University of Reading

A Nutrigenetics approach to study the impact of genetic and dietary factors on blood lipids

Submitted for the fulfilment of the degree of Doctorate of Philosophy

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DECLARATION OF AUTHORSHIP

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Israa Mohammed Shatwan

ABSTRACT

Abnormal lipid concentrations have been shown to be risk factors for cardiovascular disease, which is influenced by a complex interaction between lifestyle (such as diet) and genetic factors. Given that lipoprotein lipase (LPL) and apolipoprotein E (APOE) are key regulatory proteins in lipid metabolism, the main aims of this thesis were to examine the association of single nucleotide polymorphisms (SNPs) at the *LPL* and *APOE* genes with lipid-related outcomes and to investigate the interaction of the SNPs with dietary factors on lipids. A total of six studies with different study designs were used. These studies included a postprandial study (n=261), a case-control study (CURES, Asian India, n=1,845), three cross-sectional studies [PRECISE study (UK, n=468; Denmark, n=192) and CaPS study (UK, n=1,238)], a 16-week intervention study DIVAS (n=120) and a crossover trial (n=18). For the *LPL* gene, the SNP rs328 showed a consistent association with HDL-C concentrations in the postprandial (P=0.015) and CURES studies (P=0.0004). In addition, in the CURES, there was an interaction between *LPL* SNP rs1121923 and fat intake (energy %) on HDL-C concentrations (P=0.003). For the *APOE* gene, significant associations were detected between the *APOE* haplotype (E2, E3, and E4) and *APOE* SNP rs445925 and total cholesterol ($P=4x10^{-4}$ and P=0.003, respectively) in the PRECISE study. These associations were further replicated in the CaPS cohort. In the DIVAS study, the TT homozygotes of the *APOE* SNP rs1064725 showed a significant reduction in total cholesterol after the MUFA diet compared to the SFA (P=0.001). In the crossover trial, we examined the association vitamin D-related SNPs with lipids in 18 men with sup-optimal vitamin D status and found that the TT homozygotes of the SNP rs12785878 (T/G) at nicotinamide-adenine dinucleotide synthetase 1 gene had higher HDL-C levels compared to G allele carriers (P=0.0003). In conclusion, our findings suggest a role of dietary factors in modifying the genetic effect of *LPL* and *APOE* SNPs on lipid levels. Given the smaller sample size of some of the cohorts studied, replication of the findings is warranted.

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1. Shatwan IM, Minihane AM, Williams CM, Lovegrove JA, Jackson KG, Vimaleswaran KS (2016) Impact of Lipoprotein Lipase gene polymorphism, *S447X*, on postprandial triacylglycerol and glucose response to sequential meal ingestion. International journal of molecular sciences 17 (3):397

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5. Surendran S, Adaikalakoteswari A, Saravanan P, **Shatwan IA**, Lovegrove JA, and Vimaleswaran KS (2018) An update on vitamin B12-related gene polymorphisms and B12 status. Genes & Nutrition 13:2

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ABBREVIATIONS

25(OH) VD	25-hydroxy-vitamin D
ADIPOQ	Adiponectin
APOA-I	Apolipoprotein A-I
APOA5	apolipoprotein A5
APOB	Apolipoprotein B
APOCIII	Apolipoprotein CIII
APOE	Apolipoprotein E
AUC	Area under the curve
BMI	Body mass index
CAD	Coronary artery disease
CaPS	Caerphilly prospective study
CEPT	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CURES	Chennai Urban Rural Epidemiological Study
CVD	Cardiovascular disease
CYP24A1	Cytochrome P450, family 24, subfamily A, polypeptide 1
CYP2R1	Cytochrome P450 family 2 subfamily R member 1
DHA	Docosahexaenoic acid
DHCR7	Dehydrocholesterol reductase
DIVAS	Dietary Intervention and VAScular function study
EPA	Eicosapentaenoic acid
FFQ	Food frequency questionnaire
GC	Group-specific component vitamin D binding protein
GLM	General linear model
GWAS	Genome-wide association studies
HbA1c	Glycated haemoglobin
HDL-C	High-density lipoprotein cholesterol
HOMA-IR	Homeostasis model assessment- insulin resistance
HWE	Hardy–Weinberg equilibrium
IAUC	Incremental area under the curve
IDL	Intermediate density lipoproteins
LACT	Lecithin: cholesterol acyl transferase
LDL-C	Low-density lipoprotein cholesterol
LIPC	Hepatic lipase
LPL	Lipoprotein lipase
MetS	Metabolic syndrome
MUFA	Monounsaturated fatty acid
NADSYN1	Nicotinamide-adenine dinucleotide synthetase 1 gene
NBPF3	Neuroblastoma breakpoint family
NCEP-ATP III	National Cholesterol Education Program-Adult Treatment Panel III
NEFA	Non-esterified fatty acids
NGT	Normal Glucose Tolerance
PPARA	Peroxisome proliferator–activated receptor- alpha
PPARG	Peroxisome proliferator–activated receptor-gamma
PRECISE	Prevention of Cancer by Intervention with Selenium
PUFA	Polyunsaturated fatty acid
SD	Standard deviation

SFA	Saturated fatty acids
SNP	Single-nucleotide polymorphism
SRB-1	Scavenger receptor B-1
T2D	Type 2 diabetes
TAG	Triacylglycerol
TagSNPs	Tagging SNPs
TC	Total cholesterol
TE	Total energy
TNFA	Tumour necrosis factor-alpha
VLDL	Very-low-density lipoprotein

Chapter 1 Introduction to thesis

1.1 Introduction

Epidemiological studies have consistently shown that decreased high-density lipoprotein (HDL-C) levels accompanied by elevated levels of low-density lipoprotein (LDL-C), triacylglycerol (TAG) and total cholesterol, play a key role in the development of cardiovascular disease (CVD) risk [1, 2]. Several biomedical studies have investigated the correlation between genotype and phenotype, to identify the specific genetic variants responsible for phenotypic variation amongst individuals [3]. Many candidate genes have been studied in relation to their potential role in lipid metabolism, and an association between these genes and lipid levels has been confirmed [4, 5]. Genome-wide association studies (GWAS) have identified several single nucleotide polymorphisms (SNPs) in different genes, which are associated with CVD risk and variations in lipid and lipoprotein concentrations [6]. The SNPs identified to date, however, explain a relatively small fraction of the inherited risk of CVD. Lipid levels are not a homogeneous phenotype and are known to be responsive to changes in diet, and are dependent on the quality and quantity of fat. Controlling diet is often the first recommendation in the outpatient setting [7, 8]. Thus, it is important to examine interactive effects between dietary and genetics factors on lipids.

The nutrigenetics or gene-diet interaction approach helps to investigate how the genetic makeup of an individual influences response to diet. This approach helps to determine whether a diet can modulate genetic susceptibility by altering lipid responses, which may be beneficial in prevention and treatment of CVD [9, 10]. It also may provide evidence for tailoring optimal dietary recommendation, for individuals [11].

This chapter will: (i) discuss the evidence of the role of genes and diet separately on circulating lipids; (ii) outline the need for a nutrigenetics approach to promote cardiovascular health and reduce the burden of CVD.

1.2 CVD prevalence and public health concerns

CVD encompasses a broad range of disorders that affect the heart and blood vessels, i.e. coronary heart disease (CHD), angina, peripheral arterial disease, heart attack, congenital heart disease and rheumatic heart disease, and stroke [12, 13]. The circulation of blood to the heart, brain or body can be compromised due to a blood clot or accumulation of fat deposits inside an artery, leading to the hardening and narrowing of the artery (called atherosclerosis). Acute events usually cause heart attacks and stroke, as a result of a blockage that prevents blood from reaching the heart or brain respectively [13]. Every day, the number of individuals diagnosed with CVD is increasing, placing a huge burden on society [14]. The mortality rate of CVD in the UK alone, is one of the highest in the world, and approximately 48% of deaths in Europe are due to CVD [15]. CVD is a global health problem and has been ranked top by the World Health Organisation (Figure 1.1) [14].

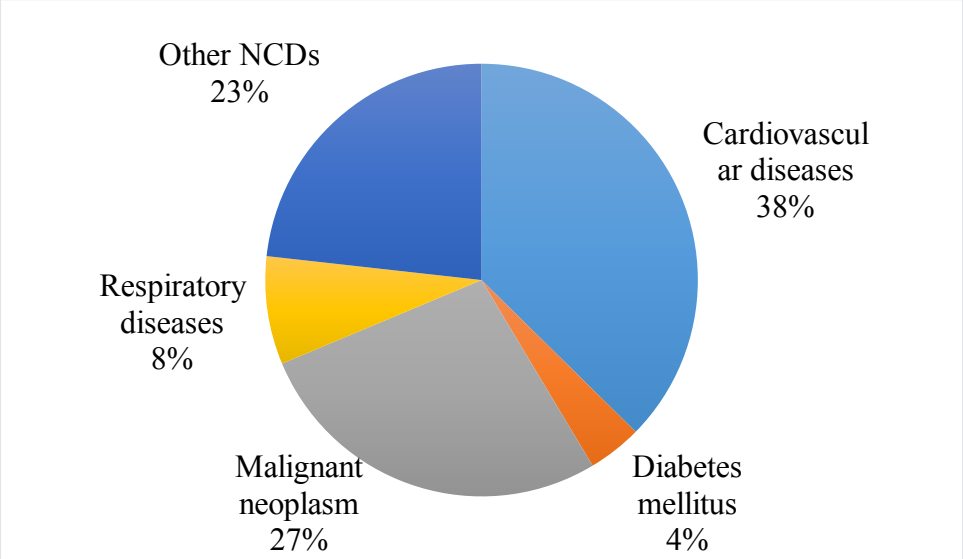

Figure 1.1 Proportion of global deaths under the age 70 years from non-communicable

diseases (source WHO, 2012).

The epidemic of CVD is increasing globally in both developed and developing countries [16]. The cost of treating CVD patients in NHS hospitals was around £6.8 billion in England (between 2012-2013). Hospital statistics indicated that around 1.6 million episodes were related to CVD in UK hospitals, among these 10.1% of all inpatient episodes were in men and 6.3% in women. The number of operations performed to treat CVD in the UK, was >90,000 which is two times higher than a decade earlier [17]. In India, CVD contributed to two thirds of total number of mortality cases due to non-communicable diseases. The mortalities from CVD under age 70 were 52% in India compared with 23% in Western populations. The cost of health care spent on treating CVD was $237 billion in a period of 10 years (from 2005-2015) [18]. Therefore, identifying risk factors for CVD and treatments against these factors will have a significant effect on disease prevention.

1.3 Lipid as a risk factor for CVD

A lipid profile that includes abnormal levels of total cholesterol, TAG and lipoprotein is a traditional CVD risk factor and biomarker [19]. These biomarkers can be measured in both fasting and postprandial states. Several observational studies have confirmed that increased fasting and postprandial TAG and TAG-rich particles are significant predictors of CVD [2]. A meta-analysis of prospective studies on a general population demonstrated that TAG increased CVD risk by approximately 30% in men and 75% in women (data for 46,413 men and 10,864 women) where the relative risk remained statistically significant after adjustment for HDL-C and other risk factors [20]. A recent meta-analysis of 61 prospective studies investigated the association between TAG levels and CVD mortality and showed that the risk ratio of CVD mortality for the lowest TAG (< 90 mg/dl) was 0.83, borderline-high TAG (150–199 mg/dl) was 1.15, and high TAG (≥ 200 mg/dl) was 1.25, which confirmed

dose dependent effect for TAG levels [21]. The association between postprandial TAG and CVD was confirmed in 86,261 participants (men and women) in the Norwegian Counties Study [22]. Therefore, these results suggest that elevated TAG concentration is a powerful risk factor for CVD [23].

Furthermore, lipoproteins (LDL-C and HDL-C) play a pivotal role in the progression of CVD. Their major function is to transport cholesterol, which is used for the maintenance of cell membranes, in the circulation system between the liver and peripheral tissues. The pathogenesis of atherosclerosis is highly correlated with LDL-C levels and is a therapeutic target to reduce the CVD risk. If there is an infection, HDL-C levels decrease rapidly; consequently, lower concentrations of HDL-C predict a serious CVD risk [24]. In a meta-analysis of seven placebo-controlled statin trials, it was shown that concentrations of LDL-C reduced by 21% and non-HDL-C reduced by 20% thereby resulting in a reduced risk of coronary heart disease (CHD). Changes in LDL-C and non-HDL-C within each trial caused a significant risk reduction of CVD risk [25]. In a prospective study on 27,791 initially healthy women in the Women's Health Study, there were 899 incidents of CVD during the ten-year follow-up period. The risk of developing CVD was 1.47 times higher for women with elevated LDL-C (121mg/dl; 3.12 mmol/L) [26]. This evidence confirmed the key association between lipoprotein levels and CVD risk.

The role of lipoproteins and lipids in the pathogenesis of CVD can be explained by increased accumulation of LDL-C particles, cholesterol, in the inner lining of the arterial wall. This leads to arterial plaques, which is the principal driver of the initiation for the pathogenesis of atherosclerosis [27]. The LDL-C and apolipoprotein B (apoB)-containing lipoproteins can pass the endothelial barrier and gain access to the sub-endothelial space, where they accumulate. Retained lipoproteins are oxidised and combined with other

atherogenic factors to promote activation of endothelial cells. The activated endothelial cells increase the recruitment of monocytes into the intima by inflammatory process and also promote the recruitment of other immune cells. The monocytes differentiate into macrophages and express receptors that mediate the internalization of very low density lipoprotein, apolipoprotein E (apoE) remnants, and modified LDL to become foam cells. In macrophage foam cells, inflammatory signalling pathways are activated resulting in more cell recruitment and LDL modification. Foam cells are considered as intermediate atherosclerotic lesions, which occupy much of the lesion volume and leads to the progression of the atherosclerosis [28, 29]. On the other hand, HDL-C and apolipoprotein A-I (apoA-I) protect against atherosclerosis via mechanisms independent of cholesterol efflux resulting in reduced inflammation [28].

Thus, this thesis aimed to study factors affecting lipid levels. Lipid concentrations are affected by a number of factors, which include modifiable factors such as diet, obesity and physical activity, and non-modifiable factors such as age, gender, and genetic factors [30, 31]. Controlling modifiable factors may reduce the risk of developing CVD. In this thesis, genetic factors and dietary factors will be investigated.

1.4 Lipid metabolism

Following the digestion and absorption of dietary lipids into free fatty acids, TAG are resynthesized within the intestinal wall and combined with cholesterol and protein to form chylomicrons. Because of insolubility of TAG and lipids, they need to combine with protein to form lipoprotein [32]. Lipoproteins consist of a mixture of apolipoproteins, phospholipid, cholesterol, and TAG. Lipoproteins are involved in the transport of lipids through the circulation from intestine and liver to other tissues that require these lipids as their energy suppliers or use them as structural materials in their membrane. Based on the density

lipoproteins are mainly divided into five major classes including chylomicrons, very low density lipoprotein (VLDL), LDL-C, intermediate-density lipoproteins (IDL), and HDL-C [33]. Chylomicrons and VLDL have higher amount of TAG within their core; in contrast, LDL-C and HDL-C have higher amount of cholesterol ester within their core [34].

Apolipoproteins (APO) coat lipoprotein particles and transport lipids in the lymphatic and circulatory systems, for instance apoA, apoB, apoC and apoE. They have a number of other functions including cofactors for enzymes and ligands for cell-surface receptors. For example, apoC-II activates the enzyme lipoprotein lipase (LPL), which is involved in the hydrolysis of TAG in chylomicrons and VLDL, to convert them to remnant particles and remove TAG from circulation [35]. Remnant chylomicrons bind to LDL receptor-related protein (LRP) receptors on the surface of hepatocytes, which recognizes apoE on the surface of remnant chylomicrons. In the liver, VLDL is synthesized from TAG in the remnant chylomicrons, cholesterol and apoB-100, apoC-II and apoE. Then, VLDL is secreted into the plasma, where they are hydrolyzed by LPL to convert to IDL and release TAG. The IDLs are hydrolyzed by hepatic lipase to form LDL-C. One of the apolipoproteins components in LDL-C is apoB-100, which facilitates binding between the lipoprotein particles and LDL-specific receptors on the surface of many cells. After binding, cholesterol in the LDL-C is used as a structural component of cell membranes or is converted to steroid hormones [34].

The HDL-C is synthesized by liver and gut. Cholesterol from peripheral tissues bind to HDL-C through apoA-I, which mediates transportation of cholesterol to the liver [36]. Several enzymes, proteins, and receptors are involved in HDL-C metabolism pathway. Lecithin cholesterol acyltransferase (LCAT) enzyme esterifies free cholesterol in nascent HDL-C to cholesteryl esters. ATP-binding cassette protein (ABCA1) is responsible for adding free cholesterol from cells to HDL-C resulting HDL-C particles that is rich in free cholesterol. Furthermore, cholesteryl esters and phospholipids are transferred from HDL-C to

apoB-containing lipoproteins, including the triglyceride rich lipoproteins (TRLs) via cholesteryl ester transfer protein (CETP). Scavenger receptor BI (SR-BI) binds to HDL-C in order to transport back cholesterol ester to the liver [37, 38].

1.5 Factors affecting lipid levels

1.5.1 Dietary factors

The last few decades have seen fundamental changes in dietary patterns in almost every region of the world [39]. This shift has raised a global concern as an unhealthy diet is a major cause of the increased burden of diseases such as CVD [40]. Therefore, nutritional science has designed and implemented strategies to reduce the incidence of diet-related diseases [41]. One of the key public health strategies for CVD prevention is to reduce saturated fatty acid (SFA) intake to < 10 % of total energy (%TE) [42], as high intake of SFA is associated with elevated LDL-C concentrations [8]. A clinical trial showed that lowering intake of SFA by 5% and 8% reduced LDL-C levels by 6.8% and 11.7%, respectively [43]. Therefore, it is recommended that SFA should be replaced with alternative macronutrients such as unsaturated fat [44]. It has been found that replacing 1% TE of SFA with either polyunsaturated fatty acids (PUFA) or cis-monounsaturated fatty acids (cis-MUFA) lowers LDL-C by 0.009 and 0.019 mmol/l respectively [8]. Furthermore, in a recent intervention study, it was reported that isoenergetic replacement of 9.5-9.6%TE from SFA with MUFA or n-6 PUFA had a beneficial effect on lipid response, with a reduction of 8.4% and 9.2% respectively in total cholesterol, 11.3% and 13.6% respectively in LDL-C, and total cholesterol to HDL-C ratio of 5.6% and 8.5% respectively [45]. In a randomised controlled trial, a moderate substitution 9%TE of SFA with MUFA for three months induced a reduction in the concentrations of cholesterol and TAG [46]. Moreover, a recent randomised controlled

trial suggested that intervention of n-6 PUFA diet (flax and safflower oils) for four weeks lowered total cholesterol [47].

High SFA intake is linked with unfavourable lipid profiles, however if carbohydrates are replaced with SFA, concentrations of TAG have been shown to increase particularly simple carbohydrates [48, 49]. Two cross-sectional studies, have indicated that higher intakes of carbohydrate was associated with lower HDL-C levels and increased TAG in healthy adults, which may have further health implications for CHD [50, 51]. A moderate carbohydrate restriction (39%TE) with low SFA (9%TE) for three weeks resulted in a reduction in TAG, small LDL mass, and total cholesterol to HDL-C ratio [52]. Some studies investigated on specific type of carbohydrates such as complex carbohydrates and whole grains, which postulated to have positive effect on lipid levels. For complex carbohydrates, a randomized crossover study conducted on 16 gestational diabetes mellitus women who were given either lower-carbohydrate/high fat diet (40% carbohydrate/45% fat/15% protein) or higher-complex carbohydrate /lower-fat diet (60 carbohydrate /25 fat /15% protein) for three days. Complex carbohydrates defined as polysaccharides and starches primarily derived from grains, vegetables, and fruits. higher-complex carbohydrate /lower-fat diet significantly lowered postprandial free fatty acids but not postprandial TAG [53].

Additionally a large cross sectional study, that included 12,745 men and women from the Polish Norwegian study, found that, among users of statin, individuals who consumed adequate amount of whole grains (≥3oz/day) showed an association with lower total cholesterol and LDL-C levels compared with individuals who consumed inadequate amount of whole grains [54]. Results from randomized control trial showed (total n=45 men and women) that healthy participants who consumed adequate amount of whole grain (corn, rice, and wheat) based on their caloric need for six weeks had decreased total, LDL, and non-HDL

cholesterol levels compared to those who received refined grains [55]. However, in a crossover study, where 33 men and women received either whole grain or refined grain (50g/1000 kcal in each diet) for 8 weeks, showed that whole grain diet significantly decreased diastolic blood pressure but not total and LDL cholesterol [56].

Few studies have investigated the effect of protein-rich diets on lipid levels. In a randomised controlled trial, a high-protein diet (20-34%TE) was shown to reduce fasted TAG, LDL-C, and total cholesterol compared with a diet high in carbohydrates and a standard protein diet [57]. Conversely, there was no significant difference in LDL-C and HDL-C levels with a high-protein diet compared to a high-carbohydrate diet [58]. A recent study investigated the effect of milk proteins on risk markers of CVD. Thirty-eight participants were given either 2 × 28 g whey protein/day, 2 × 28 g calcium caseinate/day, or 2 × 27 g maltodextrin (control)/day for 8 weeks separated by a 4-weeks washout after each study arm. Participants received both whey protein and calcium caseinate had significantly lower total cholesterol, while TAG decreased after whey protein diet only [59]. Also, another study looked at the effect of consuming either 25 g/day lupin protein isolate or 25g/day milk protein isolate for 4 weeks in 33 hypercholesterolemic participants. Findings showed that LDL-C was significantly decreased after both dietary interventions but LDL:HDL cholesterol ratio was decreased only after consuming lupin protein isolate [60].

Overall, the effect of dietary macronutrients on lipids is still controversial [61] with high individual heterogeneity in serum lipid response to the same dietary intervention [10].

1.5.2 Genetic factors

Lipid and lipoprotein metabolism are complex biological pathways containing multiple steps. A large number of nuclear factors, binding proteins, apolipoproteins, enzymes, and receptors involving hundreds of genes are involved in the lipid metabolism

pathway [4]. Between 25 % and 80 % of the inter-individual variation in lipid concentrations is explained by SNPs at several genes [62]. There is growing interest in the development of in-depth knowledge regarding how genetic factors affect the regulation of plasma lipid absorption, transport and catabolism [63]. Within the past two decades, a plethora of genes have been identified in humans, which are associated with lipid metabolism and plasma lipoprotein profiles [63].

Some of the most important genes involved in lipid metabolism are *APOA-I*, *APOE*, *LPL*, hepatic lipase (*LIPC*), and cholesteryl ester transfer protein (*CEPT*) [4, 64, 65]. It has been confirmed that the CEPT, LPL and LIPC proteins play an important role in determining the circulating HDL-C levels [66-68], and many studies have implicated that selected SNPs within these genes are the cause of changes in HDL-C concentrations [69-71]. Moreover, inheritance of the levels of the fasting and postprandial TAG has been linked to SNPs at the *LPL* gene [72, 73]. Apolipoproteins are vital components of the lipoprotein molecules due to the role they play in the metabolism of lipoproteins. Some act as ligands for receptors and play roles as cofactors, activators or inhibitors of enzymes in lipid metabolism [74]. Therefore, genes that encode these proteins have been found to have a significant impact on lipid levels. It has been confirmed that common polymorphisms at the *APOE* gene account for inter-individual variation in LDL-C, TAG and total cholesterol concentrations [75-79]. The APOA-I protein is a principal component of HDL-C particles [80]; forty-three SNPs have been reported within the gene associated with low HDL-C concentrations [37] .

Recent GWAS studies have reported a significant increase in the number of loci associated with lipid and lipoprotein profiles [81, 82]. Two large-scale GWAS study meta-analyses have identified 157 genomic loci contributing to inter-individual variation in the lipid concentrations in >100,000 individuals [83, 84]. Of these loci, 46 demonstrated the

strongest evidence of associations with HDL-C, 16 with TAG, 18 with total cholesterol, and 9 with LDL-C. Some of the identified loci affected multiple lipid outcomes, i.e. four variants showed associations with total cholesterol, HDL-C, LDL-C, and TAG [84].

Genes alone, however, do not explain the variation in lipid concentrations as lifestyle factors, i.e. dietary factors, also influence lipids. Therefore, this is investigated by identifying gene-diet interactions (also called Nutrigenetics) [85-88]. In my thesis, I aimed to investigate the interaction between dietary factors (modifiable factor) and genetic markers (non-modifiable factor) on lipid outcomes.

1.6 Nutrigenetics approach

Nutrigenetics is the study of genetic background of an individual that effects response to diet, the ultimate goal of which is to generate evidence of gene-diet interactions to personalise dietary recommendations to help prevent or delay the onset of disease [4, 89]. Investigations in nutrigenetics have undergone rapid development due to the increasing demand of new optimal dietary strategies for general public health.

1.6.1 Rational for studying gene-diet interactions

Many SNPs associated with CVD risk factors have been identified [4, 5], and represent a small fraction of the heritability of the CVD [62]. However, environmental factors including dietary factors can also modulate the effect of genes on CVD and hence, interaction between genetic and dietary factors must be taken into account [90]. Genes can trigger the incidence of CVD in individuals carrying risk alleles, when these individuals are exposed to a high-risk environmental factor [91]. Thus, findings from gene–diet interactions will contribute to the identifying the involvement of both gene and diets in the development of multifactorial disease such as CVD. This knowledge is crucial for the primary prevention of CVD, and also to develop more effective dietary strategies for preventing or delaying of CVD [90].

