.30±0.44	0.008
	360	2.88±0.43	2.98±0.65	0.670
AUC		1080.11±131.92	1180.70±237.24	0.204

P-value <0.05 considered statistically significant. Group A= (*ε2/ε3*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

Table B2: High-density lipoprotein cholesterol concentrations between the two *APOE* genotype groups after a fat meal

		Group A n=14	Group n=9	
Variables	Intervals			P-values
HDL-C (mmol/l)	0	1.18±0.30	1.12±0.17	0.560
	20	1.16±0.29	1.08±0.21	0.473
	40	1.31±0.46	1.06±0.16	0.137
	60	1.14±0.22	1.07±0.22	0.475
	120	1.15±0.24	1.05±0.21	0.318
	180	1.12±0.24	1.09±0.28	0.807
	240	1.10±0.26	1.06±0.26	0.703
	300	1.09±0.24	1.06±0.19	0.800
	360	1.12±0.27	1.04±0.24	0.466
AUC		407.79±79.66	383.64±63.46	0.453

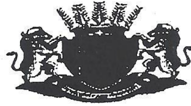
P-value <0.05 considered statistically significant. Group A= (*ε2/ε3*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

Table B3: Low-density lipoprotein cholesterol concentrations between the two *APOE*genotype groups after a fat meal

		Group A n=14	Group n=9	
Variables	Intervals			P-values
LDL-C (mmol/l)	0	1.53±0.42	1.79±0.47	0.181
	20	1.51±0.41	1.73±0.56	0.289
	40	1.53±0.57	1.82±0.62	0.263
	60	1.46±0.50	1.57±0.68	0.668
	120	1.51±0.38	1.75±1.08	0.454
	180	1.46±0.41	1.79±0.94	0.255
	240	1.46±0.36	1.59±1.00	0.661
	300	1.24±0.40	1.74±0.43	0.111
	360	1341±0.45	1.51±0.60	0.662
AUC		517.37±127.91	609.08±219.71	0.218

P-value <0.05 considered statistically significant. Group A= (*ε2/ε3*, *ε2/ε3*. *ε3/ε3*) and Group B= (*ε3/ε4*, *ε4/ε4*)

APPENDIX IV. ETHICS CLEARANCE CERTIFICATE



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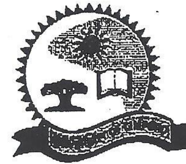
CLEARANCE CERTIFICATE
UNIVERSITY OF LIMPOPO
Polokwane/Mankweng Hospital Complex

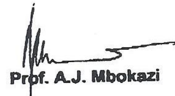


PROJECT NUMBER: 003/2008

RESEARCHER: MR S. DIKOTOPE

TITLE : Influence of Apolipoprotein (APO) E Phenotypes on Postprandial Serum Lipids in Black South African Subjects

DATE: 23 May 2008



Prof. A.J. Mbokazi

Chairman of Pietersburg Mankweng
Hospital Complex Ethics Committee

Note: The budget for research has to be considered separately. Ethics Committee is not providing any funds for projects.

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APPENDIX V. ALCOHOL CONSUMPTION QUESTIONNAIRE

CORE Alcohol Consumption (Section A)				
The next questions ask about the consumption of alcohol.				
		Response	Coding Column	
A 1a	Have you ever consumed a drink that contains alcohol such as beer, wine, spirits, [*"Black Label, Kudu, Bjala bja Sesotho, Totota*]?	Yes 01 No 02	☐☐	*If No, Go to Next Section**
A 1b	Have you consumed alcohol within the past 12 months?	Yes 01 No 02	☐☐	*If No, Go to Next Section**
A 2	In the past 12 months, how frequently have you had at least one drink? *(READ RESPONSES)* Beer, wine, spirits, [*"Black Label, Kudu, Bjala bja Sesotho, Totota*]?	5 or more days a week 01 1-4 days per week 02 1-3 days a month 03 Less than once a month 04	☐☐	
A 3	When you drink alcohol, on average, how many drinks do you have during one day?	Number Don't know 77	☐☐	
A 4	During each of the past 7 days, how many standard drinks of any alcoholic drink did you have each day? *(RECORD FOR EACH DAY* *USE SHOWCARD)*	Monday	☐☐	
		Tuesday	☐☐	
		Wednesday	☐☐	
		Thursday	☐☐	
		Friday	☐☐	
		Saturday	☐☐	
		Sunday	☐☐	