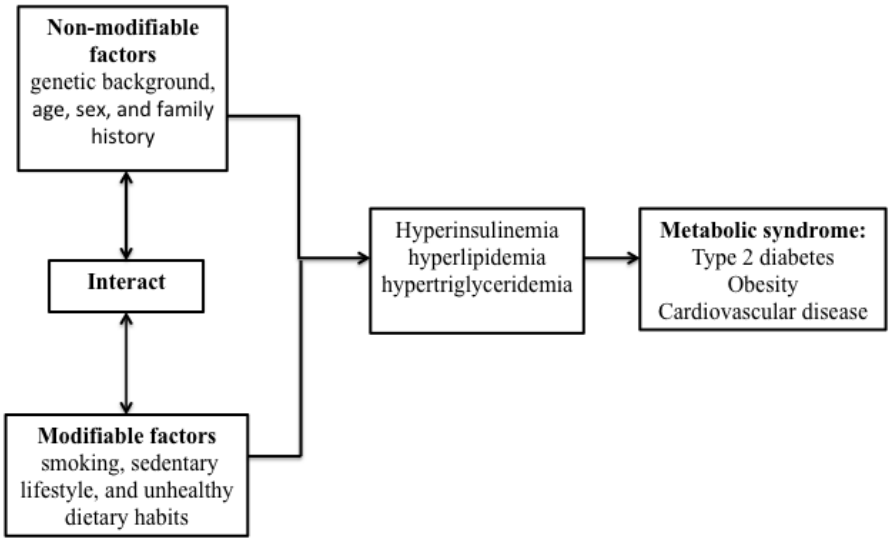

Figure 1.2 shows the interaction between genetic (non-modifiable) and dietary factors (modifiable) on lipoprotein concentrations, which eventually leads to the development of cardiometabolic diseases.

1.7 Genetic variations and ethnic background

In humans, 99.9% of the genome is identical; variations exist in the remaining 0.1% of the genome, making a person unique [92]. Approximately 85% of all SNPs are common to all human populations and only 15 % of SNPs are population-specific [93]. Therefore, if a common allele is significantly associated with a disease, it is more likely to be shared by multiple populations [94]. However, some gene frequencies vary across different ethnic groups, resulting in differences in the prevalence of disease risk across ethnicities [95]. For example, the highest frequency for the E4 allele (risk allele) at the *APOE* gene has been reported for African and North European populations compared to Indian and Asian populations [96]. Additionally, environmental factors also contribute to the disease prevalence. Moderate changes in lifestyle factors, such as reducing SFA intake, have an impact on reducing CVD risk if adopted by large groups of the population [97]. Thus, the

importance of studying different ethnic groups is to identify the effect of different lifestyle factors, mainly dietary pattern, across populations.

1.8 Selection of candidate genes for the study

This thesis focuses on two candidate genes, *LPL* and *APOE*, because of their key role in lipid metabolism (Figure 1.2). The LPL enzyme is located on the luminal surface of the vascular endothelium, and regulates the lipolytic processing of TAG-rich lipoproteins. LPL hydrolyses the circulating TAG-rich lipoproteins, resulting in the formation of chylomicron remnants and intermediate density lipoproteins (IDL), the VLDL remnants, and LDL. Therefore, LPL modulates circulating TAG and HDL-C concentrations [67, 98]. LPL has another non-enzymatic function in the binding of lipoprotein particles to cell-surface molecules, which mediate uptake of lipoproteins [99]. Furthermore, deficiencies in *LPL* enzyme expression have been implicated in the pathogenesis of hypertriacylglycerolaemia, and CVD [67, 100]. Thus, LPL is suggested to have anti-atherogenic effects through clearing circulating lipoprotein particles and pro-atherogenic effects through enhancing the uptake of potentially atherogenic TAG-rich lipoproteins by the arterial wall [101].

The APOE protein, which is a component of VLDL particles, chylomicrons, and their remnants, has an anti-atherogenic function. The APOE is a ligand for the LDL receptor-related protein, which mediates cellular removal of lipoprotein remnants and acts as a cofactor in VLDL synthesis, mainly hydrolysing VLDL remnants to produce LDL-C [102]. Thus, it is considered to be an important determinant of serum LDL-C levels [103].

Given these important functions of LPL and APOE in lipid metabolism, it would be timely to examine the interaction between SNPs of *LPL* and *APOE* genes and dietary factors on lipid outcomes.

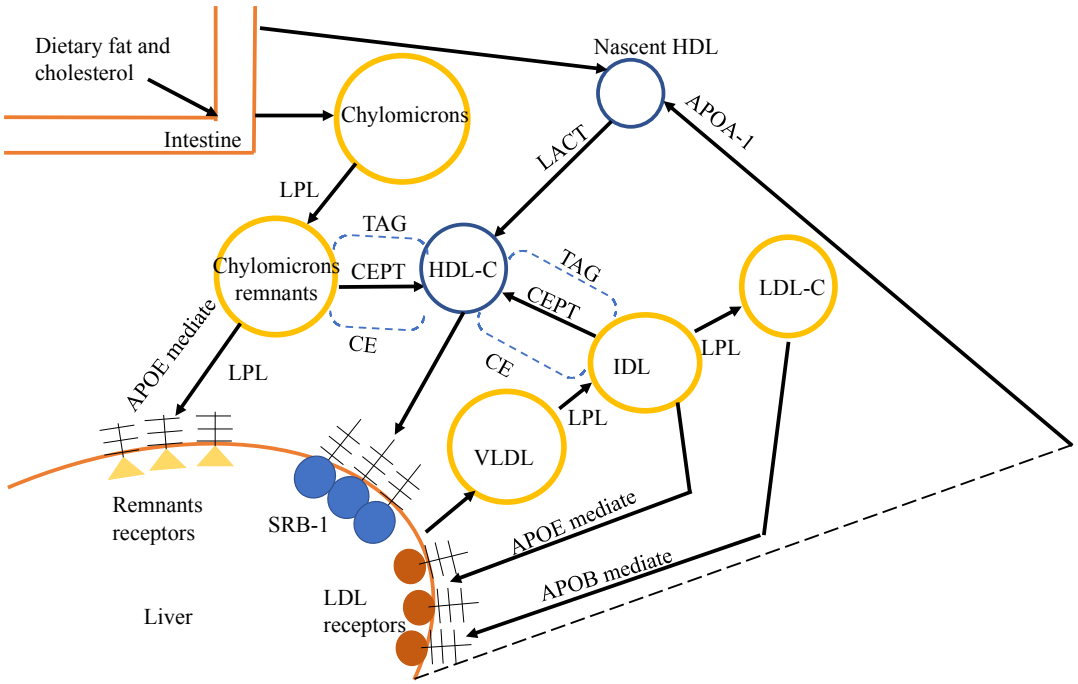

Figure 1.3 Role of lipoprotein lipase and apolipoprotein E in lipid metabolism; LPL; lipoprotein lipase, APOE; apolipoprotein E, LACT; lecithin: cholesterol acyl transferase is an enzyme that produces cholesteryl esters in plasma and promotes the formation of HDL-C; CETP; cholesteryl ester transfer protein which facilitates transfer of CE and TAG between lipoproteins, SRB-1; scavenger receptor B-1 found in liver and facilitates uptake of CE and HDL-C; IDL; intermediate density lipoprotein; CE; cholesterol ester.

1.8.1 Lipoprotein lipase

The *LPL* gene is located on chromosome 8q22 and includes 10 exons encoding 475 amino acids, of which 448 amino acids are part of the mature protein and 27 are part of the signal peptide [104]. The SNPs at this gene have previously been associated with changes in circulating TAG and HDL-C [105, 106]. Few studies have investigated the interaction between *LPL* SNPs and diet on lipids.

The interaction between the SNP rs328 (C/G) and the intake of dietary fat (energy %) on HDL-C concentrations was significant in ≈ 9,000 Caucasian Americans (men and women). The fat intake was found to be positively correlated with fasted HDL-C among CC

homozygotes and CG heterozygotes, but inversely correlated with GG homozygotes. Moreover, interactions were observed between SNP rs328 with saturated fatty acid (SFA) (as g/day) and monounsaturated fatty acid (MUFA) (as g/day and energy %) on HDL-C [85]. Unsaturated fat was shown to interact significantly with SNP rs10503669 on HDL-C concentrations in Korean participants (5,314 men and women) [107]. Furthermore, the association between SNP rs13702 (T/C) and the reduction in plasma TAG in response to unsaturated fat (30 grams/day during a 3-year intervention) was evaluated, and a greater reduction in fasted TAG was observed in C minor allele carriers [108].

A study of 452 participants consuming their habitual diet from the European LIPGENE human study showed a significant gene-nutrient interaction between *LPL* SNPs (rs328 [C/G] and rs1059611 [A/G]) and total n-6 polyunsaturated fatty acid (PUFA) intake with fasted plasma TAG concentrations. The G minor allele carriers of both SNPs were associated with lowered concentrations of TAG in individuals consuming a diet low in n-6 PUFA (35.48% of total lipid) compared with common homozygotes. This interaction was replicated in another population, where n-6 PUFA interacted with both SNPs (rs328 and rs1059611) on HDL-C [109]. In a meta-analysis based on 27,756 individuals from 10 cohorts, a significant interaction between another SNP rs13702 at the *LPL* gene and PUFA (energy %) on TAG was identified, with an inverse association between PUFA intake and TAG concentrations[110].

Data from these studies demonstrates that the strongest interaction was between *LPL* variation and PUFA intake on TAG concentrations. Replication for other fat types (SFA and MUFA) is required in addition to investigating other macronutrients (protein and carbohydrates). The diversity in ethnicity within these studies is limited and therefore, examining other ethnicities is warranted.

1.8.2 Apolipoprotein E

The most commonly studied apolipoprotein gene polymorphism is *APOE* due to its key role in lipid metabolism [111]. The human *APOE* gene is located on chromosome 19q13.2 and encodes a polymorphic protein of 299 amino acids [112]. Two SNPs in exon 4 (rs429358, and rs7412) give rise to three major isoforms encoded by three alleles: E2, E3, and E4 [113]. Most populations studied reported that the E4 allele is associated with a high risk of CVD due to an increase in TAG and LDL-C levels, whereas E2 carriers is associated with low levels of circulating LDL-C [111]. Therefore, *APOE* is considered as a potential modulator of plasma lipid response, which is altered by dietary fat composition [114].

In healthy individuals from Costa Rica, a statistically significant interaction was observed between the *APOE* common genotype (rs429358 and rs7412; E2, E3, and E4) and SFA intake on VLDL and HDL-C. The E2 allele carriers who consumed high SFA [13.5% total energy (TE)] were associated with higher VLDL and lower HDL-C concentrations, while the opposite association was observed in E4 allele carriers [87]. Moreover, an interaction was demonstrated between the *APOE* (rs405509) and dietary SFA intake on plasma LDL-C and HDL-C in an Inuit population [115]. Conversely, other studies have not shown a significant interaction between the *APOE* common genotype and dietary fat on lipid levels in Caucasian and Black American populations [116, 117].

E4/E4 individuals who followed a lower-fat-cholesterol diet (34% fat, 265 mg cholesterol/day) showed a significant reduction in total cholesterol, but there was a significant increase in total cholesterol among the E4/E4 group who followed a high-fat-cholesterol diet (38% fat, 300 mg cholesterol/day) [118]. In a chronic intervention study in which prospectively genotyped participants were randomised to a low-SFA diet (8% TE) versus a high-SFA diet (18%TE) supplemented with 3.45g docosahexaenoic acid (DHA)/day,

the E3/E3 and E4/E4 groups were reported to have lowered TAG with a high-SFA diet supplemented with DHA compared to those with a low-SFA diet [79]. In two fish oil supplementation studies (2-3g eicosapentaenoic acid (EPA)/DHA per day), the *APOE* genotype was demonstrated to have a significant effect on the plasma lipid profile [119, 120]. Individuals with the E2 allele displayed a marked reduction in postprandial TAG response [120], while men with the E4 allele exhibited a reduction in TAG [119]. The dependent effect of the *APOE* genotype on carbohydrates and MUFA has also been investigated, with lowered LDL-C found in E2 allele carriers compared with E3 and E4 allele carriers with a high-carbohydrate diet [121].

The most convincing evidence is the effect of fish oil supplementation on the *APOE* genotype. Despite extensive research on the interactions between the *APOE* genotype and dietary fat and fat composition (i.e. SFA, MUFA, and cholesterol), the reported outcomes are somewhat inconsistent.

1.9 Other genes related to lipid outcomes

This chapter, also, will summarize the findings from gene-diet interactions on lipid-related outcomes focusing on the most commonly studied candidate genes including Adiponectin (*ADIPOQ*), apolipoprotein CIII (*APOCIII*), apolipoprotein A5 (*APOA5*), apolipoprotein AI (*APOAI*), cholesteryl ester transfer protein (*CETP*), hepatitis lipase (*LIPC*), peroxisome proliferator–activated receptor (*PPARA*, and *PPARG*), and tumour necrosis factor-alpha **(***TNFA*) genes.

1.9.1 Adiponectin gene

The *ADIPOQ*, gene coding for adiponectin, is a common genetic marker associated with serum lipid level. Adiponectin is an adipocyte-derived hormone and the gene is located

on chromosome 3q27 [122]. Adiponectin has been shown to affect energy metabolism and insulin sensitivity that includes increasing mitochondrial fatty acid oxidation and enhancing TAG catabolism and fatty acid uptake, which consequently regulates lipid metabolism in skeletal muscle [123]. PPARA is also activated by adiponectin and regulates APOAI and apolipoprotein A2, leading to increased secretion of hepatic HDL-C. Therefore, plasma levels of TAG are negatively associated with adiponectin, while HDL-C concentrations are positively correlated with adiponectin. A minimum of 53 SNPs have been identified in the *ADIPOQ* gene [124]. Among these, SNPs +274G>T (rs1501299), -11377C>G (rs266729), and +45T>G (rs2241766) have been shown to be associated with CVD in previous studies [125, 126].

Recent studies have investigated the interaction between these SNPs and diet on determining lipid profile levels. A cross-sectional study has reported that three *ADIPOQ* haplotypes of SNPs rs1501299 (G/T), rs266729 (C/G), and rs2241766 (T/G) were found to interact with MUFA:SFA ratio on determining TC, and LDL-C levels (P=0.002 and 0.02, respectively) in Korean children (n=687). Individuals carrying the G-T-G haplotype exhibited higher levels of TC and LDL-C than non-carriers when the ratio of MUFA:SFA was <1 [127]. Inconsistent responses of lipids (TAG, LDL-C, HDL-C, TC) to SFA-rich diet followed by a carbohydrate-rich or MUFA-rich diet by SNP rs266729 (C/G) were not significant in healthy Caucasian individuals (n=59) [128]. Carbohydrate intake was found to interact with the *ADIPOQ* SNP rs1501299 (G/T) on HDL-C concentrations (P=0.01) in Korean patients with type 2 diabetes (n=673). High carbohydrate intake (>65%) was inversely related to HDL-C concentrations in carriers of GG homozygotes [129]. However, this finding cannot be generalized to other populations or ethnic groups given that the participants were type 2 diabetic patients. Findings from *ADIPOQ* gene- diet interaction studies were inconsistent and these discrepancies in the results could be due to the type of study design, small sample size,

and heterogeneity in age and ethnicity of the participants.

1.9.2 Apolipoprotein genes

The most widely studied genetic markers, in relation to CVD risk factors and dietary intake, are the apolipoprotein genes (*APOAI*, *APOCIII*, *APOA5*, and *APOE*). The number of published articles investigating interaction of dietary factors with these genes have increased considerably [111].

1.9.2.1 Apolipoprotein A5

The APOA5 regulates plasma TAG levels by enhancing the activity of lipoprotein lipase and inhibiting TAG-rich lipoprotein production [111, 130]. It is located on TAG-rich lipoproteins and HDL-C particles [131]. Polymorphisms in the *APOA5* gene, which is part of the apolipoprotein (*APOAI-A4-A5-CIII*) gene cluster on human chromosome 11q23, represent the most commonly studied candidate SNP with regard to lipid outcomes [132, 133]. Genetic studies have consistently demonstrated associations of *APOA5* SNPs with increased TAG and increased risk of CVD [134, 135]. The *APOA5* SNP -1131T>C (rs662799) has been shown to be a strong candidate for its relevance to plasma lipid levels [134, 136].

Several gene-diet interaction studies have investigated the impact of *APOA5* and dietary intake on plasma lipid [137, 138]. Cross-sectional studies conducted on the Caucasian population (n=1465) and Puerto Ricans (n=802) have demonstrated that SNP rs662799 (T/C) interacts with total fat intake [(TE) %, and grams] in determining TAG, TC and VLDL concentrations ($P_{interaction}$ ranges from 0.002 to 0.03) [137, 138]. Participants with the C minor allele had higher TAG levels when they consumed fat >31% of TE in Puerto Ricans, while in Spanish Caucasians, among those who consumed high fat intake, the C allele carriers had

lower TAG and VLDL levels [137, 138]. This could be because the Spanish Caucasians consumed a traditional Mediterranean Diet, which mainly consists of MUFA from olive oil. In contrast, two studies from a Slavic Caucasian population (n=5487) and Mexican population (n=200) failed to demonstrate a significant interaction between SNPs, rs662799 (T/C) and rs3135506 (C/G), and dietary fat (as total, SFA, or PUFA) on lipid [139, 140]. The lack of interaction could be because only 2% of the participants carried the minor *APOA5* gene allele, participants were relatively old [139] and the sample size of the Mexican study was very small [140].

A significant interaction between SNP rs662799 (T/C) and n-6 PUFA intake, in determining fasted TAG concentration, was demonstrated in Caucasians (n=2418) from the Framingham Study. In carriers of the C allele, higher PUFA intakes (>6 % energy) were shown to be associated with higher TAG [141]. In a 2-year longitudinal study (multi ethnic majority Caucasian) in which participants (n=734) were randomised to a high fat diet or a low-fat-diet group, G allele carriers at SNP rs964184 (C/G) were reported to have a greater decrease in TC and LDL-C, and a greater elevation in HDL-C levels than non-carriers after a low fat diet [142]. Following a diet high in carbohydrates and low in fat (70% and 15% of TE, respectively), C allele carriers of the SNP rs662799 (T/C) had higher TAG and TAG-rich lipoprotein than TT genotype carriers [143].

An interaction between dietary carbohydrate intake (as whole grain and legumes) and *APOA5* SNP rs662799 (T/C) on TAG levels was reported in Korean type 2 diabetic patients (n=185). Individuals carrying the C risk allele had higher TAG concentrations than TT genotype carriers after consuming refined rice [144]. From these reported findings, consistencies were found in *APOA5* SNP–fat interactions on lipid levels. Further studies are required to explore subgroups of dietary fat (SFA, MUFA, and PUFA) and carbohydrates in

addition to investigating other ethnicities using different study designs.

1.9.2.2 Apolipoprotein A-I

The APOA-I is the major structural apolipoprotein component of HDL-C. Its function is to activate lecithin: cholesterol acyltransferase and facilitate reverse cholesterol transport. The *APOAI* genes are located in the apolipoprotein (*APOAI-A4-A5-C3*) gene cluster on chromosome 11q23-q24 [145]. The most commonly and widely studied genetic variation is SNP rs670 (G/A) which is located in the promoter region of the gene. The presence of the minor A allele has been shown to be associated with increased HDL-C and APOA-I plasma levels [146, 147]. The studies have reported interaction between diet and *APOAI* genes and have focused mainly on dietary fat and its subgroups (SFA, MUFA and PUFA) [115, 148].

Fat intake and SFA was reported to interact significantly with SNP rs670 (G/A) and on fasted TC, LDL-C, and LDL-C/HDL-C ratio in Caucasian (n=1754) and Inuit (n=553) populations [115, 148]. Among those who consumed habitual high fat diet (>35% TE), GG genotype carriers had higher LDL-C/HDL-C ratio compared to those carrying A allele [148]. Additionally, SNP rs5070 at *APOAI* gene significantly interacted with total fat and SFA intake on LDL-C and HDL-C levels (P interaction range= 0.03 to 0.01) [115].

There was significant interaction between SNP rs670 and PUFA intake on HDL-C (P=0.005) where higher PUFA intake (>8% TE) was associated with higher HDL-C concentration in women carrying the A allele compare to those carrying GG genotype [149]. The response to a MUFA diet (22% TE) was also investigated where healthy men carrying GA heterozygous genotype had increased LDL-C levels, compared to GG genotype carriers [150]. Although, it is apparent from previous studies that A allele carriers are presented with favorable lipid levels in response to various types of fat i.e. SFA and PUFA, replication of

these findings is warranted, as well as use of randomly controlled dietary interventions, which give stronger evidence for implementing dietary recommendation.

1.9.2.3 Apolipoprotein C-III

The APOCIII is primarily produced by the liver and is a major protein of VLDL and HDL particles [111, 151]. An *in vitro* study reported that this protein play a role in inhibiting activity of LPL and hepatic lipase enzymes and also the uptake of TAG-rich lipoproteins by hepatic receptors [152]. Thus, the *APOCIII* gene strongly correlates with higher TAG concentration [153]. A number of *APOCIII* gene SNPs [-455T>C (rs2854116), -625 del, 3238C>G (rs5128)] have been described as possible genetic markers, exhibiting variability in lipid responsiveness to modified fat intake [115, 154].

Cross-sectional studies have examined the interaction between the *APOCIII* polymorphisms [rs2854116 (T/C, -455T>C), -625 del, rs5128 (C/G, 3238C>G)], total fat, and saturated fat on lipid levels. A significant interaction was found between *APOCIII* SNPs, rs2854116, rs5128, and-625 del and total fat intake and SFA intake on TC and LDL-C in Puerto Ricans (n=336) and Inuit (n=553) populations [115, 154]. Carriers of the T allele at SNPs, rs2854116 and -625T, had lower TC and LDL-C concentrations after consuming a lower SFA diet (<11 % of TE) [154]. In a Chinese population (n=56), where individuals followed a diet high in carbohydrates and low in fat (70% and 15% of TE, respectively), T allele carriers of SNP rs2854117 had higher TAG and TAG-rich lipoproteins than CC genotype carriers [143]. Inconsistencies in the effect of T allele on lipids can be explained in part by different study designs, heterogeneity in ethnicity, and possibly different sources of fats.

In response to a high MUFA diet (22% TE) for 28 days, carriers of the G allele of SNP rs5128 were reported to have a greater decrease in LDL-C (P=0.0003) and TC (P=0.009) in

young men (n=90) [155]. A significant interaction between SNP rs5128 and habitual western dietary patterns (high in fast food, salty snacks, and soft drinks) in determining HDL-C concentration (P = 0.02 in men) was demonstrated in metabolic syndrome (MetS) patients and controls from Iran. In the carriers of the G allele, higher adherence to western dietary patterns were shown to be associated with lower HDL-C compared to CC homozygotes [156]. Although there is some consistency in the reported findings regarding fat intake (SFA) and circulating lipids according to *APOCIII* gene polymorphisms, more investigations are required including a larger sample size to confirm these associations.

1.9.3 Cholesteryl ester transfer protein gene

The CETP is a plasma lipid transfer protein which plays a fundamental role in the metabolism of HDL-C. It has the ability to facilitate the transfer of cholesteryl esters from HDL to apolipoprotein B-containing lipoproteins such as TAG rich lipoproteins (CM and VLDL), with TAG, increasing HDL-C metabolism and clearance, resulting in lower circulating HDL-C concentrations [68, 157]. The most widely studied polymorphism in the *CETP* gene is the SNP rs708272 (C/T) (*TaqIB*) polymorphism, which has been shown to be associated with decreased CETP activity, resulting in greater HDL-C concentrations in Caucasian and Black individuals [158, 159]. Currently, a strong consistency exists among studies, indicating that T (B2) carriers exhibit greater HDL-C and lower CETP activity than CC (B1B1) subjects [69].

The reported interactions between *CETP* gene SNP rs708272 (C/T) and fat intake on circulating HDL-C levels have been conflicting. In diabetic Caucasian men, significant interactions were observed between SNP rs708272 (C/T) and total fat intake (P=0.003), SFA (P=0.02) and MUFA (P=0.04) intake on HDL-C [160]. However, this SNP rs708272 did not interact with total fat intake on HDL-C in the Framingham Study (n=12000) and in a Spanish

population (n=4210) [85, 161]. Conflicting findings could be due to differences in age and health status of the groups, and possibly the impact of habitual diets.

A significant interaction between another SNP at *CETP* gene rs5882 and total fat intake in determining total cholesterol concentration (P=0.04) has been demonstrated in an Inuit population (n=553) [115]. Recently, gene-diet interactions have been reported by intervention studies investigating diet high in MUFA [162]. The concentrations of HDL-C, and TAG (P=0.006, *P*=0.040 respectively) were significantly affected by the interaction between Mediterranean diet (35% TE fat, 22% TE MUFA) and SNP rs3764261 (G/T) in MetS patients (n=424), with carriers of T allele displaying higher HDL-C concentration and lower TAG concentration compared to GG common genotype carriers [162]. Findings from interactions between *CEPT* SNPs and diet (i.e. fat intake) did confirm strong evidence for interactions on HDL-C, thus, further investigations are required for strong evidence and additional randomized clinical trials are needed.

1.9.4 Hepatic lipase gene

Hepatic lipase (*LIPC*) is a lipolytic enzyme that hydrolyzes TAG and phospholipids in all major classes of lipoproteins. Also, it serves as a ligand that facilitates the binding and uptake of lipoproteins, including HDL-C, through the proteoglycan receptor pathways. Given these roles, hepatic lipase has an important impact on circulating HDL-C concentrations [66, 163], and hence, it has been studied in relation to CVD risk factors [164]. In addition, deficiency in hepatic lipase causes elevated HDL-C levels [165]. The common SNP rs1800588 (C/T; -514C→T) that is located in the promoter region of *LIPC* gene is the most commonly studied polymorphism in relation to HDL-C concentrations, where the minor T allele is associated with decreased activity of this enzyme and contributes to higher fasted HDL-C [166, 167].

Observational studies have reported a strong gene-nutrient interaction between SNP rs1800588 (C/T) at *LIPC* gene and total fat intake on determining HDL-C levels in different ethnic groups such as Caucasian, African, Inuit, and Indian populations [85, 115, 168, 169]. Among those who consumed a low-fat diet (< 30% TE), T allele carriers had higher HDL-C concentration compared to those with the CC genotype [85, 168, 169]. Additionally, SNP rs1800588 (C/T) at *LIPC* gene significantly interacted with SFA intake on HDL-C in Caucasian men with type 2 diabetes (n=780), where T allele carriers displayed elevated HDL-C concentration with low SFA intake (<11% TE) [170]. The same SNP, also, showed interactions with total fat and SFA intakes on TAG levels in Inuit and East Asian populations [115, 169].

1.9.5 Peroxisome proliferator–activated receptor genes

Peroxisome proliferator–activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily, with 3 isotypes expressed in humans and encoded by different genes (*PPARA*, *PPARG*, and *PPAR delta*) [171].

1.9.5.1 Peroxisome proliferator–activated receptor alpha genes

PPARA gene is involved in numerous biological processes, and is strongly implicated in pathways connected with lipid metabolism, including mitochondrial fatty acid beta-oxidation. Given these roles, PPARA is primarily expressed in tissues with high levels of fatty acids, such as liver, heart, and skeletal muscle. PPARA has also been stated to be associated with concentrations of TAG, HDL-C, and the overall plasma lipid profile [171]. In humans, 50 coding SNPs in *PPARA* gene have been described, and the SNP rs1800206 (C/G; Leu162Val) is the most widely studied SNP. Association between *PPARA* SNP rs1800206 and lipid traits have been examined in many studies, providing evidence for an association

with TAG, LDL-C, and HDL-C levels [124].

Fatty acids, primarily PUFA, have been identified as natural or synthetic ligands for *PPARA*, and studies have shown that high concentrations of fatty acids increase the transcriptional activation of the gene [172, 173]. Thus, extensive research has investigated the interaction between PUFA and the *PPARA* gene on lipid outcomes. A significant interaction between SNP rs1800206 (C/G) and n-6 PUFA intake on TAG concentrations (P=0.04) was shown in Caucasian Americans (n=2106). The G allele carriers had higher TAG levels than C allele carriers among those who consumed <4% TE n-6 PUFA, whereas the opposite was observed when >8% TE n-6 PUFA was consumed [174]. Also, significant gene-diet interactions were observed between SNP rs6008259 (G/A; in the untranslated region of gene) and n-6 PUFA on TC and LDL-C (P for all comparisons <0.03) in Caucasian Americans, where among those who consumed high n-6 PUFA intake (>8 g/d) AA genotype carriers had significantly lower levels of TC and LDL-C [175]. However, results obtained from randomised clinical trials were inconsistent. In response to a 4 week diet with a high PUFA:SFA ratio (10%:9% TE), G allele carries had lower TC compared to those with CC genotype in healthy men (n=20) [176]. While no significant interaction between the same SNP and n-3 PUFA (5g supplementation of fish oil for 6 weeks) on lipids was found in young healthy men (n=28) [177]. These discrepancies could be due to differences in the type of PUFA and duration of intervention.

Besides the SNP rs1800206, which is rare in Asian and African American populations [178, 179], another SNP rs1800234 (T/C; V227A) has been identified in Chinese and Japanese populations. In Chinese women, an interaction between PUFA intake and SNP rs1800234 (T/C) on HDL-C concentrations (*P*=0.049) was reported. In women carrying the C allele, increased PUFA intake (g) was inversely associated with HDL-C concentration [179].

In African Americans, significant gene-diet interactions were observed between SNP rs3892755 (C/T) and n-3 PUFA on TC and LDL-C (P= 0.03, and 0.02, respectively), where among those who consumed high n-3 PUFA intake (>0.32 g/d), TT genotype carriers had significantly lower levels of TC and LDL-C. [175].

1.9.5.2 Peroxisome proliferator–activated receptor gamma genes

Another member of the nuclear hormone receptor superfamily is PPARG, which has been shown to control the expression of genes involved in adipogenesis, regulation of insulin sensitivity and lipid homeostasis [180]. It acts by stimulating hydrolysis of the circulating TAG and the subsequent entry of fatty acids into the adipose cells [181]. PUFA is a major natural ligand of *PPARG*, which exists in two protein isoforms: *PPARG1* and *PPARG2*. *PPARG1* is expressed in many tissues in low levels, while *PPARG2* is exclusively expressed in adipose tissue.

The PPARG SNP rs1801282 (C/G; Pro12Ala) is a common polymorphism that has been extensively studied [124]. A significant effect of SNP rs1801282 (C/G) on the TAG response to a high n-3 PUFA diet (3.6g/d for 3 months) was reported, where G allele carriers had lower TAG levels compared to those with CC genotype [182]. Other studies have examined the interaction between the same SNP and PUFA:SFA ratio on lipid responses. Findings showed that G allele carriers had higher TC and LDL-C when PUFA:SFA ratio was <0.33, while G allele carriers had lower TC and TAG levels when PUFA:SFA ratio was >0.34 [183].

In an Inuit population, interaction between total fat and SFA intake and SNP rs10865710 on TC (P= 0.01, and 0.007 respectively) and LDL-C concentrations (P= 0.01, and 0.008 respectively) was observed [115]. Findings from previous studies have demonstrated robust evidence for the interaction between *PPARG* gene cluster and PUFA

intake

1.9.6 Tumour necrosis factor-alpha

The pro-inflammatory cytokine, tumour necrosis factor-A (TNFA), is secreted by both macrophages and adipocytes and has an important effect on lipid metabolism. Elevated levels of TNFA are related to increased serum TAG and VLDL-C, and decreased levels of circulating HDL-C [184]. Studies have also shown an association of TNFA with dyslipidaemia [185]. In support of this, the *TNFA* SNP rs1800629 (G/A -308G/A) has been shown to increase transcription of the *TNFA* gene; thereby increasing TNFA production [186]. This association may be mediated through interactions between dietary fat intake and polymorphisms within the *TNFA* gene [187]. The SNP rs1800629 has been shown to modulate the relationship between dietary fat intake and lipid outcomes in various populations [187, 188].

In black South African women, SNP rs1800629 (G/A) interacts with alpha linolenic acid and PUFA on modulating TC/HDL-C ratio (P= 0.036) and LDL-C (P= 0.026), respectively. Increased intake of alpha linolenic acid (% TE) was associated with increased TC/HDL-C ratio in those carrying GG genotype compared with A allele carriers, whereas increased intake of PUFA (% TE) was associated with decreased LDL-C in GG genotype carriers compared with A allele carriers [189]. In healthy and type 2 diabetic Caucasians, both SNPs rs1800629 (G/A) and rs361525 (G/A; -238G/A) significantly interacted with PUFA on HDL-C (P=0.04 and 0.0003, respectively). In carriers of the GG genotype of the SNP rs1800629, PUFA intake (%TE) was positively associated with HDL-C but negatively associated with A allele carries [187, 188]. The minor A allele carriers of the SNP rs361525 (G/A) had higher HDL-C with increase in PUFA intake [187].

In white South African women, a significant interaction between SFA intake and SNP

rs1800629 (G/A) on TC (P=0.04) was reported. Increased intake of SFA (%TE) was associated with a decrease in TC levels in those with GG genotype compared with A allele carriers [88]. Furthermore, in response to a Mediterranean diet (> 35% fat TE, mostly from MUFA) for 12 months, MetS patients with GG genotype had significantly lower TAG concentrations (P=0.005) compared with A minor allele carriers (n=507) [190]. These findings indicate that the *TNFA* gene SNPs strongly interacts with dietary fat (i.e. PUFA, SFA, and MUFA) in determining lipid levels, where GG genotype carriers were found to have favourable lipid levels.

1.10 Study designs and their role in identifying gene-diet interactions

Study designs are generally categorised as observational studies, such as cross-sectional, or case-control and experimental studies, such as randomized clinical trials [191]. One of the most common designs is a cross-sectional study, which determines the association between exposure and disease-related outcomes in specified populations at a given one time point. Disease develops over a period of time, thus, in cross sectional studies it is difficult to identify the causal relationship between a risk factor and disease, at a single point in time. Another limitation in cross sectional studies is confounding factors, which are factors associated with both the exposure and outcome. Thus, when running statistical analysis, adjustment for these confounders can be included in the regression model to minimize their confounding effects. The food frequency questionnaire (FFQ), is a questionnaire consisting of a finite list of foods and beverages. Participants indicate their usual frequency and portion size of consumption according to response categories over a period of time. The FFQ is the most popular measure of food consumption in large observational studies, easy to use, low burden on participants, and representative of a long term dietary intake [41]. Another example of an observational study is a case-control design, where a group of cases affected

with a disease of interest are collected together with a group of control individuals free from the disease at a given point of time [192]. This study design is similar to a cross sectional design, thus, it shares the same limitation. The main strength of an observational study is that a large number of samples can be collected [191]. From a nutrigenetics perspective, observational studies (cross sectional and case control) are affected by inherent bias, phenotypes have higher levels of variability over time, when testing gene-diet interactions. For example, TAG concentrations are variable over time and using fasting values at a single point is considered as a limitation [193]. Lack of replication of the initial findings is a common limitation. The other limitation includes reporting bias in FFQs, as it is self-reported by participants, can limit the study power. However, GWAS studies have identified disease-predisposing variants from cross-sectional study design, and these variants are less likely to be affected by confounding factors [194].

The other type of study design is experimental (randomized clinical trials), in which a group of volunteers, who meet specified selection/inclusion criteria, are randomly assigned to receive either the experimental treatment (intervention), or the control treatment (commonly the standard treatment for the condition). Thus, observed changes in the outcome; i.e. serum TAG, is a result of the intervention treatment. One of the main advantages of an experimental trial is the use of a blinding approach where the volunteer and/or researcher does not know whether he or she is receiving the treatment or control diet [191]. The common limitation in an intervention study is the small sample size (<200 participants) due to logistics of study management and costs, drop outs during the intervention period (especially with long periods of intervention) and insufficient volunteer adherence to their assigned treatment [41]. The crossover design is an alternative to the randomized clinical trial, which allows comparison within- and between-groups. In this type of study, half of the volunteers are randomly assigned to start with the control diet and then switch to the experimental treatment and the

other half are assigned in an opposite sequence. In this design, a washout period are generally required to avoid any carry-over influence of prior treatments [191]. The main strength of using randomized clinical trials for investigating gene-diet interaction studies is that they provide direct evidence to instruct genotype-based dietary modifications for future public health strategies. Another advantage of intervention studies is that it minimizes the effect of confounding factors, which introduces bias when exploring gene-diet interactions. However, the major concern of dietary intervention trials is the small sample size, which can reduce the power of detecting gene-diet interaction effect sizes [194].

The last study design is the postprandial study, which refers to calculation of variations in lipid, mainly TAG, values during follow up period after certain diet was given. Circulating TAG increases (postprandial lipaemia) within 2-3 hours of meal ingestion and can remain elevated for up to 5h after intake of a meal containing (30–60g fat). Given that most people usually consume fat-containing meals every 4–5h, it is clear that the most appropriate state of TAG metabolism is postprandially. The specificity of the postprandial state is an accumulation of lipoprotein particles in the circulation derived from both the liver and small intestine. During the postprandial state, TAG and cholesterol can be exchanged between lipoproteins; this is important in reverse cholesterol transport mediated by HDL-C particles and cholesterol homeostasis but it can also cause the generation of atherogenic lipid. These changes in lipid circulation during the postprandial state highlights the importance of the postprandial design in investigating gene-diet interactions [195].

1.11 Relationship between vitamins D and lipid outcomes

Vitamin D is an essential fat-soluble vitamin, which has several vital functions including formation of bone through regulation of calcium and phosphate homeostasis [196]. Besides these physiological functions, vitamin D has shown to play a role in modulating

immune function [197], blood pressure control, and prevention of CVD [198]. Also, vitamin D has been reported to be associated with lipids [199]. Lower vitamin D status has been shown to be associated with dyslipidemia (reduced HDL-C, elevated TAG) in adult men [200]. In a cross sectional study in children, it was found that each 10 nmol/L increase in vitamin D was associated with decreased concentrations of non-HDL-C, total cholesterol, and TAG [201]. This association may be, in part, explained by increased absorption of intestinal calcium, which could reduce synthesis and secretion of hepatic TAG [202]. Another explanation could be that vitamin D is associated with increased activity of LPL enzyme resulting in lower TAG concentrations and increase HDL-C [203]. Previous studies have demonstrated associations between SNPs at vitamin D binding protein (*GC*) and cytochrome P450 family 2 subfamily R member 1 (*CYP2R1*) genes and risk factors related to gestational diabetes mellitus [204].

1.12 Personalised nutrition approach

A beneficial approach from a nutrigenetics perspective will be the development of tailored nutritional advice for individuals, revising individualized dietary guidelines and developing personalised nutrition regimes based on their individual genomes, which will promote health, or prevent or reduce the incidence of disease [11]. However, at present, a large gap exists between nutrition recommendations and individual eating behaviour. Therefore, the implementation of a personalised approach could be more widely-accepted by the public. A recent study found that gene-based personalised nutrition to reduce SFA intake was more effective than general dietary guidelines for E4 allele carriers [205]. It is also important to determine whether individuals would agree to undergo genetic tests for the purpose of designing a personalised nutrition regime [15]. Ultimately, clear guidance from nutrigenetics studies is required for the implementation of personalised nutrition, which can only be achieved through the use of large, statistically powered studies, examining various

ethnic groups, considering the variety in dietary patterns worldwide, and conducting additional testing for other modifiable factors such as physical activity and smoking status.

1.13 Conclusions

The findings from these studies indicate that lifestyle (diet) modification, which attempts to optimize plasma lipid concentrations and prevent CVD, must consider underlying genetic factors. Gene-diet interaction studies contribute to elucidating the potential relationships between nutrients, genetic factors, and diseases such as CVD.

Despite growing evidence from nutrigenetics studies, consistent challenges emerge which affect decisions surrounding dietary strategies to improve health. This limitation is seen through conflicting results in the existing literature [206, 207]. An additional limitation lies in the single hypothesis test; each study investigated interactions between single genetic variants and dietary components on a single phenotype; hence, there is considerable risk of overestimating the significance of positive interactions. An additional limitation is that the majority of published literature on nutrigenetics is secondary analysis of studies which were not initially conducted to investigate gene-diet interactions [4]. Moreover, the majority of nutrigenetics studies have been conducted in Caucasian populations, with limited research in South Asian and Arab populations.

The requirements for future studies include appropriate study design including appropriate sample size. The effect of some of the gene variants may impact on postprandial metabolic stress, therefore, gene-diet interactions studies should be designed to examine the fasting and postprandial state [208].

In summary, an increased number of nutrigenetics studies are needed to determine the link between SNPs, dietary exposure, and health outcomes in order to determine consistent

data for CVD prevention. Understanding how these interactions influence metabolic pathways at the molecular level is necessary to determine mechanisms of action. Only then can a personalised dietary approach become a potential therapy for the prevention of diet-related diseases.

1.14 Aims and outline of the thesis

Based on the hypothesis that the dietary factors would influence lipid concentrations and that this may be modulated by common SNPs in the *LPL* and *APOE* genes, the aims of this thesis were

- to examine the association between selected common SNPs at the *LPL* and *APOE* genes with lipid outcomes (TAG, HDL-C, LDL-C, and total cholesterol),
- to examine the interaction between these SNPs and dietary factors (fat, carbohydrate, and protein as total energy %) on lipids using several study designs: 1) chronic dietary intervention studies, 2) acute postprandial dietary intervention studies, 3) case-control, 4) cohort-based cross sectional 5) dietary intervention crossover on two different ethnic populations (Caucasian, and Asian Indian).

The aims and the hypothesis of each chapter are outlined below.

Chapter 2: It was hypothesized that *LPL* gene was associated with postprandial lipid concentration and this association might provide a more physiological perspective of disturbances in lipoprotein homeostasis compared to assessment in the fasting state. Therefore, the aim of this chapter was to investigate the influence of two commonly studied *LPL* polymorphisms (rs320, *HindIII*; rs328, *S447X*) on postprandial lipaemia in 261

participants using a standard sequential meal challenge.

Chapter 3: It was hypothesized that *LPL* SNPs interacted with dietary factors on determining lipid concentrations. Also, given that there were no gene-diet interaction studies, to date, in Asian Indian populations, this chapter examined the association of four common *LPL* SNPs (rs1121923, rs328, rs4922115, and rs285) with lipid outcomes and investigated the interactions of these four polymorphisms with dietary factors on lipid in up to 1,845 Asian Indian participants (788 type 2 diabetes cases and 1,057 controls) from the cross-sectional Chennai Urban Rural Epidemiological Study (CURES).

Chapter 4: The hypothesis of this chapter was that *APOE* and *LPL* SNPs were associated with lipid levels and this association might be modulated by dietary factors. Therefore, this chapter investigated the association of two common SNPs (rs320 and rs328) at *LPL*, seven tagSNPs (rs405509, rs769450, rs439401, rs445925, rs405697, rs1160985, and rs1064725) at the *APOE* gene, and a common *APOE* haplotype (rs429358, and rs7412; E2, E3, and E4) with blood lipid. It also examined the interaction of these SNPs with dietary factors in 660 Caucasians from the baseline data of the Prevention of Cancer by Intervention with Selenium (PRECISE) study. The findings of the PRECISE study were replicated using 1,238 individuals from the Caerphilly cohort.

Chapter 5: It was hypothesized that the replacement of energy from SFA with MUFA or PUFA would have a beneficial effect on lipid levels and that *LPL* and *APOE* genotypes would contribute to the individual variability. It has been revealed, however, that inter-individual variability in lipid responses to dietary fat intake is influenced by genetic variation. Therefore, the aim of this chapter was to determine whether the *LPL* and *APOE* genotypes modified lipid responses after substituting SFAs with MUFAs or n-6 PUFAs in adults with moderate CVD risk. A retrospective analysis was conducted on 120 participants in the

Dietary Intervention and VAScular function (DIVAS) study.

Chapter 6: The hypothesis of this chapter is that SNPs of genes involved in vitamin D may affect lipid levels, and this effect may be influenced by vitamin D supplementation. Therefore, this chapter aimed to evaluate the association of the SNPs at *LPL*, *APOE* and 4 SNPs related to vitamin D genes with lipid and investigated the interaction between vitamin D fortified dairy drink and SNPs on lipid outcomes in 18 men with sub-optimal vitamin D status.

Chapter 7: This chapter will focus on the discussion, which will be based on the findings from all the chapters, and the future prospects of this PhD work.

Table 1.1 Summary of SNPs that were examined in each chapter

Chapters	**Study design**	**SNPs analyzed**
Chapter 2 Impact of Lipoprotein Lipase gene polymorphism, *S447X*, on postprandial triacylglycerol and glucose response to sequential meal ingestion	Postprandial study	*LPL* SNPs (rs320 and rs328) and seven *APOE* tagSNPs (rs405509, rs769450, rs439401, rs445925, rs405697, rs1160985, and rs1064725)
Chapter 3 High fat diet modifies the association of *LPL* gene polymorphism with HDL-C in an Asian Indian population	Case-control study	*LPL* SNPs (rs1121923, rs328, rs4922115 and rs285) *APOE* SNPs were not available in the dataset
Chapter 4 Apolipoprotein E and lipoprotein lipase gene polymorphisms, dietary factors and blood lipids	Cross sectional study	*LPL* SNPs (rs320 and rs328), seven *APOE* tagSNPs (rs405509, rs769450, rs439401, rs445925, rs405697, rs1160985, and rs1064725), and one *APOE* haplotype (rs7412 and rs429358),
Chapter 5 Apolipoprotein E gene polymorphism modifies fasting total cholesterol concentrations in response to replacement of dietary saturated with monounsaturated fatty acids in adults at moderate cardiovascular disease risk	Intervention study	*LPL* SNPs (rs320 and rs328) and seven *APOE* tagSNPs (rs405509, rs769450, rs439401, rs445925, rs405697, rs1160985, and rs1064725)
Chapter 6 Impact of polymorphisms in genes related to vitamin D	Crossover study	*LPL* SNPs (rs320 and rs328), seven *APOE* tagSNPs (rs405509, rs769450, rs439401, rs445925, rs405697, rs1160985, and rs1064725), and four vitamin D-related

metabolism and serum lipids on vitamin D concentrations and lipid responses to vitamin D fortified test meals.		SNPs *NADSYN1* SNP rs12785878, *CYP24A1* SNP rs6013897, *GC* SNP rs2282679, *CYP2R1* SNP rs12794714

Table 1.2 Summary of studies that have investigated gene-diet interactions on lipids

Gene name	**Gene symbol**	**SNP**	**Study population, Sample size, N (healthy, disease)**	**Age**	**Ethnicity**	**Study design**	**Lipid outcomes**	**Dietary factors**	**P value for interaction**	**Reference**
1-acylglycerol-3-phosphate O-acyltransferase 4	*AGPAT 4*	rs3798943 rs9458172	N=210 (healthy overweight)	18-50	Caucasian (Canadian)	Intervention study	TAG	Fish oil supplement	P=0.02 P=0.01	[209]
Adiponectin	*ADIPOQ*	rs266729	N=59 (healthy)	21-25	Caucasian (European, Spanish)	Intervention study	TAG, LDL-C, HDL-C, TC	SFA-rich diet followed by CHO-rich diet or MUFA-rich diet	Not significant	[128]
		rs2241766 rs1501299 rs266729 (haplotype)	N=687 (healthy Children)	7-11	Korean	Cross sectional study	TC LDL-C	MUFA:SFA intake ratio	P=0.002 P=0.02	[127]
		rs1501299	N= 673 (Type 2 diabetes patients)	40-85	Korean	Cross sectional study	HDL-C	CHO intake	P=0.01	[129]

Angiopoietin-like 4	*ANGPTL4*	rs116843064	N=8511 (healthy)	45-64	Caucasian (U.S)	Cross sectional study	HDL-C	CHO intake	P=0.04 in men only	[210]
Angiotensinogen	*AGT*	rs699	N=553 (healthy)	36-38	Inuit population	Cross sectional study	TC	Total fat intake	P=0.006	[115]
							TC	SFA intake	P=0.002	
							LDL-C	Total fat intake	P=0.01	
							LDL-C	SFA intake	P=0.003	
Apolipoprotein A5 *APOA5*		rs662799	N= 2148 (healthy)	49-51	Caucasian (U.S)	Cross-sectional study	TAG	n-6 PUFA intake	P=0.001	[141]
							VLDL		P=0.006	
		rs662799	N=802 (healthy)	45-75	Puerto Ricans	Cross-sectional study	TC	Total fat intake	P=0.03	[138]
							TAG		P=0.03	
		rs662799	N=1465 (healthy, overweight and obese)	20-65	Caucasian (European, Spanish)	Cross-sectional study	TAG	Total fat intake	P=0.003	[137]
							VLDL		P=0.002	
		rs964184	N=734 (healthy, overweight and obese)	30-70	Multi-ethnic (majority Caucasian)	Intervention study	TC	Fat diet (low compared to high)	P=0.007	[142]
							LDL-C		P=0.01	
							HDL-C		P=0.006	

		rs662799 rs3135506	N=5487 (healthy)	45-69	Caucasian (European, Czech)	Cohort study	TC, TAG, HDL-C	Total fat intake	Not significant	[139]
		rs662799	N= 185 (type 2 diabetic patient)	48-52	Korean	Intervention study	TAG	Dietary CHO source (whole grains and legumes)	P=0.001	[144]
		rs662799 rs3135506	N=200 young healthy	18-25	Mexican	Cross sectional	TAG, TC, HDL-C, LDL-C	Total fat intake and SFA intake	Not significant	[140]
		rs662799	N=56 young adult	22-26	Chines	Intervention study	TAG TAG-rich lipoprotein	High CHO low fat diet	P=0.05 P=0.01	[143]
Apolipoprotein B	*APOB*	rs693	N=87 (healthy)	40-65	Caucasian (European, Finnish)	Intervention study	VLDL HDL-C	Low SF diet, low cholesterol diet	P=0.05 P=0.05	[211]
		rs693	N=72 (Healthy men)	19-23	Caucasian (European, Spanish)	Intervention study	TAG	High MUFA diet	P=0.03	[212]
		rs693	N=553 (healthy)	36-38	Inuit population	Cross sectional study	TC	Total fat intake	P=0.02	[115]

		rs1042034	N=1128 (healthy, and carotid artery disease)	49-82	Caucasian (American)	Case-control study	TC	Dietary cholesterol intake	P=0.01	[213]
Apolipoprotein A1	*APOA1*	rs670	N=50 (healthy men)	19-29	Caucasian (European, Spanish)	Intervention study	LDL-C	MUFA diet	P=0.01	[150]
		rs670	N=1577 (healthy)	28–79	Caucasian (American)	Cross sectional study	HDL-C	PUFA intake	P=0.005 in women	[149]
		rs670	N=1754 (MetS and control)	35-60	Caucasian (European, French)	A prospective Case control study	LDL-C/HDL-C	Total fat intake	P=0.05	[148]
		rs670	N=553 (healthy)	36-38	Inuit population	Cross sectional study	TC	SFA intake	P=0.02	[115]
							LDL-C	Total fat intake	P=0.02	
							LDL-C	SFA intake	P=0.02	
		rs5070					HDL-C	Total fat intake	P=0.03	
							HDL-C	SFA intake	P=0.01	