APPENDIX VI. TOBACCO USE QUESTIONNAIRE

CORE Tobacco Use (Section S)					
Now I am going to ask you some questions about various health related habits. This includes things like smoking, drinking alcohol, eating fruits and vegetables and physical activity. Let's start with smoking.					
		Response		Coding Column	
S 1a	Do you currently smoke any tobacco products, such as cigarettes, cigars or pipes? Cigarette • Commercial eg "Lucky Strike • Rolled (newspaper + BB/Horseshoe tobacco	Yes No	01 02	☐☐	*If No, go to Expanded Section"*
S 1b	<u>If Yes,</u> Do you currently smoke tobacco products daily?	Yes No	01 02	☐☐	*If No, go to Expanded Section"*
S 2a	How old were you when you first started smoking daily?	Age (years) Don't remember	77	☐☐	*If Known, go to S 3*
S 2b	Do you remember how long ago it was?	In Years		Years ☐☐	
		OR in Months		Months ☐☐	
	(CODE 77 FOR DON'T REMEMBER)	OR in Weeks		Weeks ☐☐	
S 3	On average, how many of the following do you smoke each day?	Manufactured cigarettes		☐☐	
	(RECORD FOR EACH TYPE)	Hand-rolled cigarettes		☐☐	
		Pipes full of tobacco		☐☐	
	(CODE 88 FOR NOT APPLICABLE)	Cigars, cheroots, cigarillos		☐☐	
	☐☐☐☐☐☐☐☐	← Other (please specify):		☐☐	

APPENDIX VII. MEDICATION QUESTIONNAIRE

Questionaire about medication

Are you on any medication? YES/NO

If yes, indicate for which medical condition---

APPENDIX VII: APOE GENOTYPES ON GEL

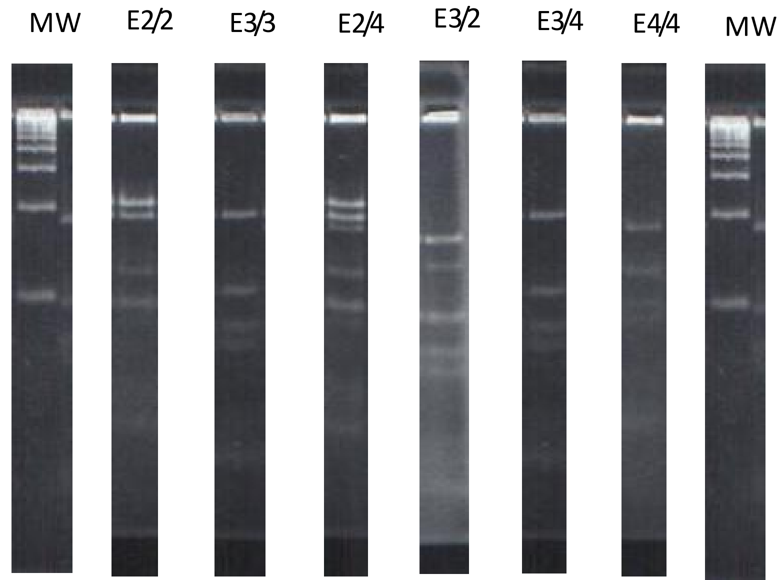

Figure 4.25. APOE genotypes of subjects on Meta-Phore gel after digestion of PCR products with HhAI endonuclease. Fragments have been extracted and compiled from different gels ran at different times.

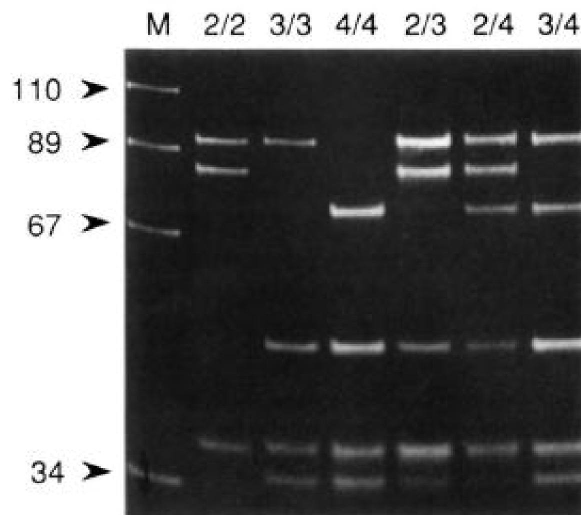

Figure 4.26. APOE genotypes extracted from Hixon and Vernier, (1990) used as reference for identification of genotypes.