Apolipoprotein A4	*APOA4*	rs675	N=41 (healthy men)	19-23	Caucasian (European, Spanish)	Intervention study	TC	SFA diet, low fat diet, MUFA diet	P=0.03	[214]
							LDL-C		P=0.03	
		rs5110					TC		P=0.03	
							LDL-C		P=0.031	
		rs675	N=91 (healthy)	21-42	Caucasian (American)	Intervention study	TAG	Egg consumption	P=0.0001	[215]
Apolipoprotein E	*APOE*	rs429358 rs7412	N=103 (healthy)	22-65	Black and Caucasian (American)	Intervention study	TC LDL-C	Change in SFA diet	Not significant	[116]
		rs429358 rs7412	N=420 (healthy)	20-65	Costa Ricans	Cross sectional study	VLDL	SFA intake	P=0.03	[87]
							HDL-C		P=0.02	
		rs429358 rs7412	N=65 (healthy men)	26-48	Caucasian (Canadian)	Intervention study	LDL-C	High CHO diet	P=0.04	[121]
		rs429358 rs7412	N=90 (healthy)	35-70	Caucasian (British)	Intervention study	TAG	High SFA-DHA diet	P=0.03	[79]

	rs429358 rs7412	N=996 (healthy)	25-46	Caucasian (European, Lithuanian)	Cross sectional study	TC LDL-C	SFA intake	Not significant	[216]
	rs405509	N=553 (healthy)	36-38	Inuit population	Cross sectional study	LDL-C HDL-C HDL-C	Total fat intake Total fat intake SFA intake	P=0.02 P=0.04 P=0.01	[115]
Apolipoprotein C-III *APOCIII*	rs5128	N=90 (healthy men)	18-26	Caucasian (European, Spanish	Intervention study	TC LDL-C	High MUFA diet	P=0.009 P=0.0003	[155]
	rs2854116 -625 del	N=336	29-53	Costa Rica (a mixture of Caucasian and Amerindian ethnic groups).	Cross sectional study	TC LDL-C	SFA intake	P=0.0004 P=0.01	[154]
	rs5128	N=553 (healthy)	36-38	Inuit population	Cross sectional study	LDL-C	Total fat intake SFA intake	P=0.03 P=0.03	[115]

		rs5128	N=1510 (MetS, and control)	29-55	Iran	Case-control study	HDL-C	Western dietary patterns intake	P=0.02 in men	[156]
		rs2854117	N=56 young adult	22-26	Chines	Intervention study	TAG TAG-rich lipoprotein	High CHO low fat diet	P=0.01 P=0.05	[143]
ATP-binding cassette (ABC) transporter	*ABCA1*	rs9282541	N=3591 (healthy)	34-62	Mexican	Cross sectional study	HDL-C	CHO intake	P=0.03 in premenopausal women	[217]
		rs2230806	N=553 (healthy)	36-38	Inuit population	Cross sectional study	TAG	SFA intake	P=0.04	[115]
ATP-binding cassette, sub-family G	*ABCG1*	rs4148102	N=1941 (healthy)	21-85	Caucasian (European, Spanish)	Cross sectional study	TC LDL-C	PUFA intake	P=0.006 P=0.003	[218]
acyl-CoA oxidase 1, palmitoyl	*ACOX1*	rs17583163	N= 208 (healthy)	22-38	Caucasian (Canadian)	Intervention study	TAG	PUFA diet	P=0.004	[219]

Cholesteryl ester transfer protein	*CETP*	rs708272	N=780 diabetic men	40-75	Caucasian (American)	Cross sectional study	HDL-C	Total fat intake SFA intake MUFA intake	P=0.003 P=0.02 P=0.04	[160]
		rs708272	N=12000 (healthy)	45-64	Caucasian (American) and African American	Cohort study	HDL-C	Total fat intake	Not Significant	[85]
		rs708272 -4,502C>T	N=4210 high CVD risk individuals	55-80	Caucasian (European, Spanish)	Cross sectional study	HDL-C	Total fat intake	Not Significant	[161]
		rs5882	N=553 (healthy)	36-38	Inuit population	Cross sectional study	TC	Total fat intake	P=0.04	[115]
		rs3764261	N=424 (MetS patients)	20-75	Caucasian (European, Spanish)	Intervention study	HDL-C TAG	Mediterranean diet	P=0.006 P=0.04	[162]
Cytochrome P450, family 1, subfamily A, polypeptide 1	*CYP1A1*	rs1048943	N=553 (healthy)	36-38	Inuit population	Cross sectional study	TC	Total fat intake	P=0.03	[115]

CD36 molecule	*CD36*	rs6969989	N=4210 (healthy women)	39-70	Korean	Cross sectional study	HDL-C	Oily fish intake	P=0.0001	[220]
							TAG		P=0.0001	
Endothelial lipase	*LIPG*	rs2000813	N=497 (healthy)	17-79	Caucasian (Canadian)	Cohort Study	HDL_3-C	Total fat intake	P=0.03 in women	[221]
							HDL_3-C	PUFA intake	P=0.003 in women	
Fatty acid desaturase	*FADS1*	rs174546	N=3575 (healthy)	30-50	Caucasian (European, Dutch)	Cohort Study	TC	n-3 PUFA intake	P=0.006	[222]
							HDL-C	n-6 PUFA intake	P=0.004	
		rs174547	N=4,635 (healthy)	45–68	Caucasian (European, Swedish)	Cohort Study	LDL-C	n-3 PUFA intake	P=0.01	[223]
							HDL-C	Ratio of n-3 PUFA and n-6 PUFA intakes	P=0.03	
Glycerol-3-phosphate acyltransferase	*GPAM*	rs17129561	N=210 (healthy overweight)	18-50	Caucasian (Canadian)	Intervention study	TAG	Fish oil supplement	P=0.04	[209]
		rs2792751							P=0.004	

Glucokinase regulatory protein	*GCKR*	rs780094	N=20986 (healthy)	40-79	Caucasian (European)	Cohort study	TAG TC	Mediterranean diet	Not significant	[224]
Hepatic lipase	*LIPC*	rs1800588	N=2130 (healthy)	28-79	Caucasian (U.S)	Cross-sectional study	HDL-C	Total fat intake	P=0.001	[168]
		rs1800588	N=2170 (healthy)	29-51	Chinese, Malaysian and Asian Indians	Cross-sectional study	TAG HDL-C/TG HDL-C (only with Indian)	Total Fat intake	P=0.001 P=0.001 P=0.01	[169]
		rs1800588	N=780 (Type 2 diabetic men)	40–75	Caucasian (American)	Cross-sectional study	HDL-C	SFA intake	P=0.003	[170]
		rs1800588	N=12000 (healthy)	45-64	Caucasian (American) and African American	Cohort study	HDL-C	Total fat intake	P=0.01	[85]
		rs1800588	N=553 (healthy)	36-38	Inuit population	Cross sectional study	TAG SFA intake	Total fat intake	P=0.02 P=0.03	[115]

Interleukin 6	*IL-6*	rs1800796	N=581 (healthy)	40-65	Chinese	Cross-sectional study	HDL-C	n-3 PUFA intake	P=0.02 in men	[225]
		rs2069845	N=268 (healthy and obese women)	18-45	Black and Caucasian (South African)	Cross-sectional study	TAG	Total fat intake	P=0.04 (in black)	[226]
							TC/HDL-C ratio	Total fat intake	P=0.02 (in black)	
		rs1800795					TAG	n-3PUFA intake	P=0.04 (in white)	
							TC/HDL-C	EPA intake	P=0.02 (in white)	
							TC/HDL-C	DHA intake	P=0.01	
Lipoprotein lipase	*LPL*	rs328	N=12000 (healthy)	45-64	Caucasian (American) and African American	Cohort study	HDL-C	Total fat intake	P=0.002 in white	[85]
		rs328	N=452 (MetS) replicated in 1754	35-70	Caucasian (European, 8 countries)	Cohort study	TAG	n-6 PUFA intake	P=0.04	[109]
		rs1059611					TAG	n-6 PUFA intake	P=0.04	

		rs13702	Ten cohorts study 27,756 individuals	32-77	Caucasian (European, and American)	Meta-analysis	TAG	PUFA intake	P=0.001	[110]
		rs13702	N=7187 (healthy)	55-80	Caucasian (European, Spanish)	Intervention study	TAG	MUFA intake, baseline Unsaturated fat intake, baseline After intervention, with Mediterranean diet	P=0.03 P=0.03 P=0.02	[108]
Liver X receptorα	*LXRα*	rs12221497	N=732 (healthy)	40-60	Caucasian (French-Canadian)	Cross sectional study	TC LDL-C	Dietary cholesterol intake	P=0.04 P=0.02	[227]
		rs3758674					TC LDL-C	Dietary cholesterol intake	P=0.03 P=0.02	
		-840C>A					TC LDL-C	Dietary cholesterol intake	P=0.04 P=0.02	

Lipin 1	*LPIN1*	rs4315495	N=373 (healthy, obese or overweight)	34-48	Caucasian (European)	Intervention study	TAG	Protein diet	P=4x10^{-6}	[228]
Monoglyceride lipase	*MGLL*	rs782440 rs6776142 rs555183 rs782444 rs6787155 rs1466571	N=210 (healthy)	18-50	Caucasian (Canadian)	Intervention study	LDL-C	n-3 PUFA supplementation	P=0.01 P=0.008 P=0.04 P=0.04 P=0.02 P=0.02	[229]
Methylene-tetrahydrofolate reductase	*MTHFR*	rs1801133	N=574 (healthy)	31-59	Caucasian (European, Greece)	Cross sectional study	Oxidized LDL	Mediterranean diet	P=0.001	[230]
Nitric oxide synthase 3	*NOS3*	rs1799983	N=450 (MetS patients)	53-55	Caucasian (European, 8 countries)	Intervention study	TAG	n-3 PUFA diet	P=0.01	[231]
Niemann-Pick C1-like 1	*NPC1L1*	rs2072183	N=1128 (healthy, and carotid artery disease)	49-82	Caucasian (American	Case control study	TC	Dietary cholesterol intake	P=0.01	[213]

Nuclear factor kappa B	*NFKB1*	rs28362491	N = 593 (healthy)	20-29	Caucasian (Canadian)	Cross sectional study	HDL-C	PUFA intake	P=0.02	[232]
			N = 103 (type 2 diabetes)	42–75			HDL-C	PUFA intake	P=0.005	
Paraoxonase	*PON1*	rs662	N=654 (healthy men)	25-74	Caucasian (European, Spanish)	Cross-sectional study	HDL-C	Oleic acid intake	Not significant	[233]
Peroxisome proliferator-activated receptor-alpha	*PPARA*	rs1800206	N=2106 (healthy)	44-64	Caucasian (U.S)	Cross-sectional study	TAG	n-6 PUFA intake	P=0.01	[174]
		rs1800206	N=20 (healthy men)	23-49	Caucasian (Canadian	Intervention study	TC	PUFA: SFA diet ratio	P=0.04	[176]
		rs1800234	N=2899 (healthy)	25-49	Chinese	Cross-sectional study	HDL-C	PUFA intake	P=0.04 (in women.)	[179]
		rs1800206	N=28 (healthy men)	21-53	Caucasian (Canadian)	Intervention study	TC HDL-C LDL-C TAG	n-3 PUFA diet	No significant	[177]

		rs6008259	N=13614 (healthy)	45-64	Caucasian (American) and African American	Cross-sectional study	TC	n-6 PUFA intake	P=0.03 in white	[175]
							LDL-C	n-6 PUFA intake	P=0.03 in white	
		rs3892755					TC	n-3 PUFA intake	P=0.03 in African	
							LDL-C	n-3 PUFA intake	P=0.02 in African	
Peroxisome Proliferator-Activated Receptor Gamma	*PPARG*	rs1801282	N=151 (healthy)	30-65	Caucasian (European 4 countries, and Australian)	Intervention study	TAG	n-3 PUFA diet compared to high fat diet	P=0.003	[182]
							TAG	n-3 PUFA diet compared to SFA diet	P=0.006	
		rs1801282	N=347(healthy)	30-70	Caucasian (British)	Intervention study	TC	PUFA: SFA diet ratio	P=0.02	[183]
							LDL-C		P=0.002	
							TAG		P=0.02	
		rs10865710	N=553 (healthy)	36-38	Inuit population	Cross sectional study	TC	Total fat intake	P=0.01	[115]
							TC	SFA intake	P=0.01	
							LDL-C	Total fat intake	P=0.007	
							LDL-C	SFA intake	P=0.008	

Peroxisome proliferator-activated receptor delta	*PPAR-delta*	rs2016520	N=340 (healthy)	37-60	Caucasian (French Canadians)	Cross sectional study	HDL-C TC\HDL-C ratio	Total Fat intake	P=0.003 P=0.04	[234]
Retinoid X receptor, α	*RXRA*	rs11185660 rs11185660 rs10881576 rs12339187	N= 208 (healthy)	18-50	Caucasian (Canadian)	Intervention study	TAG TAG TAG TAG	Total fat diet SFA diet SFA diet SFA diet	P=0.004 P=0.002 P=0.004 P=0.01	[219]
Toll-like receptor 4	*TLR4*	rs5030728	N= 676 (healthy)	20 - 29	Caucasian (Canadian)	Cross sectional study	HDL-C	SFA intake	P=0.003	[235]
Transcription factor 7-like 2	*TCF7L2*	rs7903146	N=1083 (healthy)	17-92	Caucasian (European American)	Intervention study	VLDL Postprandial TAG	PUFA diet	P=0.01 P=0.02	[236]
Tumor necrosis factor-alpha	*TNFA*	rs361525 rs1800629	N=109 (Type 2 diabetes)	42–75	Caucasian (Canadian)	Cross sectional study	HDL-C HDL-C	PUFA intake PUFA intake	P=0.003 P=0.001	[187]
		rs1800629 rs361525	N=595 (healthy)	20-29	Caucasian (Canadian)	Cross sectional study	HDL-C	PUFA intake	P=0.04	[188]

		rs1800629	N=223 (healthy and obese women)	18-45	Black	Cross sectional study	TC/HDL-C ratio	α -linolenic acid intake	P=0.03	[189]
							LDL-C	PUFA intake	P=0.02	
		rs1800629	N=148 (healthy and obese women)	18-45	Caucasian (South Africa)	Cross sectional study	TC	SFA intake	P=0.04	[88]
		rs1800629	N=507 (MetS patients)	20-75	Caucasian (European, Spanish)	Intervention study	TAG	Mediterranean diet	P=0.005	[190]
Vascular endothelial growth factor A	*VEGFR 2*	rs2071559	N=136 (healthy)	30-65	Japanese	Cross-sectional study	LDL-C	Western Diet	P=0.01	[237]
		rs1870377	N=179 (healthy)		Chinese Malaysian		LDL-C	Meat, rice and noodles diet	P=0.001	

Abbreviations TAG; triacylglycerol, HDL-C; high-density lipoprotein, LDL-C; low-density lipoprotein, TC; total cholesterol, VLDL; very low density lipoprotein, SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, PUFA; polyunsaturated fatty acids, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid; CHO; carbohydrates, MetS; metabolic syndrome.

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Chapter 2

For this study, I was involved in getting the dataset from the collaborators and cleaning the dataset. I ran the entire statistical analysis using the SPSS software and wrote the first draft of the manuscript. I revised the manuscript based on the comments from all the co-authors before the manuscript was submitted to the journal. I was also involved in drafting the responses to the comments from reviewers.

For the *APOE* genetic analysis, I had to prepare the DNA samples to be transported to the LGC Genomics company in London to run the genotyping analysis of the candidate SNPs from the *APOE* gene.

Chapter 2 Impact of Lipoprotein Lipase gene polymorphism, *S447X*, on postprandial triacylglycerol and glucose response to sequential meal ingestion

Published

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2.1 Abstract

Lipoprotein lipase (LPL) is a key rate-limiting enzyme for the hydrolysis of triacylglycerol (TAG) in chylomicrons and very low-density lipoprotein. Given that postprandial assessment of lipoprotein metabolism may provide a more physiological perspective of disturbances in lipoprotein homeostasis compared to assessment in the fasting state, we have investigated the influence of two commonly studied *LPL* polymorphisms (rs320, *HindIII*; rs328, *S447X*) on postprandial lipaemia, in 261 participants using a standard sequential meal challenge. *S447* homozygotes had lower fasting HDL-C (P=0.015) and a trend for higher fasting TAG (P=0.057) concentrations relative to the *447X* allele carriers. In the postprandial state, there was an association of the *S447X* polymorphism with postprandial TAG and glucose, where *S447* homozygotes had 12% higher TAG area under the curve (AUC) (P=0.037), 8.4% higher glucose-AUC (P=0.006) and 22% higher glucose-incremental area under the curve (IAUC) (P=0.042). A significant gene-gender interaction was observed for fasting TAG (P=0.004), TAG-AUC ($P_{interaction}$=0.004) and TAG-IAUC ($P_{interaction}$=0.016), where associations were only evident in men. In conclusion, our study provides novel findings of an effect of *LPL S447X* polymorphism on the postprandial glucose and gender-specific impact of the polymorphism on fasting and postprandial TAG

concentrations in response to sequential meal challenge in healthy participants.

2.2 Introduction

Large prospective cohort studies have identified elevated non-fasting triacylglycerol (TAG) concentrations as an independent risk factor for cardiovascular disease (CVD) [1]. The Copenhagen City Heart Study [2] and US Women's Health Study [3] showed that non-fasting TAG concentrations of ≥5.0 mmol/L were associated with myocardial infarction and the Norwegian Counties Study [4] showed that non-fasting TAG levels of >3.5 mmol/L were associated with a 5-fold increase in risk of death from coronary heart disease. Endothelial associated Lipoprotein lipase (LPL) (E.C. 3.1.1.34) plays an important role in the metabolism and clearance of triacylglycerol (TAG)-rich lipoproteins from the circulation [5] and atherogenesis, where it influences the interaction between atherogenic lipoproteins and receptors on the vascular wall [6]. Hence, enzymes such as lipoprotein lipase (LPL) (E.C. 3.1.1.34) that regulate lipoprotein metabolism in the postprandial state [7] are of interest to the prevention of CVDs.

Several polymorphisms in the *LPL* gene have been shown to lead to a reduction in enzyme synthesis and activity and, to date, rs320 [*HindIII* (T/G)] and rs328 [*Serine 447 Stop S447X* (C/G)] have been the most extensively studied. These variants with a prevalence of 40-75% (rs320) and 17-22% (rs328) among Caucasians [8, 9], respectively, have been shown to be associated with coronary artery disease, myocardial infarction [10-12] and pronounced fasting hypertriacylglycerolemia [13, 14]. Only limited number of studies has examined their impact on postprandial lipaemia [15-17] and these studies have used only a single test meal, which does not reflect the habitual eating pattern in humans.

Given that we spend nearly 75% of the time in a postprandial state, the normal physiological pattern of meal intake and the impact of *LPL* gene polymorphisms on the

clearance of dietary TAG may be more evident after a sequential meal challenge. Hence, in the present study, we investigated the association of the two commonly studied *LPL* polymorphisms [rs320 (*HindIII*) and rs328 (*S447*)] with fasting and postprandial lipid concentrations by using a standard sequential meal challenge and examined the penetrance of genotype according to gender, with gender previously shown to be a modulator of the impact of other variants on postprandial TAG handling [18].

2.3 Experimental Section

2.3.1 Subjects

All individuals included in this study were obtained from postprandial studies using identical inclusion/exclusion criteria, and all underwent the same sequential meal postprandial protocol, at University of Reading between 1997-2007, as previously described [19]. Briefly, 261 participants (109 women and 153 men) aged 22–71 years and BMI 17.6-37.3 kg/m^2, were included in the dataset. The postprandial study excluded participants who had CVD, including angina stroke; diabetes or fasting glucose > 6.5 mmol/l, liver or other endocrine dysfunction; were pregnant or lactating; who were smoking > 15 cigarette per day; doing aerobic exercise more than three times per week; who had hemoglobin levels < 130g/L for men and 120g/L for women or taking medication or supplements. The University of Reading Ethics and Research Committee and the West Berkshire Health Authority Ethics Committees approved the experimental protocol. Informed consent to participate in the study was obtained.

2.3.2 Sequential test meal protocols

Details of the postprandial protocol have been described previously [19]. Briefly, study participants were asked to refrain from alcohol or organised exercise regimens on the

previous day and were provided with a relatively low fat (<10 g fat) evening meal to standardise short-term fat intake. After a 12 h overnight fast, the participants were cannulated and fasting blood sample was taken. Following a standard test breakfast (0 min; 3.9 MJ energy, 111 g carbohydrate, 19 g protein and 49 g fat) and lunch (330 min; 2.3 MJ energy, 63 g carbohydrate, 15 g protein and 29 g fat), blood samples were taken from the cannula at 30–60 min intervals until 480 min after the test breakfast.

2.3.3 Biochemical measurements

Plasma glucose and lipid concentrations were measured using an automated analyzer assay (Instrumentation Laboratory (UK) Ltd, Warrington, UK). In the fasting sample, HDL-C was estimated in the supernatant following precipitation of the apolipoprotein B (apoB)-containing lipoproteins with a dextran-manganese chloride reagent. The LDL-C concentration was calculated using the Friedewald formula. Insulin levels were determined by ELISA (Dako Ltd, High Wycombe, UK). The total area under the curve (AUC, 0-480 min) was calculated using the trapezium rule, and incremental area under the curve (IAUC) calculated by subtracting the fasting levels from the total AUC. For NEFA, AUC and IAUC were calculated from the time of suppression until the end of the postprandial period (120-480 min) due to initial drop in NEFA concentrations after the meal. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using formula: [fasting insulin (pmol/l) x fasting glucose (mmol/l)]/135.

2.3.4 DNA extraction and genotyping

DNA was isolated from the buffy coat layer of 10 ml of EDTA blood using the Qiagen DNA Blood Mini Kit (Qiagen Ltd, Crawley, UK). Allelic discrimination of two *LPL* gene polymorphisms (rs320, *HindIII* and rs328, *S447*) was conducted using a 'Assay-on-Demand' SNP genotyping assays (Applied Biosystems, Warrington, UK).

2.3.5 Statistical analyses

The statistical significance of associations between biochemical data and genotypes was established using a general linear model adjusted for covariates such as age, gender, and BMI, which are highly correlated to lipid concentrations. Genotype distribution for *LPL* SNPs was assessed using the Hardy-Weinberg equilibrium. Given the small number of rare homozygotes, we applied a dominant model in which carriers of 1 or 2 copies of the minor allele of the two polymorphisms *HindIII* and *S447X* were grouped and compared with major allele homozygotes. Interaction between gender and polymorphisms on outcomes was examined by introducing the interaction terms into the linear regression analysis models with adjustment to same variables in association test. All data presented in the text and tables represents mean ±SD. We used SPSS software (version 21; SPSS Inc, Chicago, IL) for all statistical analyses. Probability values under 0.05 were considered significant.

2.4 Results

The study participants included 153 men (mean±SD, age 53±10 years; BMI 27.3±3.1 kg/m^2) and 109 women (mean±SD, age 52±11 years; BMI 25.4±3.5 kg/m^2). The prevalence of *S447* homozygotes was 81% (n=213) versus 18% *447X* minor allele carriers (n=48). The frequency of carriers of *447X* in this study was consistent with published reports in Caucasian population [20]. 56% of participants were homozygous for the H1 major allele for *HindIII* (n=131) with 43% H2 minor allele carriers (n=100).

Table 2.1 describes the baseline characteristics of participants according to the *S447X* polymorphism. There was a borderline genotype-related association with fasting serum TAG levels after adjusting for age, gender, and BMI (P=0.057). Circulating HDL-C concentrations were markedly lower in *S447* homozygotes than *447X* allele carriers (P=0.015). None of the

other fasting biochemical parameters were significantly associated with *S447X* (**Table 2.1**) or *HindIII* polymorphisms (**Table 2.2**) (all P >0.05).

A greater TAG AUC was observed in *S447* allele homozygotes following the sequential meals (P=0.037). The AUC and IAUC of the glucose response was 8.4% (P=0.006) and 22.6% (P=0.042) lower in *447X* allele carriers (P=0.006), respectively than common homozygotes. The postprandial summary measures did not show any association with *HindIII* genotypes (**Table 2.2**).

The *S447X* polymorphism showed a significant interaction with gender on fasting TAG (P=0.004) (**Figure 2.1**), TAG AUC (P=0.004) (**Figure 2.2**) and TAG IAUC (P=0.016) (**Figure 2.3**), with the major *S447* allele homozygotes at *S447X* in men showing higher values for fasting and postprandial TAG compared with X minor allele carriers.

Given the strong linkage disequilibrium between the two *LPL* polymorphisms [15], we also examined the combined effects of the polymorphisms on baseline characteristics and postprandial TAG and glucose. Nine possible genotype combinations were generated. However, given the small sample size, only 3 combinations [*S447S* - H1/H1 (n=131), *S447S* – H1/H2 (n= 55) and *S447X* – H1/H2 (n=45)] were available in our study participants. The frequencies of these three genotype combinations are presented in the **Table 2.3**. In the genotype-genotype analysis, we found that individuals with the *S447S* genotype irrespective of HindIII alleles (i.e., SS/H1H1 and SS/ H1H2) had higher TAG AUC (P=0.040) and glucose AUC (P=0.034) levels than *447X* allele carriers (**Table 2.4**) suggesting that the associations are driven mainly by the *S447X* polymorphism.

Table 2.1: Baseline and postprandial characteristics of the participants according to *LPL-S447X* polymorphism

Participants characteristics	**S/S (n=213)**	**S/X (n=48)**	**$P_{association}$**
Age (years)	53 ±11	52 ±11	0.648
Men/Women	125/88	27/21	-
BMI (kg/m^2)	26.3 ±3.4	27.4 ±3.2	**0.015**
Baseline characteristics			
TC (mmol/l)	5.78 ±1.05	5.62 ±1	0.285
TAG (mmol/l)	1.67 ±0.90	1.48 ±0.51	0.057
HDL-C (mmol/l)	1.29 ±0.42	1.40 ±0.33	**0.015**
LDL-C (mmol/l)	3.73 ±1.02	3.53 ±0.92	0.167
Glucose(mmol/l)	5.16 ±0.66	5.14 ±0.45	0.534
Insulin (pmol/l)	48.3 ±31.2	50.3 ±26.2	0.517
NEFA (µmol/l)	519 ±184	477 ±170	0.102
HOMA-IR	1.96 ±1.40	1.99 ±1.09	0.376
Postprandial summary measures			
TAG AUC (mmol/l × 480 min)	1193 ±593	1046 ± 429	**0.037**
TAG IAUC (mmol/l × 480 min)	353 ±228	305 ±210	0.149
NEFA AUC mmol/l × 300 min	153 ±45	149 ±32	0.410
NEFA IAUC (mmol/l × 300 min)	96 ±39	100 ± 26	0.792
Glucose AUC (mmol/l × 480 min)	3114 ±460	2850 ± 763	**0.006**
Glucose IAUC (mmol/l × 480 min)	595 ±284	460 ± 238	**0.042**
Insulin AUC (nmol/l × 480 min)	139 ±94	123 ±38	0.858
Insulin IAUC (nmol/l × 480 min)	114 ± 89	100± 34	0.876

Values are mean ± standard deviation. P-values are from a linear model testing the association with *LPL-S447X*, adjusted for age, gender, BMI. Abbreviations: TC, total cholesterol; TAG, triacylglycerol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; NEFA, non-esterified fatty acids; HOMA-IR, homeostasis model assessment - insulin resistance. For the baseline analysis, the insulin and HOMA-IR values were available for 166 participants (men = 124, women = 42). For the postprandial analysis, the insulin AUC and IAUC data was available for 79 participants (men = 68,

women = 11).

Table 2.2: Baseline and postprandial characteristics of the participants according to *LPL- HindIII* polymorphism

Participants characteristics	**H1 (n=131)**	**H1/H2 (n=100)**	**$P_{association}$**
Age (years)	53 ±11	52 ±10	0.567
BMI (kg/m^2)	26.0 ±3.4	26.6 ±3.1	0.095
Baseline characteristics			
TC (mmol/l)	5.62 ±1.02	5.67 ±1	0.517
TAG (mmol/l)	1.53 ±0.87	1.55 ±0.65	0.972
HDL-C (mmol/l)	1.35 ±0.42	1.36 ±0.39	0.526
LDL-C (mmol/l)	3.59 ±0.96	3.59 ±0.98	0.872
Glucose(mmol/l)	5.14 ±0.69	5.10 ±0.50	0.413
Insulin (pmol/l)	45.9 ±30.0	52.2 ±31.9	0.414
NEFA (µmol/l)	530 ±200	498 ±173	0.177
HOMA-IR	1.87 ±1.36	2.06 ±1.35	0.680
Postprandial summary measures			
TAG AUC (mmol/l × 480 min)	1086 ±549	1098 ±464	0.947
TAG IAUC (mmol/l × 480 min)	321 ±212	335 ±230	0.677
NEFA AUC mmol/l × 300 min	155 ±40	153 ±49	0.600
NEFA IAUC (mmol/l × 300 min)	99 ±33	94 ±45	0.231
Glucose AUC (mmol/l × 480 min)	3109 ±408	2958 ±705	0.065
Glucose IAUC (mmol/l × 480 min)	590 ±287	558 ± 269	0.648
Insulin AUC (nmol/l × 480 min)	128 ±59	134 ±130	0.782
Insulin IAUC (nmol/l × 480 min)	105 ±54	109±125	0.879

Values are mean ± standard deviation. P-values are from a linear model testing the association with *LPL-S447X*, adjusted for age, gender, BMI. Abbreviations: TC, total cholesterol; TAG, triacylglycerol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; NEFA, non-esterified fatty acids; HOMA-IR, homeostasis model assessment - insulin resistance. For the baseline analysis, the insulin and HOMA-IR values were available for 166 participants (men = 124, women = 42). The fasting and

postprandial insulin and HOMA-IR were not available for all participants (n= 151).

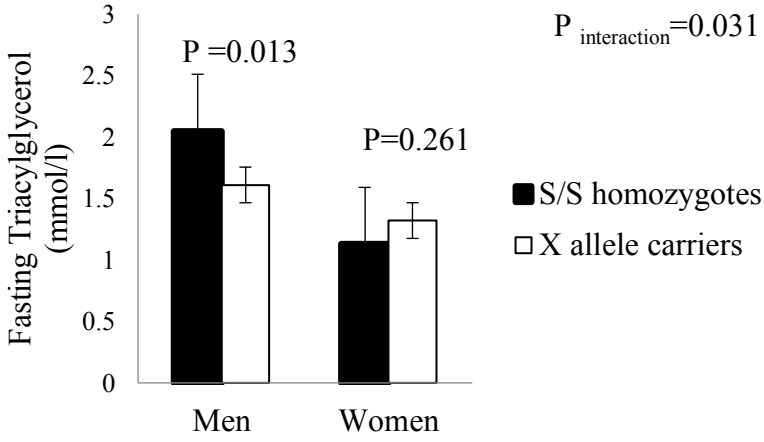

Figure 2.1 Mean (SEM) for fasting triacylglycerol (TAG) according to *S447X* polymorphism in men and women. Carriers of one or two copies of X minor allele are combined and presented by white bars. Gene-gender interaction was statistically significant for fasting TAG levels ($P_{interaction}$=0.031).

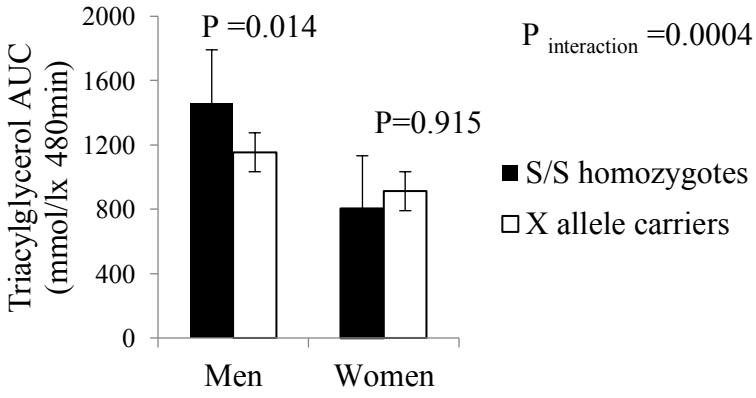

Figure 2.2 Mean (SEM) for the AUC TAG response according to *S447X* polymorphism after consumption of a test breakfast (49 g fat) at 0 min and a test lunch (29 g fat) at 330 min. *S447* homozygotes (n=213) had 12% higher TAG area under the curve (AUC) (P=0.037) compared to *447X* carriers (n=48) for men. Carriers of one or two copies of X minor allele are combined and presented by white bars. Gene-Gender interaction was statistically

significant for area under the TAG curve values ($P_{interaction}$=0.004).

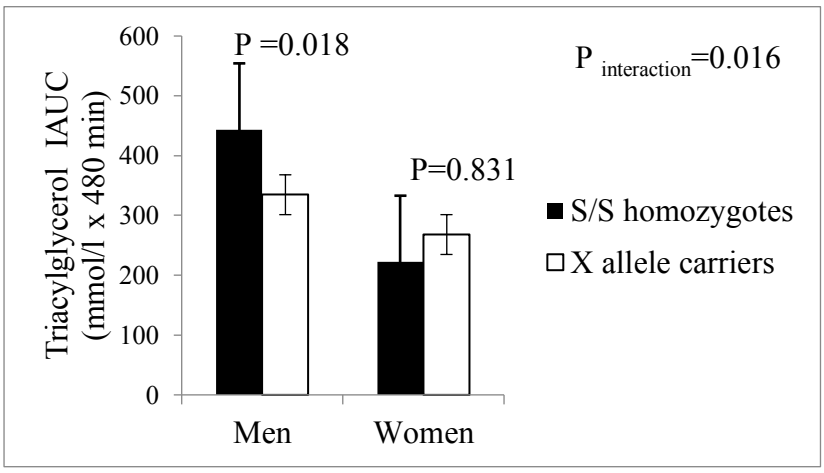

Figure 2.3 Mean (SEM) for the IAUC TAG response according to *S447X* polymorphism after consumption of a test breakfast (49 g fat) at 0 min and a test lunch (29 g fat) at 330 min in men, and women. Carriers of one or two copies of X minor allele are combined and presented by white bars. Gene-Gender interaction was statistically significant for IAUC TAG ($P_{interaction}$=0.016).

Table 2.3: Distribution of study participants according to combined *LPL HindIII* and *S447X* markers

S447X-HindIII	**S/S**	**S/X**	**X/X**
H1/H1	131	0	0
H1/H2	55	45	0
H2/H2	0	0	0

Table 2.4 Association between the combined genotypes of *LPL S447X-HindIII* polymorphisms and fasting and postprandial characteristics.

Participants characteristics	SS/H1 (n=131)	SX/H1H2 (n=45)	SS/H1H2 (n=55)	$P_{association}$
Age (years)	53 ±11	52 ±11	52 ±9	0.570
BMI (kg/m^2)	25.9 ±3.4	27.2 ±3.1	26.1±2.9	0.052
Baseline characteristics				
TC (mmol/l)	5.62 ±1.02	5.58 ±0.99	5.75±1.01	0.649
TAG (mmol/l)	1.53 ±0.87	1.44 ±0.47	1.64±0.76	0.230
HDL-C (mmol/l)	1.35 ±0.42	1.41 ±0.33	1.32±0.43	0.128
LDL-C (mmol/l)	3.59 ±0.96	3.50 ±0.91	3.67±1.04	0.596
Glucose(mmol/l)	5.14 ±0.69	5.12 ±0.49	5.09±0.53	0.708
Insulin (pmol/l)	45.9 ±30.0	49.9 ±26.6	54.18±36	0.298
NEFA (μmol/l)	530 ±200	471 ±171	519±173	0.145
HOMA-IR	1.87 ±1.36	1.97 ±1.11	2.13±1.54	0.434
Postprandial summary measures				
TAG AUC (mmol/l × 480 min)	1086 ±549	995 ±369	1183±518	0.040
TAG IAUC (mmol/l × 480 min)	321 ±212	302 ±215	361±241	0.262
NEFA AUC mmol/l × 300 min	155 ±40	149 ±33	156±59	0.554
NEFA IAUC (mmol/l × 300 min)	99 ±33	100 ±27	89±55	0.333
Glucose AUC (mmol/l × 480 min)	3109 ±408	2831 ±797	3057±617	0.034
Glucose IAUC (mmol/l × 480 min)	590 ±287	454 ± 248	636±260	0.051
Insulin AUC (nmol/l × 480 min)	128 ±60	119 ±38	145±169	0.948
Insulin IAUC (nmol/l × 480 min)	105 ±54	95±303	118±164	0.967

Abbreviations: TC, total cholesterol; TAG, triacylglycerol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; NEFA, non-esterified fatty acids; HOMA-IR, homeostasis model assessment - insulin resistance; AUC, area under the curve; IAUC, incremental area under the curve.

2.5 Discussion

Our postprandial study using a standard sequential meal challenge demonstrates that individuals homozygous for the common allele of the *S447X* polymorphism had significantly lower fasting HDL-C levels and a significantly elevated postprandial TAG and glucose response relative to *447X* allele carriers. In addition, a gender-specific association between the *S447X* polymorphism and fasting and postprandial TAG concentrations was observed, where the effect of the genotype was evident only in men.

Several studies have demonstrated the association between *S447* genotype and elevated fasting plasma TAG levels and lower HDL-C levels [21, 22]. Our study also has shown a borderline association of the *S447* allele with higher TAG and a significant association with lower HDL-C levels, which is in accordance with a meta-analysis (n=45,079) that showed 0.05 mmol/L lower HDL-C levels and 0.15 mmol/L higher TAG among the *S447* homozygotes [12]. Furthermore, the association of the *S447* genotype with postprandial TAG in our study has also been confirmed in previous studies (**Table 3.5**). However, the mechanism by which this polymorphism affects lipid levels still remains obscure. Functional studies have demonstrated that the *447X* variant that results in a 2 amino acid truncation on the carboxyl-terminal domain of the LPL increases the ability of the cell surface receptors to bind with TAG-lipoproteins [8]; but it is not clear how this truncation increases the ability of LPL to bind TAG. While a few studies have reported that *S447X* polymorphism might increase or decrease the LPL activity [23-25], some have failed to show a significant effect [24, 26]. The probable mechanism by which *LPL S447* allele lowers HDL-C could be related to higher TAG concentrations. A delayed clearance of triglyceride-rich lipoproteins (TRLs) drives the transfer of TAG from TRLs to both LDL and HDL by cholesteryl ester transfer protein (CETP), which makes them suitable substrates for lipases. This leads to the formation

of smaller denser HDL particles, which are rapidly removed from the circulation, thus decreasing HDL-C concentrations [27].

We also observed a novel association between *LPL S447X* polymorphism and postprandial glucose using the sequential meal challenge, where *S447* homozygotes had higher glucose AUC and IAUC. Previous postprandial studies have shown higher postprandial glucose concentrations in response to three meals [28, 29]. Given that the postprandial studies examining the effects of *LPL* polymorphisms have used only a single meal [15-17], it is possible that the effects of *LPL* polymorphisms on postprandial glucose have been missed previously. One of the reasons for higher postprandial glucose could be due to the decreased insulin sensitivity in the *S447* homozygotes [30]. LPL has been considered as a link between insulin resistance and atherosclerosis, given its role in controlling the delivery of free fatty acids to muscle, adipose tissue and vascular wall macrophages, wherein lipid uptake influences insulin sensitivity [31]. It was also shown in the Quebec Family study that the *LPL* markers (*HindIII* and *S447X*) combination influenced the insulin AUC during an oral glucose tolerance test [32]. In our study, we did not find any association with fasting and postprandial insulin and HOMA-IR, which might be due to limited power for this analysis as insulin concentrations were not available for all participants ($n<166$). However, given that low HDL cholesterol and high TAG are frequently found with insulin resistance [33], it is possible that decreased insulin sensitivity could be a possible mechanism for higher postprandial glucose concentrations in the *S447* homozygotes. This was also shown in a previous study where individuals with insulin resistance had elevated fasting and postprandial TAG, and lower HDL-C levels [34].

Our results also demonstrated gender-specific effect of *LPL S447X* polymorphism on fasting and postprandial TAG, where the association was significant only in men. The gender-specific effects of other SNPs (*LEPR* [35], *APOA5* [18] and *APOB* [36]) in men have

already been shown in our postprandial cohort which is characteristic of men with higher BMI, fasting TAG, insulin and lower HDL-C than women irrespective of the genotype. A postprandial study in 63 men also showed that those with low fasting HDL-C and high TAG concentrations had higher postprandial TAG [37]. It is possible that men from our cohort were at a greater metabolic stress which may be a contributory factor in the gender-specific effect of *S447* genotype on the fasting and postprandial responses.

Previous studies have shown associations between the *HindIII* polymorphism and elevated lipids [10]. In this study, we did not find any significant difference in fasting and postprandial lipid levels across the genotypes of the polymorphism. However, the combination of *HindIII* and *S447X* markers revealed significant associations with TAG and glucose AUC, which might be due to the strong linkage disequilibrium between the *HindIII* and *S447X* markers. In addition, previous studies have investigated the effect of this polymorphism on postprandial lipids using only a single meal [15]. Hence, our finding with *HindIII* polymorphism requires a replication using a sequential meal challenge, which reflects the habitual eating pattern.

In conclusion, our study provides novel findings of an effect of *LPL S447X* polymorphism on the postprandial glucose and gender-specific impact of the polymorphism on fasting and postprandial TAG levels in response to sequential meal challenge in healthy participants. The elevated fasting and postprandial TAG and postprandial glucose and lower fasting HDL-C concentrations are likely to result in prolonged appearance of lipids, in particular remnant particles, which might be one of the reasons for the increased prevalence of CVD in participants carrying the *S447* allele. Further studies are required to confirm our gender-specific associations between the *LPL* polymorphism and fasting and postprandial TAG levels with an assessment of serum LPL concentrations and activity using a sequential

meal challenge. This will further shed light on the size-effect of *LPL* polymorphisms on lipid and glucose metabolism in population subgroups.

Author Contributions

Israa M Shatwan and KSV **analyzed the data and wrote the paper**. CJW, KGJ, AMM and JAL designed the postprandial experiments. KSV, CJW, KGJ, AMM and JAL critically analyzed the paper.

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Table 2.5 List of postprandial studies determining the effects of *LPL* gene polymorphisms (*S447X and HindIII*) on fasting and postprandial lipids.

Reference	Sample	Meal Composition Size	LPL Polymorphism	Outcome	Association (P Value)
Lopez-Miranda et al. 2004 [15]	51	High fat meal (60 g fat and 420 mg cholesterol—60% fat, 15% protein, and 25% carbohydrate)	HindIII	Postprandial Small triacylglycerol-rich lipoproteins (TRL)-retinyl palmitate (RP)	P=0.030
			S447X	Small TRL-RP	P=0.028
				Large TRL-B48	P=0.046
				Small TRL-B48	P=0.048
			HindIII-S447X	Fasting Triacylglycerol (TAG)	P=0.047
				Large TRL-TG	P=0.048
				Large TRL-RP	P= 0.004
				Small TRL-RP	P=0.014
				Large TRL-B48	P=0.036
Humphries et al. 1998 [17]	2181	Oral liquid lipid load (42 g saturated fat, 22 g protein, 56 g carbohydrate, and 417 mg cholesterol)	H-/X447	Fasting TAG	P=0.01
				Postprandial TAG	P< 0.05

Anagnostopoulou et al. 2009 [16]	80	High fat meal (5.3 g protein- 2.5%, 24.75 g carbohydrate- 14.0%, 240 mg cholesterol, and 65.2 g fat—83.5% fat)	S447X	Fasting / Postprandial TAG	No association

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Chapter 3

For this study, I was involved in getting the dataset from the collaborators and cleaning the dataset. I ran the entire statistical analysis using the SPSS software and wrote the first draft of the manuscript. As part of the initial stages of statistical analysis, I had run the analyses separately in controls and cases; however, for final version of the manuscript, I had to run the analysis on total sample size to increase the statistical power of the study findings. I revised the manuscript based on the comments from all the co-authors before the manuscript was submitted to the Nutrition and metabolism journal. I was also involved in drafting the responses to the comments from reviewers.

Chapter 3 High fat diet modifies the association of lipoprotein lipase gene polymorphism with high density lipoprotein cholesterol in an Asian Indian population

Published

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*Equally contributed

3.1 Abstract

Background:

Single nucleotide polymorphisms (SNPs) in lipoprotein lipase gene (*LPL*) have been shown to influence metabolism related to lipid phenotypes. Dietary factors have been shown to modify the association between *LPL* SNPs and lipids; however, to date, there are no studies in South Asians. Hence, we tested for the association of four common *LPL* SNPs with plasma lipids and examined the interactions between the SNPs and dietary factors on lipids in 1,845 Asian Indians.

Methods:

The analysis was performed in 788 Type 2 diabetes cases and 1,057 controls randomly

chosen from the cross-sectional Chennai Urban Rural Epidemiological Study. Serum triacylglycerol (TAG), serum total cholesterol, and high-density lipoprotein cholesterol (HDL-C) were measured using a Hitachi-912 autoanalyzer (Roche Diagnostics GmbH, Mannheim, Germany). Dietary intake was assessed using a semi-quantitative food frequency questionnaire. The SNPs (rs1121923, rs328, rs4922115 and rs285) were genotyped by polymerase chain reaction followed by restriction enzyme digestion and 20% of samples were sequenced to validate the genotypes obtained. Statistical Package for Social Sciences for Windows version 22.0 (SPSS, Chicago, IL) was used for statistical analysis.

Results:

After correction for multiple testing and adjusting for potential confounders, SNPs rs328 and rs285 showed association with HDL-C (P=0.0004) and serum TAG (P=1x10^{-5}), respectively. The interaction between SNP rs1121923 and fat intake (energy %) on HDL-C (P=0.003) was also significant, where, among those who consumed a high fat diet (28.4 ± 2.5 %), the T allele carriers (TT + XT) had significantly higher HDL-C concentrations (P=0.0002) and 30% reduced risk of low HDL-C levels compared to the CC homozygotes. None of the interactions on other lipid traits were statistically significant.

Conclusion: Our findings suggest that individuals carrying T allele of the SNP rs1121923 have increased HDL-C levels when consuming a high fat diet compared to CC homozygotes. Our finding warrants confirmation in prospective studies and randomized controlled trials.

3.2 Introduction

The Asian Indian population has a greater predisposition to non-communicable diseases such as type 2 diabetes (T2D) [1, 2] and cardiovascular disease (CVD) [3] compared to Europeans. Despite low body mass index (BMI), Indians are characterized by a higher frequency of hyperinsulinemia [4], insulin resistance [5], dyslipidemia with hypertriacylglycerolemia and low high-density lipoprotein cholesterol (HDL-C) levels [6] and increased visceral fat, which are referred to as 'Asian Indian Phenotype' or 'Atherogenic Lipoprotein Phenotype' [7, 8]. Blood lipid levels are heritable phenotypes and findings from previous studies show that the blood concentrations of HDL-C, low density lipoprotein cholesterol (LDL-C) and triacylglycerol (TAG) have a strong inheritance [9].

Genetic studies have implicated several gene loci in the predisposition to dyslipidemia in Asian Indians [10-13], one of which is lipoprotein lipase (*LPL*) [14-16]. LPL plays an important role in the metabolism of HDL-C, where it has been shown to hydrolyze TAG in TAG-rich lipoproteins such as chylomicrons and very low density lipoproteins [17]. It has been postulated that increased activity of LPL enzyme enhances the release of components of TAG-rich lipoproteins which are then transferred to HDL to raise HDL levels; conversely, lack of LPL can retard the transfer of these components to HDL [18]. Several candidate gene studies have shown an association between single nucleotide polymorphisms (SNPs) in *LPL* and lipid traits in various populations including Asian Indians [11, 12, 19-22]. Genome wide association studies have also demonstrated strong evidence for the association of *LPL* polymorphisms with HDL-C concentrations [23-25]. A few studies have examined the *LPL* gene–diet interactions in association with HDL-C

[26-30]; however, the findings have been quite inconsistent due to variations in sample size, dietary factors and the selection of *LPL* polymorphisms.

Given that there are no gene-diet interaction studies, to date, in Asian Indian populations, we examined the association of four common *LPL* SNPs [Val135Val C/T (rs1121923), Ser447Ter C/G (rs328), G/A (rs4922115) and *Pvu II* C/T (rs285)] with HDL-C and investigated the interactions of these four polymorphisms with dietary carbohydrate, fat and protein percentage on HDL-C in up to 1,845 participants (788 T2D cases and 1,057 controls) from the cross-sectional Chennai Urban Rural Epidemiological Study (CURES). In addition, we examined the genetic associations and interactions for other lipid traits such as TAG, LDL-C and total cholesterol in these participants.

3.3 Methods

3.3.1 Study population

One thousand eight hundred and forty five participants comprising 788 cases with T2D and 1,057 controls with normal glucose tolerance (NGT) were randomly chosen from the urban component of the Chennai Urban Rural Epidemiological Study (CURES), an epidemiological study conducted on a representative population (age >20 years) of Chennai (formerly Madras), the fourth largest city in India. The detailed methodology of the study participants is published elsewhere [31]. Briefly, in Phase 1 of CURES, 26,001 individuals were recruited based on a systematic random sampling technique. Participants with self-reported diabetes taking drug treatment for diabetes were classified as "known diabetes subjects." All known diabetes participants (n = 1,529) were invited to visit the center for detailed studies. In addition, every 10th

individual of the 26,001 individuals without known diabetes was invited to undergo oral glucose tolerance tests using a 75-g oral glucose load (dissolved in 250 ml of water) (Phase 3 of CURES). Those who were confirmed by oral glucose tolerance test to have 2-h plasma glucose value ≥11.1 mmol/l based on World Health Organization (WHO) consulting group criteria were labeled as "newly detected diabetes subjects" and those with 2-h plasma glucose value <7.8 mmol/l as being NGT [32]. CURES participants who were on lipid lowering drugs such as statins, fibrates and niacin were excluded from the study (n=134). On the basis of the National Cholesterol Education Program-Adult Treatment Panel III (NCEP-ATP III) guidelines [33] the study population was divided into those with normal HDL-C (≥1.03 mmol/l for men; ≥1.3 mmol/l for women) and low HDL-C (<1.03 mmol/l for men; <1.3 mmol/l for women). Written informed consent was obtained from each study participant, and the study was approved by the Madras Diabetes Research Foundation Institutional Ethics Committee.

3.3.2 Phenotype measurements

Anthropometric measurements including weight, height, and waist were obtained using standardized techniques. The BMI was calculated as weight (in kg) divided by the square of height (in m). Biochemical analyses were performed on a Hitachi-912 Auto Analyzer (Hitachi, Mannheim, Germany) using kits supplied by Roche Diagnostics (Mannheim). Fasting plasma glucose (glucose oxidase–peroxidase method), serum total cholesterol (cholesterol oxidase-phenol-4-amino-antipyrene peroxidase method), serum TAG (glycerol phosphatase oxidase-phenol-4-amino-antipyrene peroxidase method), and HDL-C (direct method; polyethylene glycol-pretreated enzymes) were measured.

Low-density lipoprotein cholesterol was calculated using the Friedewald formula [34]. Glycated haemoglobin (HbA1c) was estimated by high-performance liquid chromatography using a Variant™ machine (Bio-Rad, Hercules, CA, USA). Serum insulin concentration was estimated using an enzyme-linked immunosorbent assay (Dako, Glostrup, Denmark).

3.3.3 Dietary assessment

Dietary intakes were assessed using a previously validated and published [35] interviewer administered semi-quantitative food frequency questionnaire (FFQ) containing 222 food items to estimate food intake over the past year. Briefly, individuals were asked to estimate the usual frequency (number of times per day, week, month or year/never) and the usual serving size of the portion of the various food items in the FFQ. Common household measures such as household cups, bowls, ladles, spoons (for the cooked foods like vegetables), wedges, circles of different diameter and visual atlas of different sizes of fruits (small, medium, large) were shown to assist the individuals in estimating portions. A detailed description of the development of FFQ and the data on reproducibility and validity had been published [35]. EpiNu, an in-house database was used to assess the average daily food and nutrient intake.

3.3.4 SNP selection and Genotyping

Four common SNPs in the *LPL* gene (rs285, rs328, rs4922115 and rs1121923) were chosen for the present study. The SNPs rs328 and rs285 were chosen based on their previous associations with lipid outcomes in several populations [11, 12, 22, 36]. The

SNPs rs1121923 and rs4922115 were identified from the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) based on their location in the exon 3 and 3'UTR regions, respectively, assuming that variations in the coding and regulatory regions might confer a functional effect on the gene expression. The SNPs were genotyped by polymerase chain reaction on a GeneAmp® PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA) followed by restriction enzyme digestion (New England Biolabs, Inc., Beverly, MA). The program usually had the following steps: initial denaturation at 95°C for 10 min, 30-35 cycles of denaturation at 95°C for 45 sec, primer-annealing at 58°C for rs1121923 and rs285 SNPs and 60°C for rs328 and rs4922115 SNPs for 45 sec, and primer extension at 72°C for 45 sec, followed by a final extension at 72°C for 5 min. The restrictions enzymes used for genotyping the SNPs were *Sau96I* for rs1121923, *Mnl I* enzyme for rs328, the *Eco RV* enzyme for rs4922115 and *Pvu II* for rs285. Agarose gel electrophoresis was used to detect the amplification of PCR reaction and the restriction enzyme digested products. To ensure that the genotyping was of adequate quality, we performed random duplicates in 10% of the samples. The assays were performed by a technician who was masked to the phenotype, and there was 98% concordance in the genotyping. Variants were also confirmed by direct sequencing using an ABI 3500 genetic analyzer (Applied Biosystems, Foster City, CA). Population stratification was performed using a case-control approach at 6 unlinked marker loci believed to be unrelated to the disease under study, but known to have allelic diversity among different populations [37].

3.3.5 Statistical analysis

Statistical Package for Social Sciences for Windows version 22.0 (SPSS, Chicago, IL) was used for statistical analysis. The effects of the variants on quantitative and categorical variables were analyzed. Allele frequencies were estimated by gene counting. Agreement with Hardy–Weinberg equilibrium (HWE) expectations was tested using a $\chi 2$goodness-of-fit test. Comparison of the means between the two groups was analyzed by independent *t*-test. The $\chi 2$ test was used to compare the proportions of genotypes or alleles. Dominant model was used, given the low frequency of minor allele homozygotes. Linear regression was used to examine the association of the *LPL* SNPs with various lipid outcomes. The SNP-diet interactions on lipid traits were tested by including the interaction term in linear regression models. Models were adjusted for age, gender, BMI (as continuous), T2D status and total energy intake wherever appropriate. Multiple testing correction using Bonferroni method was applied separately for the testing of main and interaction effects (i.e., association of the four SNPs with HDL-C and interaction with dietary factors on HDL-C levels) [$P \leq 0.003$ (= 0.05/20) was considered statistically significant] and additional analyses (i.e., association of the four SNPs with other lipid traits and interaction with dietary factors on other lipid traits) [$P \leq 0.001$ (= 0.05/48) was considered statistically significant].

3.4 Results

Table 3.1 shows the anthropometric and biochemical characteristics of NGT and T2D participants. T2D cases had markedly increased levels of TAG, LDL-C and total cholesterol, while HDL-C was significantly lower in cases compared to controls

(P<0.003 for all comparisons). In our study population, the genotype distributions for the four *LPL* polymorphisms were GG: 68.5%, GA: 27.5% and AA: 4.0% (rs4922115); CC: 87.5%, CT: 12.0% and TT: 0.5% (rs1121923); CC: 72.8%, CG: 25.2% and GG: 2.00% (rs328); and CC: 40.9%, CT: 45.6% and TT: 13.5% (rs285). All the four SNPs were in HWE (P>0.05).

The association between *LPL* SNPs and HDL-C levels is presented in **Table 3.2**. Of the four *LPL* variants, the SNP rs328 alone showed a significant and a consistent association with HDL-C concentrations [both as continuous and categorical variable (stratified based on NCEP ATP III guidelines)] under a dominant model after correction for multiple testing (P=0.0004 for the continuous variable and P=0.001 for the categorical variable). The minor allele (G) carriers of the SNP rs328 had 5% higher HDL-C compared to the homozygous carriers of the common 'C' allele.

In the interaction analysis, after correction for multiple testing, none of the interactions were statistically significant except for the interaction between SNP rs1121923 and fat intake (energy %) on HDL-C (P=0.003) (**Table 3.3**), where among those who consumed a high fat diet (3rd tertile: 28.4± 2.5%), the T allele carriers had significantly higher HDL-C concentrations compared to the CC homozygotes (P=0.0002) (**Figure 3.1**). To test whether this interaction was significant on HDL-C as a categorical variable, we stratified the data based on normal and low HDL-C levels according to the NCEP ATP III guidelines for dyslipidemia [33] and found that among those who consumed a high fat diet, the individuals who carried the T allele had 30% reduced risk of low HDL-C levels compared to the CC homozygotes (P=0.001) (**Figure 3.2**). We further investigated the interaction of the SNP with various fat

subclass intakes on HDL-C but none of the interactions were statistically significant [monounsaturated (MUFA) (P=0.36), polyunsaturated (PUFA) (P=0.22) and saturated fatty acids (SFA) (P=0.46)].

Table 3.1: Baseline characteristics of the CURES study participants

	Participants with Normal Glucose Tolerance (N=1,057)	**Participants with Type 2 diabetes (N=788)**	**P value**
Age (year)	38.5 ± 13.6	50.6 ± 11.1	<0.0001
Gender (men / women)	608/ 449	433/ 355	0.2 *
BMI (kg/m^2)	23.2± 4.5	25.2 ± 4.4	<0.0001
Fasting Glucose (mmol/l)	4.7± 0.5	8.8± 3.8	<0.0001
Fasting Insulin (lIU/mL)	8.2 ± 5.6	11.6 ± 7.0	<0.0001
Total serum Cholesterol (mmol/l)	4.6± 0.9	5.2± 1.1	<0.0001
Serum TAG (mmol/l)	1.3± 0.7	1.9± 1.3	<0.0001
HDL-C (mmol/l)	1.12± 0.25	1.08 ± 0.24	0.003
LDL-C (mmol/l)	2.9± 0.8	3.2± 0.9	<0.0001
Glycated hemoglobin (%)	5.56 ± 0.47	8.64± 2.26	<0.0001
Systolic pressure (mmHg)	117.0 ± 17.4	128.9 ± 21.5	<0.0001
Diastolic pressure (mmHg)	73.2 ± 11.2	77.1 ±12.0	<0.0001
Protein intake (energy %)	11.3 ± 1.2	11.4 ±1.2	0.03
Carbohydrate intake (energy %)	64.4 ± 6.4	64.9 ± 5.8	0.1
Fat intake (energy %)	23.5 ± 4.7	23.4 ± 4.6	0.8

Total energy intake (kcal)	2627.2 ± 725.4	2533.5 ± 907.2	0.02
Total saturated fat intake (%)	2.4 ± 0.9	2.1 ± 0.9	<0.0001
Total monounsaturated fat (%)	1.9 ± 0.7	1.7 ± 0.8	0.0002
Total polyunsaturated fat (%)	1.6 ± 0.8	1.7 ± 0.9	0.04

Data shown are represented as means ± SD, wherever appropriate

P values for the differences in the means/ proportions between cases and controls

P values were calculated by using Independent t test.

* P value was calculated using a Chi-square test.

CURES, Chennai Urban Rural Epidemiological Study; TAG, triacylglycerol; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein cholesterol

Table 3.2: Association of the lipoprotein lipase single nucleotide polymorphisms (SNPs) with HDL-C levels

Association of the SNPs with HDL-C levels (continuous variable)	
SNP	**HDL-C levels (means ± SD)**
SNP rs4922115	
GG	1.1 ± 0.3
GA	1.1 ± 0.2
AA	1.1 ± 0.2
Dominant model(GG vs GA+AA)	
(P value)	0.02
SNP rs1121923	
CC	1.1 ± 0.3
CT	1.2± 0.3
TT	1.0 ± 0.2
Dominant model(CC vs CT+TT)	
(P value)	0.02
SNP rs328	
CC	1.1 ± 0.3
CG	1.2 ± 0.3
GG	1.2 ± 0.2
Dominant model(CC vs CG+GG)	
(P value)	**0.0004**
SNP rs285	
CC	1.1± 0.3
CT	1.1 ± 0.2
TT	1.2 ± 0.2
Dominant model(CC vs CT+TT)	
(P value)	0.03

Association of the SNPs with HDL-C (categorical variable)		
	Low HDL-C levels Number (%)	**Normal HDL-C levels Number (%)**
SNP rs4922115		
GG	366 (65%)	497 (71.3%)
GA	171 (30.4%)	167 (25.3%)
AA	26 (4.6%)	24 (3.4%)
Dominant model (GG vs GA+AA)		
(P value)	0.02	
SNP rs1121923		
CC	530 (91.7%)	642 (84.7%)
CT	45 (7.8%)	112 (14.8%)
TT	3 (0.5%)	4 (0.5%)

Dominant model (CC vs CT+TT)			
(P value)		0.1	
SNP rs328			
CC	336 (78.3%)		540 (69.8%)
CG	88 (20.5%)		215 (27.8%)
GG	5 (1.2%)		19 (2.5%)
Dominant model (CC vs CG+GG)			
(P value)		**0.001**	
SNP rs285			
CC	336 (45.6%)		421 (38%)
CT	330 (44.8%)		515 (46.5%)
TT	71 (9.6%)		171 (15.4%)
Dominant model (CC vs CT+TT)			
(P value)		**0.001**	

HDL-C: High density lipoprotein cholesterol

P values are adjusted for age, gender, body mass index, and Type 2 diabetes status

Those p values that are in bold implicates those values that are significant after Bonferroni correction

Table 3.3: Interaction between lipoprotein lipase single nucleotide polymorphisms and dietary factors on HDL-C levels

Beta coefficients ± standard error ($P_{interaction}$) for interaction on HDL-C (continuous variable)		
Interaction between rs4922115 fat energy intake (%)*	*Interaction between rs4922115* protein energy intake (%)*	*Interaction between rs4922115* carbohydrate energy intake (%)*
-0.01 ± 0.002 (0.1)	-0.02 ± 0.01 (0.2)	0.01 ± 0.02 (0.06)
Interaction between rs1121923 fat energy intake (%)*	*Interaction between rs1121923* protein energy intake (%)*	*Interaction between rs1121923* carbohydrate energy intake (%)*
-0.01 ± 0.01 (**0.003**)	-0.3 ± 0.01 (0.02)	0.01 ± 0.002 (0.05)
Interaction between rs328 fat energy intake (%)*	*Interaction between rs328* protein energy intake (%)*	*Interaction between rs328* carbohydrate energy intake (%)*
-0.01 ± 0.002 (0.16)	-0.02 ± 0.01 (0.12)	0.01 ± 0.002 (0.07)
Interaction between rs285 fat energy intake (%)*	*Interaction between rs285* protein energy intake (%)*	*Interaction between rs285* carbohydrate energy intake (%)*
0.01 ± 0.002	0.02 ± 0.01	-0.01 ± 0.002

(0.05)	(0.04)	(0.03)

HDL-C, High density lipoprotein cholesterol

$P_{interaction}$ values adjusted for age, gender, body mass index, type 2 diabetes and total energy intake

(*) refers to the interaction between SNP and dietary factor

Those p values that are in bold implicates those values that are significant after Bonferroni correction

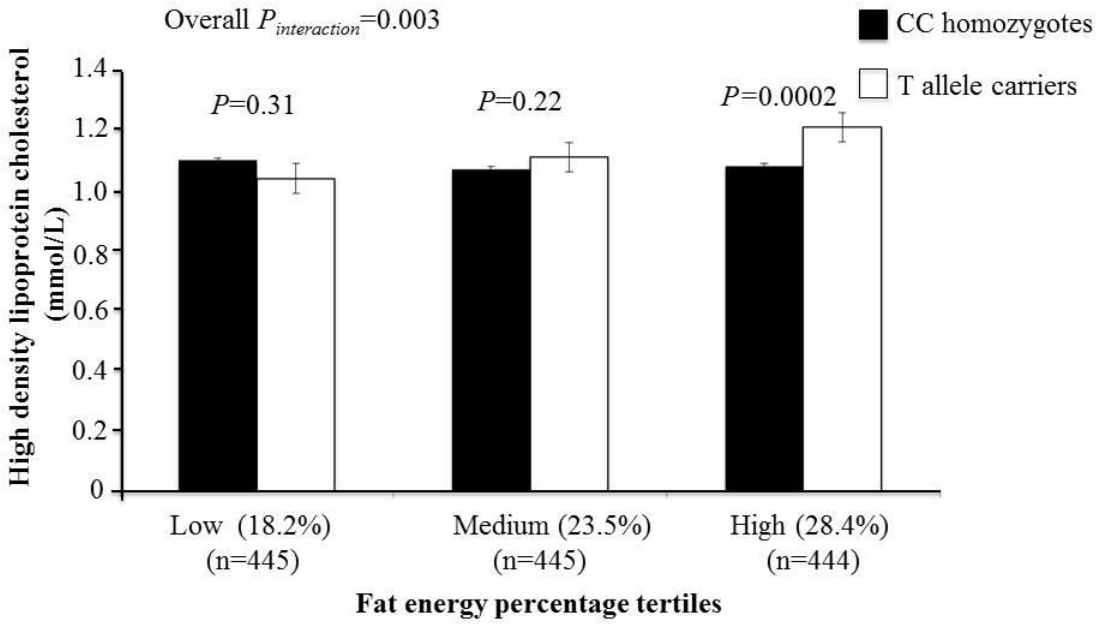

Figure 3.1 Interaction between Lipoprotein lipase gene SNP rs1121923 and fat energy intake (%) on HDL-C concentrations ($P_{interaction}$=0.003). Among those who consumed a high fat diet, T allele carriers had significantly higher levels of HDL-C compared to the CC homozygotes (P=0.0002).

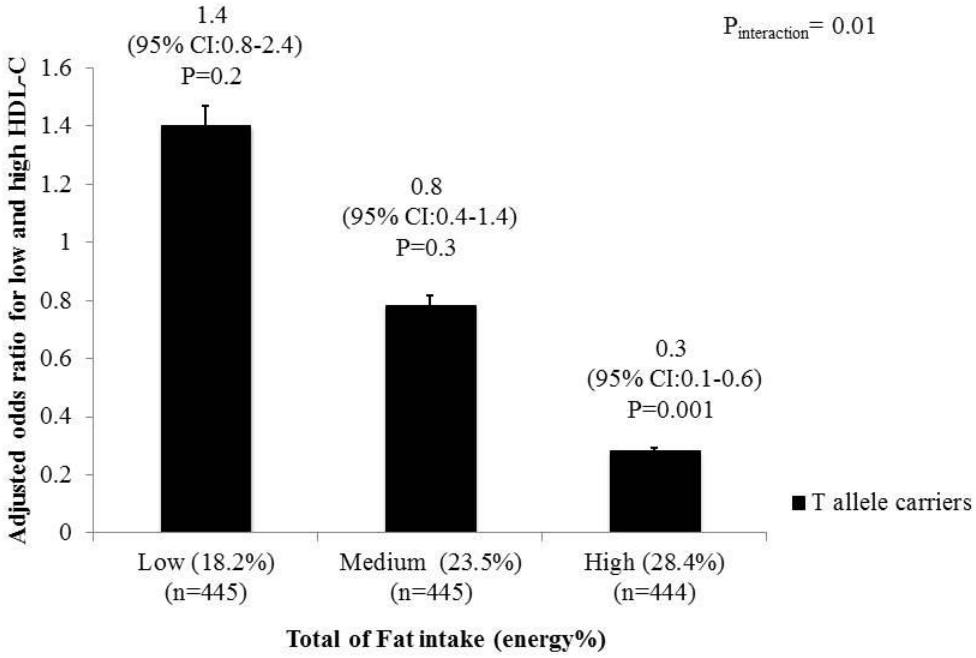

Figure 3.2 Interaction between Lipoprotein lipase gene SNP rs1121923 and fat energy intake (%) on HDL-C as a categorical variable ($P_{interaction}$=0.01). Among those who consumed a high fat diet (28.4%), the individuals who carried the T allele have 30% reduced risk of low HDL-C levels compared to those who carry the CC genotype (P=0.001). Data shown are represented as means ± SE. $P_{interaction}$ values adjusted for age, gender, body mass index, type 2 diabetes and total energy intake.

The SNP rs285 alone showed a significant association with serum TAG ($P=1x10^{-5}$), where CC genotype carriers had higher TAG concentrations than T allele carriers (**Table 3.4**). In the interaction analysis, there was an interaction of the *LPL* SNP rs4922115 with fat intake (energy %) on TAG, where, among those who consumed a low (1st tertile: 18.1 ± 2.6 %) or medium fat (2nd tertile: 23.4 ± 1.1 %) diet, individuals carrying the GG genotype had significantly lower TAG concentrations compared to 'A' allele carriers (P=0.01 for low fat intake; P=0.02 for medium fat intake). However, after correction for multiple testing, this interaction was not statistically significant. None of the other interactions between the SNPs and dietary factors on total cholesterol, serum TAG and LDL-C were statistically significant (**Table 3.5**).

Table 3.4: Association between single nucleotide polymorphisms (SNPs) at lipoprotein lipase gene and lipid traits

SNPs	Total cholesterol mmol/l	Triglycerides mmol/l	LDL-C mmol/l
SNP rs4922115			
GG	4.9 ± 1.1	1.6 ± 1.0	3.1 ± 0.9
GA	4.8 ± 1.1	1.9 ± 1.8	3.0 ± 0.9
AA	4.7 ± 0.9	1.7± 0.8	2.9 ± 0.8
Dominant model (GG vs GA+AA) (P value)	0.4	0.001	0.1
SNP rs1121923			
CC	4.8 ± 1.1	1.7 ± 1.4	3.0 ± 0.9
CT	5.1 ± 1.0	1.7± 1.0	3.2 ± 0.9
TT	4.9 ± 0.6	1.4 ± 0.4	3.3 ± 0.4
Dominant model (CC vs CT+TT) (P value)	0.4	0.3	0.9
SNP rs328			
CC	4.9 ± 1.0	1.7± 1.1	3.1 ± 0.9
CG	4.9 ± 1.1	1.5± 0.9	3.1 ± 0.9
GG	5.0 ± 0.9	1.4± 0.5	3.2 ± 0.8
Dominant model (CC vs CG+GG) (P value)	0.3	0.1	0.3
SNP rs285			
CC	4.8 ± 1.1	1.7± 1.2	3.0 ± 0.8
CT	4.8 ± 1.1	1.5± 0.9	3.1 ± 0.9
TT	4.8 ± 1.0	1.4± 0.8	3.0 ± 0.8
Dominant model (CC vs CT+TT) (P value)	0.7	**0.00009**	0.4

LDL-c, Low density lipoprotein cholesterol Results are expressed as mean ± SD. P values adjusted for age, gender, body mass index and type 2 diabetes Those p values that are in bold implicates those values that are significant after Bonferroni correction

Table 3.5 Interaction between single nucleotide polymorphisms (SNPs) at lipoprotein lipase gene and dietary factors on lipids traits

Beta coefficients (standard error) $P_{interaction}$* for interaction of SNP rs4922115 with dietary factors on total cholesterol		
Interaction between rs4922115 fat energy intake*	*Interaction between rs4922115* protein energy intake*	*Interaction between rs4922115* carbohydrate energy intake*
-0.1 (0.5)	-0.2 (1.9)	-0.01 (0.4)
0.8	0.9	0.9
Beta coefficients (standard error) $P_{interaction}$* for interaction of SNP rs4922115 with dietary factors on serum triglycerides		
Interaction between rs4922115 fat energy intake*	*Interaction between rs4922115* protein energy intake*	*Interaction between rs4922115* carbohydrate energy intake*
-3.5 (1.4)	7.4 (5.4)	-0.8 (1.1)
0.01	0.2	0.5
Beta coefficients (standard error) $P_{interaction}$* for interaction of SNP rs4922115 with dietary factors on LDL-C		
Interaction between rs4922115 fat energy intake*	*Interaction between rs4922115* protein energy intake*	*Interaction between rs4922115* carbohydrate energy intake*
-0.03 (0.4)	0.61 (1.6)	-0.08 (0.3)
0.9	0.7	0.8
Beta coefficients (standard error) $P_{interaction}$* for interaction of SNP rs1121923 with dietary factors on total cholesterol		
Interaction between rs1121923 fat energy intake*	*Interaction between rs1121923* protein energy intake*	*Interaction between rs1121923* carbohydrate energy intake*
-0.2 (0.7)	0.5 (2.4)	0.03 (0.5)
0.8	0.8	0.9
Beta coefficients (standard error) $P_{interaction}$* for interaction of SNP rs1121923 with		

dietary factors on serum triglycerides		
Interaction between rs1121923* fat energy intake (%)	Interaction between rs1121923* protein energy intake (%)	Interaction between rs1121923* carbohydrate energy intake (%)
2.5 (1.9)	3.8 (6.8)	-2.5 (1.5)
0.2	0.6	0.09
Beta coefficients (standard error) $P_{interaction}$* for interaction of SNP rs1121923 with dietary factors on LDL-C		
Interaction between rs1121923* fat energy intake (%)	Interaction between rs1121923* protein energy intake (%)	Interaction between rs1121923* carbohydrate energy intake (%)
-0.2 (0.6)	0.9 (1.9)	0.1 (0.4)
0.7	0.6	0.8
Beta coefficients (standard error) $P_{interaction}$* for interaction of SNP rs328 with dietary factors on total cholesterol		
Interaction between rs328* fat energy intake (%)	Interaction between rs328* protein energy intake (%)	Interaction between rs328* carbohydrate energy intake (%)
0.5 (0.5)	0.1 (0.2)	-0.1 (0.4)
0.3	0.7	0.8
Beta coefficients (standard error) $P_{interaction}$* for interaction of SNP rs328 with dietary factors on serum triglycerides		
Interaction between rs328* fat energy intake (%)	Interaction between rs328* protein energy intake (%)	Interaction between rs328* carbohydrate energy intake (%)
0.6 (1.3)	-0.6 (5.0)	-0.7 (0.9)
0.6	0.9	0.5
Beta coefficients (standard error) $P_{interaction}$* for interaction of SNP rs328 with dietary factors on LDL-C		
Interaction between rs328*	Interaction between rs328*	Interaction between rs328* carbohydrate energy intake

fat energy intake (%)	protein energy intake (%)	(%)
0.7 (0.5)	-0.2 (0.2)	-0.4 (0.3)
0.1	0.3	0.3
Beta coefficients (standard error) $P_{interaction}$* for interaction of SNP rs285 with dietary factors on total cholesterol		
Interaction between rs285* fat energy intake (%)	Interaction between rs285* protein energy intake (%)	Interaction between rs285* carbohydrate energy intake (%)
-0.2 (0.4)	-0.8 (1.5)	0.3 (0.3)
0.5	0.6	0.3
Beta coefficients (standard error) $P_{interaction}$* for interaction of SNP rs285 with dietary factors on serum triglycerides		
Interaction between rs285* fat energy intake	Interaction between rs285* protein energy intake	Interaction between rs285* carbohydrate energy intake
-1.6 (0.9)	-1.0 (3.4)	1.2 (0.7)
0.1	0.8	0.08
Beta coefficients (standard error) $P_{interaction}$* for interaction of SNP rs285 with dietary factors on LDL-C		
Interaction between rs285* fat energy intake	Interaction between rs285* protein energy intake	Interaction between rs285* carbohydrate energy intake
-0.09 (0.3)	-1.0 (1.3)	0.1 (0.3)
0.8	0.5	0.7

$P_{interaction}$ values adjusted for age, gender, body mass index, type 2 diabetes and total energy intake

3.5 Discussion

To our knowledge, this is the first genetic epidemiological study to investigate the interaction between SNPs at *LPL* gene and dietary factors on blood lipids in an Asian Indian population. Our study provides evidence for a novel interaction between SNP rs1121923 and fat intake (energy %) on HDL-C, where the T allele carriers had significantly higher levels of HDL-C compared to the CC homozygotes among those who consumed a high fat diet. Given that the total fat intake has increased in India in the last few decades and Asian Indians are characterized by altered lipid levels and at a higher risk of premature coronary artery disease (CAD) [38], our study findings have significant public health implications.

Several *LPL* polymorphisms have been extensively studied in association with various lipid traits [12, 22, 25, 26]. The most notable of these known functional common polymorphisms is rs328, also known as S447X (premature truncation at codon 447). SNP rs328 is a gain-of-function polymorphism that has been shown to be consistently associated with higher HDL-C [12]. Our study has also shown a significant association of the SNP rs328 with HDL-C concentrations, where the minor 'G' allele carriers had significantly higher HDL-C compared to those with common CC genotype. The rs285 (*Pvu II)* variant located in the intron 6 of the *LPL* gene has been shown to be associated with dyslipidemic phenotypes such as low HDL-C and high TAG among Caucasians [39], which is in accordance with our study findings in Asian Indians where the CC genotype carriers of the SNP rs285 had significantly higher TAG than T allele carriers. The 'A' allele of the SNP rs4922115 (located in the 3' UTR region) and 'C' allele of the SNP rs1121923 (Val135Val) (located in the exon 3) were also associated

with lower HDL-C levels in our study; however, after correction for multiple testing they were not significant. Our findings confirm the previously reported associations and reveal that *LPL* SNPs play an important role in lipid metabolism in this Asian Indian population.

Total fat intake has increased considerably in India in the last few decades [40]. The National Sample Survey Organization survey has reported that the fat intake of urban component of Indian populations has increased from 42.0 g/d/capita in 1993–1994 to 52.5 g/d/capita in 2011–2012 [41]. Interestingly our study in this South Indian population has identified an interaction between *LPL* SNP rs1121923 and fat intake (energy %) on HDL-C, where, among those who consumed a high fat diet (28.4%), individuals carrying the T allele had significantly higher HDL-C concentrations compared to the CC genotype carriers. Even though our study is the first to report this gene-diet interaction, previous studies in developing countries have shown that the quantity of dietary fats can affect the lipid profile and is directly related to the development of metabolic diseases such as obesity and diabetes [42, 43]. Quality of dietary fat has also shown to alter blood lipid levels [43] but the present study failed to identify an interaction of the *LPL* SNP with MUFA, PUFA and SFA, respectively, on lipid traits. Vegetable oils used in Indian cooking represent 80% of the visible fat consumed [44] and a study in an Indian population showed that the higher ratio of n_6:n_3 was attributed to the type and quantity of oil used [45]. Previous studies have shown that dietary fat increases HDL-C [46], which is partially explained by our study findings where the T allele carriers of the SNP rs1121923, who consumed a high fat diet, had higher HDL-C concentrations compared to CC genotype carriers. Even

though the exact mechanism by which T allele contributes to the increase in HDL-C levels under conditions of high fat diet is unknown, the finding is suggestive of the complex inheritance pattern of the HDL-C levels [47], where several genes/polymorphisms are likely to contribute to the alteration of HDL-C levels through gene-gene and gene-diet interactions. Our findings are supported by animal studies [48] where mice that are challenged with a high fat diet showed a strong correlation between LPL activity and HDL cholesterol suggesting the link between LPL, fat intake and HDL levels.

Besides HDL-C, there were interactions of the *LPL* SNP rs4922115 with fat energy intake (%) on TAG, where, among those who consumed a low or medium fat diet, individuals carrying the GG genotype had significantly lower TAG concentrations compared to 'A' allele carriers. The SNP rs4922115 has not been studied previously in other populations except in Hispanics [49], where this SNP was identified by direct sequencing. However, the study did not explore the individual effect of the SNP on blood lipids and hence we are unable to compare our findings with the Hispanic study. Even though the interaction between SNP rs4922115 and fat energy intake (%) was not statistically significant after Bonferroni correction in our study, replication of these interactions in another large cohort is highly warranted.

One of the main limitations of the study is the small sample size. Given that there are no previously reported effect sizes for the *LPL* SNP-diet interaction on blood lipids, we are unable to calculate the statistical power for our study. However, we were still able to identify significant gene-diet interactions on HDL-C even after correction for multiple testing. The interaction was significant only with total fat intake (energy %)

but not when split as PUFA, MUFA and SFA, which might be due to small sample size. Nevertheless, our study is well powered to identify the effect sizes for association between the SNPs and lipids. To increase the statistical power, individuals with and without T2D were included and hence it is possible that the T2D status could have introduced a bias in our study as the dietary pattern is likely to be changed among 'known' diabetic participants. However, T2D status was adjusted in all the analyses and the interaction findings were borderline significant even after excluding the 'known' diabetic participants from our analysis (data not shown), which could also be due to the small sample size after exclusion. Another limitation is that, our study was cross-sectional and therefore was unable to examine the casual relationship between fat intake and lowering of HDL-C levels; randomized controlled trials with prospective genotyping are required to explore the causality using genetic markers. The main strength of the present study is that a validated interviewer-administered FFQ was used to measure the usual long-term intake of the population. Furthermore, the sampling is representative of the overall population of Chennai. Indeed, the intake of major foods in our study was similar to the findings of the pooled urban data of the National Nutrition Monitoring Bureau for ten states in India [50] and hence, the results of the present study could be reasonably extrapolated to urban Indian population.

3.6 Conclusions

Our study confirms the association between *LPL* SNP rs328 and HDL-C concentrations and also provides an evidence for a novel interaction between SNP rs1121923 and fat intake (energy %) on HDL-C levels in this Asian Indian population.

Given that Asian Indians have altered lipid profile and an increased predisposition to premature CAD [38, 40], our study suggesting that individuals carrying T allele of the SNP rs1121923 have increased HDL-C levels when consuming a high fat diet has significant public health implications. This finding warrants confirmation in prospective studies and randomized controlled trials.

Author contributions: Israa Shatwan performed the statistical analysis and drafted the manuscript; AKA performed the genotyping and statistical analysis; BD, LRB and SV assisted with the statistical analysis; MV, ARM, SV and RV designed the CURES study; RV designed the genetic study; BD, ARM, SV, JAL, MV and RV critically reviewed the manuscript; VKS conceived the nutrigenetics study, performed the statistical analysis and wrote the manuscript. All authors contributed to and approved the final version of the manuscript.

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3.7 References

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Chapter 4

For this study, I was involved in cleaning the dataset to perform statistical analysis. I prepared the analysis plan for running the statistical analysis and did the entire literature review of the topic. I ran the statistical analysis using the SPSS software and wrote the first draft of the manuscript. I revised the manuscript based on the comments from all the co-authors before the manuscript was submitted to the Lipids in Health and disease journal. I was also involved in drafting the responses to the comments from reviewers.

Chapter 4 Apolipoprotein E and lipoprotein lipase gene polymorphisms, dietary factors and blood lipids

Under review in Lipids in Health and disease

4.1 Abstract

Background: Several candidate genes have been identified in relation to lipid metabolism, and among these, lipoprotein lipase (*LPL*) and apolipoprotein E (*APOE*) gene polymorphisms are major sources of genetically determined variation in lipid concentrations. This study investigated the association of two single nucleotide polymorphisms (SNPs) at *LPL*, seven tagging SNPs at the *APOE* gene, and a common *APOE* haplotype (two SNPs) with blood lipids, and examined the interaction of these SNPs with dietary factors. **Methods**: The population studied for this investigation included 660 individuals from the Prevention of Cancer by Intervention with Selenium (PRECISE) study who supplied baseline data. The findings of the PRECISE study were further replicated using 1,238 individuals from the Caerphilly Prospective cohort (CaPS). Dietary intake was assessed using a validated food-frequency questionnaire (FFQ) in PRECISE and a validated semi-quantitative FFQ in the CaPS. Interaction analyses were performed by including the interaction term in the linear regression model adjusted for age, body mass index, sex and country. **Results**: There was no association between dietary factors and blood lipids after Bonferroni correction and adjustment for confounding factors in either cohort. In the PRECISE study, after correction for multiple testing, there was a statistically significant association of the *APOE* haplotype (rs7412 and rs429358; E2, E3, and E4) and *APOE* tagSNP rs445925 with total cholesterol ($P=4x10^{-4}$ and P=0.003, respectively). Carriers of the E2 allele had lower total cholesterol

concentration (5.54± 0.97 mmol/L) than those with the E3 (5.98± 1.05 mmol/L) (P=0.001) and E4 (6.09± 1.06 mmol/L) ($P=2x10^{-4}$) alleles. The association of *APOE* haplotype (E2, E3, and E4) and *APOE* SNP rs445925 with total cholesterol ($P=2x10^{-6}$ and $P=3x10^{-4}$, respectively) was further replicated in the CaPS. Additionally, significant association found between *APOE* haplotype and *APOE* SNP rs445925 with low density lipoprotein cholesterol in CaPS ($P=4x10^{-4}$ and P=0.001, respectively). After Bonferroni correction, none of the cohorts showed a statistically significant SNP-diet interaction with lipid outcomes. **Conclusion**: In summary, our findings from the two cohorts confirm that genetic variations at the *APOE* locus influence plasma total cholesterol concentrations, however, the gene-diet interactions on lipids require further investigation in larger cohorts.

4.2 Background

Cardiovascular diseases (CVD) are common multifactorial conditions characterized by dyslipidaemia, type 2 diabetes and hypertension [1, 2]. Elevated triacylglycerol (TAG) and reduced high density lipoprotein cholesterol (HDL-C) concentrations are associated with an increased risk of developing CVD [3-5]. Furthermore, several studies have reported that certain genetic variants influence susceptibility to altered circulating lipid concentrations, leading to an increased risk of CVD events [6-8]. Genetic variations have been shown to be associated with lipid outcomes, while dietary factors appear to modulate the effect of such genes on lipid concentrations [9, 10]. Previous studies have shown that single nucleotide polymorphisms (SNPs) of the apolipoprotein E (*APOE*) [6, 11] and lipoprotein lipase (*LPL*) [12-14] genes contribute to significant variation in lipid concentrations.

The APOE protein plays a key role in the transport and metabolism of cholesterol

and TAG containing particles by serving as a receptor-binding ligand that mediates the clearance of dietary derived chylomicrons, and hepatically derived very low density lipoprotein (VLDL) and their remnants from the circulation [6]. The three most recognized alleles of the *APOE* gene are E2, E3 and E4, with carriage of E4 associated with CVD risk factors and increased low density lipoprotein cholesterol (LDL-C) concentrations [11, 15, 16], and hence increased CVD risk [17, 18].

Genetic variations in the *LPL* gene have been reported to be involved with lipid metabolism and partly explain the phenotypic variation in blood lipid levels [19]. LPL is a lipolytic enzyme that catalyses hydrolysis of TAG in all of the major classes of TAG-rich lipoproteins [20]. High enzyme activity is associated with favourable lipid levels, including relatively low TAG concentrations [21]. The two most widely studied *LPL* SNPs, rs328 (S447X) and rs320 (HindIII) [22, 23]. The 'G' minor alleles of both the SNPs, rs328 and rs320, are associated with decreased TAG concentrations and increased HDL-C concentrations, whereas the opposite association was found for the 'C' allele and 'T' allele respectively [24-26].

Data from several studies supports the role of genetic factors in lipid metabolism [27]; however, only a few studies have examined the effects of lifestyle factors such as diet on the association of polymorphisms with lipid-related outcomes [10, 28, 29]. Therefore, the present study aimed to investigate the effect of seven *APOE* tagSNPs (rs405509, rs769450, rs439401, rs445925, rs405697, rs1160985, and rs1064725), one *APOE* haplotype (rs7412 and rs429358), and two commonly studied *LPL* SNPs (rs328 and rs320) on blood lipids profile in 660 participants (baseline data) from the Prevention of Cancer by Intervention with Selenium (PRECISE) study. As diet type and intake is

also known to modify lipid levels [30-32], the potential impact of the interaction between these SNPs and dietary factors on lipid levels was also investigated. To confirm the findings, the Caerphilly Prospective Study (CaPS; n=1,238) was used as a replication cohort.

4.3 Material and methods

4.3.1 PRECISE cohort

4.3.1.1 Participants and methods

Baseline data of 660 individuals from the PRECISE study, conducted in two populations [UK (n=468) and Denmark (n=192)] were used for the analysis [33, 34]. Briefly, study participants were selected from four general practices (study centres) in various areas of the UK that were affiliated with the Medical Research Council General Practice Research Framework (MRC GPRF). Between June 2000 and July 2001, research nurses recruited similar numbers of men and women from each of three age groups: 60–64, 65–69 and 70–74 years. The Danish participants were men and women recruited from the same three age groups from the County of Funen in Denmark.

The UK study obtained approval from the appropriate UK Local Research Ethics Committees [South Tees (ref: 99/69), Worcestershire Health Authority (ref: LREC 74/99), Norwich District (ref: LREC 99/ 141), Great Yarmouth and Waveney (under reciprocal arrangements with Norwich District LREC)], and the participants provided written informed consent. The regional Danish Data Protection Agency and Scientific Ethical Committees of Vejle and Funen counties approved the Danish study (Journal number. 19980186).

4.3.1.2 Dietary information

Information about each participant's usual dietary intake was obtained using validated EPIC food frequency questionnaires (FFQ) [35]. Total energy intake and macronutrient composition were analysed using the FETA software program [36].

4.3.1.3 Anthropometric measurements and biochemical analysis

Body mass index (BMI) was calculated as body weight in kilograms divided by height in square metres (kg/m^2). Participants provided non-fasting blood samples for biochemical analysis and these samples were stored at −80°C. Total cholesterol and HDL-C concentrations in lithium-heparin plasma were measured using an Architect c16000 analyser (Abbott) with dedicated reagents. Measurements were performed by enzymatic colorimetric analysis. Traceability for total cholesterol and HDL-C was ensured through participation in the National Reference System for Cholesterol (NRS/CHOL), as established by the Clinical and Laboratory Standards Institute, with isotope dilution-MS used as the reference method, and reference material taken from the National Institute of Standard and Technology. Evidence of equivalence in the analytical performance of the cholesterol-oxidase assays performed in the UK and Denmark from a comparison of total cholesterol on forty-four serum samples which produced a limit of variation of 2% [33].

4.3.1.4 SNP selection

The *APOE* gene is located on chromosome 19q13.32. It comprises four exons, which are transcribed into the *APOE* mRNA which is 1,180 nucleotides long. The seven

tagSNPs for the *APOE* gene were chosen based on International HapMap Phase II collected from individuals of Northern and Western European ancestry (CEU) (HapMap Data release 27 Phase 2+3, Feb 09, NCBI B36 assembly, dbSNP b126). The Haploview software V3.3 (http://www.broadinstitute.org/haploview/haploview-downloads) was used to assess the linkage disequilibrium between SNPs. Tagger software was used to select tagSNPs with the 'pairwise tagging only' option. Two criteria were used to filter the SNPs included in the analysis, minor allele frequency ≥5% and Hardy–Weinberg equilibrium P-value >0.01. In total, seven tagSNPs [rs405509 (G>T), rs1160985 (C>T), rs769450 (G>A), rs439401 (C>T), rs445925 (G>A), rs405697 (G>A), and rs1064725 (T>G)] representing the entire common genetic variations across the *APOE* gene were selected for the study. The *APOE* haplotype/SNPs [6, 11, 37-44] and *LPL* [12, 13] SNPs were chosen based on their previous association with various lipid outcomes.

4.3.1.5 DNA isolation and genotyping

The genotyping for the selected SNPs using a KASP assay with a competitive allele-specific PCR assay® was performed on DNA samples by LGC Genomics (Hoddesdon, Herts, UK). The eleven SNPs were in Hardy Weinberg Equilibrium (HWE) (P>0.05 for all comparisons) (Table 4.6).

4.3.2 Caerphilly Prospective Study (CaPS)

4.3.2.1 Participants and methods

The CaPS was used to replicate the findings from the PRECISE study. The phase 1 (July 1979 to September 1983) recruitment for the CaPS included 2,512 men aged 45-

59 years who were living in the town of Caerphilly and five of its adjacent villages in the UK; these participants were followed up at regular intervals [45, 46]. The follow-up data collection included periods from 1984 to1988 (phase 2), from 1989 to 1993 (phase 3), from 1993 to 1997 (phase 4), and from 2002 to 2005 (phase 5). For the current study, the data analysed were taken from phase 3 (n=1,238), which had the maximum number of samples and variables appropriate to this analysis (total cholesterol and dietary information), and from phase 5 (n=529) (HDL-C and LDL-C). Ethical approval was obtained from the South Wales Research Ethics Committee D, and each subject provided written informed consent.

4.3.2.2 Dietary information

Participants completed validated semi-quantitative FFQ in phase 3 [47, 48]. The FFQ included 50 typical food items in the British diet in order to estimate the mean daily energy intake and macronutrients and micronutrients consumption.

4.3.2.3 Anthropometric measurements and biochemical analysis

Height and weight was recorded in order to calculate the BMI. Height was measured on a staidiometer and weight was measured on a beam balance. Plasma prepared from blood samples taken after an overnight fast were transported at 4°C to the laboratories on the day of venepuncture. Total cholesterol and HDL-C, LDL-C concentrations were measured using enzymatic procedures [49]. and the LDL-C levels were calculated using the Friedewald Formula [50].

4.3.2.4 DNA isolation and genotyping

DNA was extracted from blood samples collected during the period 1992–1994. SNP information was obtained from the Illumina Cardio Metabochip, which includes data on 200,000 SNPs from regions previously identified for associations with risk factors for cardiometabolic disease [51]. Imputation was conducted against the 1000-genomes reference panel, providing information on approximately two million typed or imputed SNPs. Duplicate samples were genotyped to compute the error rate. Quality control on genotyped samples has been previously reported [52] and the SNPs had a call rate of >98%. The SNPs were in HWE (P>0.05) (Table 4.6).

4.3.3 Statistical analysis

Statistical analysis was performed using the SPSS software package, version 22.0. Univariate linear regression analysis was applied to test for association of the SNPs with total cholesterol and HDL-C, controlling for age, sex, BMI and country. SNP-diet interactions on total cholesterol and HDL-C were investigated using a univariate general linear model. In this model, total cholesterol and HDL-C were the dependent variables, SNPs were fixed factors, and dietary factors (fat energy %, protein energy %, carbohydrate energy %), sex, age BMI, and country were covariates. The dominant model was applied for all SNPs with minor allele frequency ≤0.3 and the additive model applied for SNPs with minor allele frequency ≥0.4. For analytical purposes, the six *APOE* genotype groups (E2/E2, E2/E3, E3/E3, E3/E4, E4/E4, and E2/E4) were classified into three groups. The E3/E3 genotype was classified as a group as it occurs at high frequency in the population (wild type). The E2/E2 and E2/E3 genotypes were combined and presented as E2 carriers. The E3/E4 and E4/E4 genotypes were also combined, and

presented as E4 carriers [29]. Previous studies have shown that the impact of the E2 allele on serum lipids is greater than that of the E4 allele [17], therefore, the E2/E4 genotype was excluded from the analysis. The Bonferroni correction was applied separately for association and interaction analyses. For association between phenotypic and dietary factors, the Bonferroni-corrected P value was 0.008 (2 lipid outcomes* 3 dietary factors) for the PRECISE study and P value was 0.01 for CaPS (total cholesterol was the only variable available). For association between SNPs and lipids, the Bonferroni corrected P value was 0.003 (10 SNPs*2 lipid outcomes = 20 tests). For interactions, the Bonferroni corrected P value was 0.001 (10 SNPs*2 lipid outcomes*3 dietary factors = 60 tests). In the replication analysis, the Bonferroni corrected P value for association was 0.002 (10 SNPs*3 lipid outcomes = 30 tests), while for interactions it was 0.001 (10 SNPs*1 lipid outcome* 3 dietary factors = 30 tests).

4.4 Results

4.4.1 Participant characteristics

The general characteristics of the participants by sex are presented in **Table 4.1**. In the PRECISE study, women were found to have significantly higher total cholesterol and HDL-C concentrations than men ($P=2.31x10^{-10}$ and $P= 2.71x10^{-16}$, respectively). The consumption of carbohydrates ($P=1.42x10^{-9}$) and protein (energy %) ($P=5x10^{-5}$) were higher in women than in men, whereas the consumption of fat (energy %) and total energy intake were lower in women than in men (P=0.01). Characteristics of the individuals from CaPS are given in **Table 4.1**. Elevated total cholesterol levels were observed among men at phase 3. Dietary-pattern data showed higher consumption of

energy from total fat.

4.4.2 Association between dietary factors and blood lipids

In both the PRECISE and CaPS, there was no association between the dietary factors and total cholesterol or high-density lipoprotein after Bonferroni correction and adjustment for confounding factors (**Table 4.2**).

4.4.3 Genotypes and serum lipid levels in the PRECISE study

As shown in **Table 4.3**, of the seven tagSNPs at *APOE*, tagSNP rs445925 was significantly associated with total cholesterol (P=0.003) after correction for multiple testing. The 'A' allele carriers (5.65± 0.98 mmol/L) had 5% lower levels of total cholesterol than GG homozygotes (5.99± 1.06 mmol/L).

The levels of HDL-C were significantly different among the *LPL* SNP genotypes, rs328 (P=0.04) and rs320 (P=0.02), where the carriers of the 'G' minor allele of both SNPs had higher levels of HDL-C (1.68 ± 0.41 mmol/L for rs328 and 1.66 ±0.40 mmol/L for rs320) than CC homozygotes (rs328) and TT homozygotes (rs320) (1.61 ± 0.38 and 1.60 ±0.39 mmol/L) respectively. However, these associations were not statistically significant after Bonferroni correction.

Table 4.1: Baseline characteristics of the PRECISE and Caerphilly Prospective studies participants

	PRECISE study			Caerphilly Prospective study (CaPS)
Characteristics	Men (N=248 UK, 95 Danish)	Women (N=220 UK, 97 Danish)	P value	Men (N=1,238)
Age (years)	67 ± 4	67± 4	0.12	62± 4
Body mass index (kg/m^2)	27.2± 4.9	27.3± 4.9	0.82	26.8± 3.7
Total Cholesterol (mmol/L)	5.6± 0.9	6.2± 1.1	$2.31x10^{-10}$	6.1± 1.1
High density lipoprotein cholesterol (mmol/L)*	1.5± 0.3	1.7± 0.4	$2.71x10^{-16}$	1.3± 0.3
Protein intake (total energy %)	17.6± 3.7	18.8± 3.7	$5X10^{-5}$	14.9± 2.7
Carbohydrate intake (total energy %)	42.8± 13.3	48.2± 8.7	$1.42x10^{-9}$	48.4± 7.5
Fat intake (total energy %)	35.3± 7.1	33.9± 6.9	0.01	36.5± 6.9
Total energy intake (kcal)	2256 ± 658	1992± 613	$2.63x10^{-7}$	1964 ± 625
Total energy intake (MJ)	9.4± 2.7	8.3± 2.6	$2.63x10^{-7}$	8.2± 2.6

Data shown are represented as means ± SD, wherever appropriate. P values are for the differences in the means between men and women. P values were calculated by using independent t-test.

*For CaPS, HDL-C levels were obtained from phase 5 while all other variables were obtained from phase 3.

Table 4.2: Association between dietary factors and lipids in PRECISE and Caerphilly Prospective studies

PRECISE study		
Association between dietary factors and total cholesterol		
Fat total energy % intake Beta (± S.E), $P_{association}$	Protein total energy % intake Beta (± S.E), $P_{association}$	Carbohydrate total energy % intake Beta (± S.E), $P_{association}$
0.01 (0.01) 0.47	-0.01 (0.01) 0.13	-0.004 (0.01) 0.40
Association between three dietary factors and HDL-C high density lipoprotein		
Fat total energy % intake	Protein total energy % intake	Carbohydrate total energy % intake
-0.002 (0.002) 0.29	-0.002 (0.004) 0.59	-0.004 (0.002) 0.02
Caerphilly Prospective study		
Association between three dietary factors and total cholesterol		
Fat total energy % intake Beta (± S.E), $P_{association}$	Protein total energy % intake Beta (± S.E), $P_{association}$	Carbohydrate total energy % intake Beta (± S.E), $P_{association}$
0.01 (0.004) 0.06	-0.01 (0.01) 0.26	-0.01 (0.004) 0.17

P values were obtained using linear regression adjusted for age, sex, body mass index and country. HDL-C high density lipoprotein cholesterol.

4.4.4 *APOE* haplotype and serum lipid levels in the PRECISE study

The effects of *APOE* haplotypes (E2, E3, and E4) on serum lipids are shown in **Table 4.3**. These haplotypes (E2, E3, and E4) were significantly associated with total cholesterol ($P=4x10^{-4}$) after correction for multiple testing. The carriers of the E2 allele (5.54± 0.97 mmol/L) had lower total cholesterol concentrations than the carriers of the E3 (P=0.001) (5.98± 1.05 mmol/L) and E4 alleles (6.09± 1.06 mmol/L) ($P=2x10^{-4}$).

4.4.5 Interactions between genotypes and dietary factors on serum lipids in the PRECISE study

None of the dietary factors significantly interacted with the *APOE* SNPs, haplotypes and *LPL* SNPs with plasma lipids after correction for multiple testing (P >0.001) (**Table 4.4**).

4.4.6 Replication analysis: Effect of SNPs at *APOE* and *LPL* on serum lipids in the CaPS

The associations of *APOE* and *LPL* SNPs with blood lipids in the CaPS are presented in **Table 4.3**. The association of *APOE* haplotype (E2, E3, and E4) and *APOE* SNP rs445925 with total cholesterol ($P=2x10^{-6}$ and $P=3x10^{-4}$, respectively) was replicated. The 'A' allele carriers of *APOE* SNP rs445925 had lower total cholesterol (5.96±1.24 mmol/l) than 'GG' genotypes (6.24±1.08 mmol/L). In the *APOE* haplotype analysis, the carriers of the E2 allele had 5% and 14% lower total cholesterol than carriers of the E3 ($P=4x10^{-4}$) and E4 alleles ($P=3x10^{-6}$), respectively. Additionally, significant association was seen between *APOE* haplotypes (E2, E3, and E4) and *APOE* SNP rs445925and LDL-C ($P=4X10^{-4}$, 0.001, respectively).

There was an interaction between fat (% energy) and *APOE* haplotype (E2, E3, and E4) on total cholesterol (P=0.038) in CaPS. However, after correction for multiple testing, all the SNP-diet interactions were consistent with chance variation (**Table 4.5**).

Table 4.3: Association of *APOE* and *LPL* SNPs with HDL-C, LDL-C and total cholesterol levels in the PRECISE and Caerphilly prospective studies

SNP	MAF	HDL-C (mmol/L)	Total Cholesterol (mmol/L)	LDL-C * (mmol/L)
		PRECISE		
LPL				
rs320	0.26			
TT		1.6 ±0.3	5.9 ±1.1	
T/G		1.7 ±0.4	5.8 ±1.0	
P value		0.02	0.19	
rs328	0.10			
CC		1.6 ± 0.3	5.9 ±1.1	
C/G		1.7 ± 0.4	5.7 ± 0.9	
P value		0.04	0.06	
APOE				
rs405509	0.47			
GG		1.7± 0.4	5.8± 1.1	
GT		1.5± 0.3	5.8± 1.1	
TT		1.6± 0.3	6.1± 1.0	
P value		0.07	0.23	
rs769450	0.39			
GG		1.6± 0.3	5.9± 1.1	
A allele		1.6± 0.4	5.9± 1.1	
P value		0.72	0.97	
rs439401	0.33			
CC		1.6± 0.4	5.9± 1.1	
T allele		1.6± 0.3	5.9± 1.1	
P value		0.43	0.51	
rs445925	0.11			
GG		1.6± 0.3	5.9± 1.1	
A allele		1.7± 0.4	5.6± 0.9	
P value		0.25	**0.003**	
rs405697	0.25			
GG		1.6± 0.4	5.9± 1.1	
A allele		1.6± 0.3	5.9± 1.0	
P value		0.71	0.96	
rs1160985	0.43			
CC		1.6± 0.3	5.9± 1.1	
CT		1.6± 0.4	5.8± 1.0	
TT		1.7± 0.4	5.9± 1.1	
P value		0.12	0.44	
rs1064725	0.04			
TT		1.6± 0.4	5.9± 1.0	

G allele		1.7± 0.3	6.1± 1.2	
P value		0.17	0.38	
(**rs7412-rs429358**)				
E2, E3, and E4				
E3		1.6± 0.3	5.9± 1.1	
E4		1.5± 0.3	6.1± 1.1	
E2		1.7± 0.4	5.5± 0.9	
P value		0.09	**$4X10^{-4}$**	
		Caerphilly		
LPL				
rs320	0.26			
TT		1.3± 0.3	6.1± 1.1	2.7± 0.8
T/G		1.4± 0.3	6.2± 1.2	2.8± 0.8
P value		0.05	0.55	0.05
rs328	0.10			
CC		1.3± 0.3	6.1± 1.1	2.7± 0.8
C/G		1.3± 0.3	6.1± 1.1	2.9± 0.9
P value		0.63	0.71	0.05
APOE				
rs405509	0.46			
GG		1.4± 0.3	6.0± 1.1	2.7± 0.9
GT		1.3± 0.3	6.2± 1.1	2.8± 0.8
TT		1.3± 0.3	6.3± 1.1	2.9± 0.9
P value		0.16	0.02	0.29
rs769450	0.41			
GG		1.3± 0.2	6.1± 1.2	2.8± 0.9
A allele		1.4± 0.3	6.2± 1.1	2.8± 0.8
P value		0.10	0.41	0.82
rs439401	0.35			
CC		1.4± 0.3	6.2± 1.1	2.8± 0.9
T allele		1.3± 0.3	6.1± 1.1	2.7± 0.8
P value		0.72	0.42	0.32
rs445925	0.11			
GG		1.3± 0.3	6.2± 1.1	2.8± 0.8
A allele		1.3± 0.3	5.9± 1.2	2.5± 0.9
P value		0.99	**$3X10^{-4}$**	**0.001**
rs405697	0.26			
GG		1.4± 0.4	6.1± 1.1	2.8± 0.9
A allele		1.3± 0.3	6.1± 1.1	2.8± 0.8
P value		0.30	0.88	0.9
rs1160985	0.45			
CC		1.34± 0.29	6.2± 1.1	2.8± 0.9
CT		1.35± 0.35	6.2± 1.2	2.7± 0.8
TT		1.37± 0.40	6.1± 1.0	2.8± 0.8
P value		0.61	0.30	0.73

rs1064725	0.01			
TT		1.3± 0.3	6.2± 1.1	2.8± 0.8
G allele		1.4± 0.3	6.1± 1.1	2.8± 0.7
P value		0.18	0.60	0.68
(rs7412-rs429358) E2, E3, and E4				
E3		1.4± 0.4	6.2± 1.1	2.8± 0.8
E4		1.4± 0.3	6.4± 1.1	3.0± 0.9
E2		1.3± 0.3	5.8± 1.3	2.4± 0.8
P value		0.95	**$2X10^{-6}$**	**$4X10^{-4}$**

Values are given as mean ± SD. P values for differences between genotypes were obtained using linear regression model adjusted for age, sex, body mass index, and country.

Bonferroni corrected P value <0.003 was considered statistically significant.

MAF; minor allele frequency. HDL-C high-density lipoprotein cholesterol, LDL-C; low-density lipoprotein cholesterol.
* LDL-C values available only in Caerphilly prospective study.

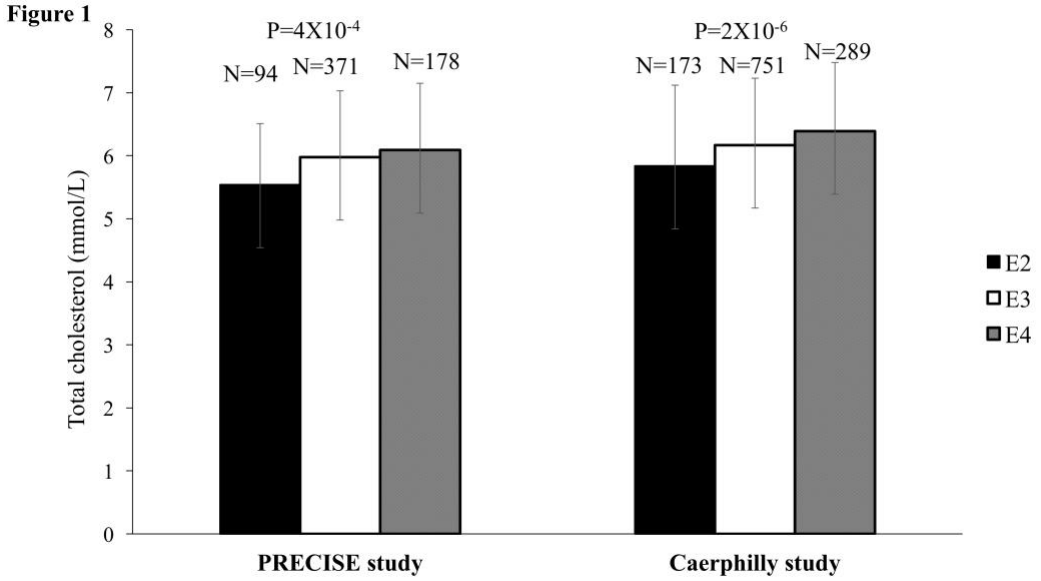

Figure 4.1 Association of *APOE* haplotypes (E2, E3, and E4) with total cholesterol concentrations in the Prevention of Cancer by Intervention with Selenium (PRECISE) study and Caerphilly Prospective study (CaPS). E2 allele carriers have significantly lower levels of total cholesterol than E3 (P=0.001 and $P=4x10^{-4}$ in the PRECISE and CaPS, respectively) and E4 ($P=2x10^{-4}$ and $P=3x10^{-6}$ in the PRECISE and CaPS, respectively) allele carriers.

Table 4.4: Interaction between *APOE* and *LPL* SNPs and dietary factors on HDL-C and total cholesterol in the PRECISE study

Interaction between rs320 at *LPLdietary factors on HDL-C**		
Interaction between SNP rs320* fat energy % intake	Interaction between SNP rs320* protein energy % intake	Interaction between SNP rs320* carbohydrate energy % intake
0.003 (0.004) 0.46	0.002 (0.01) 0.76	-0.0004 (0.002) 0.87
Interaction between rs320 at *LPL* *dietary factors on Total Cholesterol		
Interaction between SNP rs320* fat energy % intake	Interaction between SNP rs320* protein energy % intake	Interaction between SNP rs320* carbohydrate energy % intake
0.01(0.01) 0.27	-0.03 (0.02) 0.13	-0.01 (0.01) 0.06
Interaction between rs328 at *LPL* *dietary factors on HDL-C		
Interaction between SNP rs328* fat energy % intake	Interaction between SNP rs328* protein energy % intake	Interaction between SNP rs328* carbohydrate energy % intake
0.01 (0.01) 0.09	-0.001 (0.01) 0.89	0.001 (0.003) 0.63
Interaction between rs328 at *LPL* *dietary factors on Total Cholesterol		
Interaction between SNP rs328* fat energy % intake	Interaction between SNP rs328* protein energy % intake	Interaction between SNP rs328* carbohydrate energy % intake
-0.002 (0.02) 0.88	0.003 (0.03) 0.90	-0.01 (0.01) 0.55
Interaction between rs405509 at *APOEdietary factors on HDL-C**		
Interaction between SNP rs405509* fat energy % intake	Interaction between SNP rs405509* protein energy % intake	Interaction between SNP rs405509* carbohydrate energy % intake
0.01 (0.01) 0.11	-0.001 (0.01) 0.75	-0.01 (0.003) 0.09
Interaction between rs405509 at *APOE* *dietary factors on Total Cholesterol		
Interaction between SNP rs405509* fat energy % intake	Interaction between SNP rs405509* protein energy % intake	Interaction between SNP rs405509* carbohydrate energy % intake
0.02 (0.02)	-0.04 (0.03)	-0.01 (0.01)

0.39	0.26	0.59
Interaction between rs769450 at *APOE* *dietary factors on HDL-C		
Interaction between SNP rs769450* fat energy % intake	Interaction between SNP rs769450* protein energy % intake	Interaction between SNP rs769450* carbohydrate energy % intake
-0.001 (0.004) 0.88	0.001 (0.01) 0.88	0.003 (0.003) 0.19
Interaction between rs769450 at *APOE* *dietary factors on Total Cholesterol		
Interaction between SNP rs769450* fat energy % intake	Interaction between SNP rs769450* protein energy % intake	Interaction between SNP rs769450* carbohydrate energy % intake
-0.001 (0.01) 0.94	0.01 (0.02) 0.63	0.01 (0.01) 0.51
Interaction between rs439401 at *APOE* *dietary factors on HDL-C		
Interaction between SNP rs439401* fat energy % intake	Interaction between SNP rs439401* protein energy % intake	Interaction between SNP rs439401* carbohydrate energy % intake
0.01 (0.004) 0.11	0.01 (0.01) 0.39	-0.001 (0.003) 0.64
Interaction between rs439401 at *APOE* *dietary factors on Total Cholesterol		
Interaction between SNP rs439401* fat energy % intake	Interaction between SNP rs439401* protein energy % intake	Interaction between SNP rs439401* carbohydrate energy % intake
0.003 (0.01) 0.79	-0.02 (0.02) 0.37	-0.001 (0.01) 0.89
Interaction between rs445925 at *APOE* *dietary factors on HDL-C		
Interaction between SNP rs445925* fat energy % intake	Interaction between SNP rs445925* protein energy % intake	Interaction between SNP rs445925* carbohydrate energy % intake
-0.003 (0.01) 0.53	0.01 (0.01) 0.52	0.0003 (0.003) 0.93
Interaction between rs445925 at *APOE* *dietary factors on Total Cholesterol		
Interaction between SNP rs445925* fat energy % intake	Interaction between SNP rs445925* protein energy % intake	Interaction between SNP rs445925* carbohydrate energy % intake
-0.03 (0.01) 0.05	0.01 (0.03) 0.66	0.01 (0.01) 0.36
Interaction between rs405697 at *APOE* *dietary factors on HDL-C		

Interaction between SNP rs405697* fat energy % intake	Interaction between SNP rs405697* protein energy % intake	Interaction between SNP rs405697* carbohydrate energy % intake
0.01 (0.004) 0.06	-0.002 (0.01) 0.80	-0.004 (0.002) 0.16
Interaction between rs405697 at *APOE* *dietary factors on Total Cholesterol		
Interaction between SNP rs405697* fat energy % intake	Interaction between SNP rs405697* protein energy % intake	Interaction between SNP rs405697* carbohydrate energy % intake
0.01 (0.01) 0.22	-0.03 (0.02) 0.19	-0.003 (0.01) 0.72
Interaction between rs1160985 at *APOE* *dietary factors on HDL-C		
Interaction between SNP rs1160985* fat energy % intake	Interaction between SNP rs1160985* protein energy % intake	Interaction between SNP rs1160985* carbohydrate energy % intake
-0.01 (0.01) 0.08	-0.002 (0.01) 0.97	0.01 (0.004) 0.03
Interaction between rs1160985 at *APOE* *dietary factors on Total Cholesterol		
Interaction between SNP rs1160985* fat energy % intake	Interaction between SNP rs1160985* protein energy % intake	Interaction between SNP rs1160985* carbohydrate energy % intake
-0.01 (0.01) 0.58	0.05 (0.03) 0.28	-0.001 (0.01) 0.19
Interaction between rs1064725 at *APOE* *dietary factors on HDL-C		
Interaction between SNP rs1064725* fat energy % intake	Interaction between SNP rs1064725* protein energy % intake	Interaction between SNP rs1064725* carbohydrate energy % intake
-0.001 (0.01) 0.90	0.004 (0.02) 0.77	-0.002 (0.004) 0.73
Interaction between rs1064725 at *APOE* *dietary factors on Total Cholesterol		
Interaction between SNP rs1064725* fat energy % intake	Interaction between SNP rs1064725* protein energy % intake	Interaction between SNP rs1064725* carbohydrate energy % intake
0.03 (0.03) 0.28	0.02 (0.04) 0.62	-0.01 (0.01) 0.48
Interaction between *APOE* (E2, E3, and E4)*dietary factors on HDL-C		
Interaction between SNP *APOE* (E2, E3, and E4)* fat energy % intake	Interaction between SNP *APOE* (E2, E3, and E4)* protein energy % intake	Interaction between SNP *APOE* (E2, E3, and E4)* carbohydrate energy % intake

-0.01 (0.01) 0.39	0.001 (0.01) 0.99	0.002 (0.003) 0.17
Interaction between *APOE* (E2, E3, and E4)*dietary factors on Total Cholesterol		
Interaction between SNP *APOE* (E2, E3, and E4)* fat energy % intake	Interaction between SNP *APOE* (E2, E3, and E4)* protein energy % intake	Interaction between SNP *APOE* (E2, E3, and E4)* carbohydrate energy % intake
-0.03 (0.02) 0.18	-0.02 (0.04) 0.32	0.01 (0.01) 0.51

Values represented β regression coefficients (± S.E), and $P_{interaction}$. P values were obtained by using a general linear model adjusted for age, sex, body mass index, country and total energy intake, wherever appropriate.

Bonferroni corrected P value <0.001 was considered statistically significant.

HDL-C; high density lipoprotein cholesterol.

Table 4.5: Interaction between *APOE* and *LPL* SNPs and dietary factors on total cholesterol in the Caerphilly prospective study

Interaction between rs320 at *LPL* *dietary factors on Total Cholesterol		
Interaction between SNP rs320* fat energy % intake	Interaction between SNP rs320* protein energy % intake	Interaction between SNP rs320* carbohydrate energy % intake
0.01 (0.01) 0.48	-0.01 (0.03) 0.57	-0.004 (0.01) 0.64
Interaction between rs328 at *LPL* *dietary factors on Total Cholesterol		
Interaction between SNP rs328* fat energy % intake	Interaction between SNP rs328* protein energy % intake	Interaction between SNP rs328* carbohydrate energy % intake
-0.01 (0.01) 0.58	-0.04 (0.03) 0.17	0.01 (0.01) 0.29
Interaction between rs405509 at *APOE* *dietary factors on Total Cholesterol		
Interaction between SNP rs405509* fat energy % intake	Interaction between SNP rs405509* protein energy % intake	Interaction between SNP rs405509* carbohydrate energy % intake
0.03 (0.01) 0.11	-0.04 (0.04) 0.52	-0.02 (0.01) 0.31
Interaction between rs769450 at *APOE* *dietary factors on Total Cholesterol		
Interaction between SNP rs769450* fat energy % intake	Interaction between SNP rs769450* protein energy % intake	Interaction between SNP rs769450* carbohydrate energy % intake
-0.01 (0.01) 0.10	0.05 (0.02) 0.04	0.01 (0.01) 0.42
Interaction between rs439401 at *APOE* *dietary factors on Total Cholesterol		
Interaction between SNP rs439401* fat energy % intake	Interaction between SNP rs439401* protein energy % intake	Interaction between SNP rs439401* carbohydrate energy % intake
-0.003 (0.01) 0.77	-0.01 (0.03) 0.68	0.004 (0.01) 0.65
Interaction between rs445925 at *APOE* *dietary factors on Total Cholesterol		
Interaction between SNP rs445925* fat energy % intake	Interaction between SNP rs445925* protein energy % intake	Interaction between SNP rs445925* carbohydrate energy % intake
-0.0003 (0.01) 0.97	-0.02 (0.03) 0.55	0.002 (0.01) 0.87

Interaction between rs405697 at *APOE* *dietary factors on Total Cholesterol		
Interaction between SNP rs405697* fat energy % intake	Interaction between SNP rs405697* protein energy % intake	Interaction between SNP rs405697* carbohydrate energy % intake
0.01 (0.01) 0.51	-0.03 (0.03) 0.24	-0.002 (0.01) 0.84
Interaction between rs1160985 at *APOE* *dietary factors on Total Cholesterol		
Interaction between SNP rs1160985* fat energy % intake	Interaction between SNP rs1160985* protein energy % intake	Interaction between SNP rs1160985* carbohydrate energy % intake
-0.01 (0.01) 0.13	-0.004 (0.03) 0.19	0.01 (0.01) 0.43
Interaction between rs1064725 at *APOE* *dietary factors on Total Cholesterol		
Interaction between SNP rs1064725* fat energy % intake	Interaction between SNP rs1064725* protein energy % intake	Interaction between SNP rs1064725* carbohydrate energy % intake
-0.01 (0.03) 0.66	0.05 (0.11) 0.62	0.01 (0.03) 0.74
Interaction between *APOE* (E2,E3, and E4)*dietary factors on Total Cholesterol		
Interaction between SNP *APOE* (E2, E3, and E4)* fat energy % intake	Interaction between SNP *APOE* (E2, E3, and E4)* protein energy % intake	Interaction between SNP *APOE* (E2, E3, and E4)* carbohydrate energy % intake
-0.02 (0.02) 0.038	0.02 (0.04) 0.83	0.01 (0.01) 0.08

Values represented β regression coefficients (± S.E), and $P_{interaction}$.

P values were obtained by using a general linear model adjusted for age, sex, body mass index, country and total energy intake, wherever appropriate.

Bonferroni corrected P value <0.001 was considered statistically significant.

Table 4.6: Genotype distribution of SNPs at *LPL* and *APOE* genes and Hardy Weinberg Equilibrium P values

SNP	Common homozygous N (%)	Heterozygous N (%)	Rare homozygous N (%)	Chi square	HWE P value
PRECISE					
rs320	354 (0.53)	271 (0.40)	39 (0.05)	1.8	0.17
rs328	522 (0.80)	127 (0.19)	3 (0.005)	2.6	0.11
rs405509	183 (27.6%)	330 (49.8%)	149 (22.5%)	0.0001	0.99
rs769450	228 (34.5%)	339 (51.4%)	92 (13.8%)	3.66	0.06
rs439401	291 (44.2%)	290 (44.1%)	76 (11.5%)	0.08	0.77
rs445925	506 (77.3%)	142 (21.7%)	6 (0.9%)	1.33	0.24
rs405697	365 (54.9%)	257 (38.7%)	42 (6.3%)	0.13	0.71
rs1160985	200 (30.2%)	344 (52%)	117 (17.7%)	2.18	0.13
rs1064725	606 (91.4%)	56 (8.4%)	1 (0.1%)	0.06	0.81
Caerphilly Prospective study					
rs320	721 (0.53)	536 (0.39)	86 (0.06)	1.05	0.31
rs328	1068 (0.79)	266 (0.19)	9 (0.006)	3.01	0.08
rs405509	381 (0.28)	675 (0.50)	287 (0.21)	0.13	0.71
rs769450	452(0.33)	672(0.50)	219(0.16)	1.35	0.24
rs439401	560 (0.41)	615 (0.45)	168 (0.12)	0.0018	0.96
rs445925	1056 (0.78)	271 (0.20)	16 (0.01)	0.08	0.77
rs405697	728 (0.54)	513 (0.38)	102 (0.07)	0.77	0.38
rs1160985	394 (0.29)	688 (0.51)	261 (0.19)	1.61	0.20
rs1064725	1076 (0.96)	35 (0.03)	0	0.28	0.59

HWE; Hardy Weinberg Equilibrium

4.5 Discussion

Our findings demonstrated significant associations between the *APOE* haplotype (E2, E3, and E4) and *APOE* SNP rs445925 with total plasma cholesterol and LDL-C (only CaPS) concentration, which were further replicated in an independent UK Caucasian cohort. The levels of total cholesterol were significantly lower in carriers of the *APOE* E2 allele and the 'A' allele of the SNP rs445925 than carriers of E3, E4 and 'GG' genotype of the *APOE* SNP rs445925, respectively. Given that our findings confirm that genetic polymorphisms of *APOE* influence the inter-individual variation in total plasma cholesterol, a marker of dyslipidemia, changes in dietary consumption to reduce disease susceptibility could be implemented for individuals at genetic risk.

The effects of *APOE* polymorphisms on lipid concentrations have previously been investigated in different ethnic groups [11, 53, 54] and studies have shown that the *APOE* gene variants contributed to 7% variability in total cholesterol [55]. The results of the current study were in line with previously reported findings that *APOE* haplotypes (E2, E3, and E4) are associated with serum total cholesterol and LDL-C, with E4 carriers associated with increased concentrations compared with E3/E3 wildtype and particularly E2 carriers [16, 53, 56]. One of the primary roles of APOE is binding the low density lipoprotein receptor (LDLR) and the LDLR-related protein, to facilitate cellular uptake of lipoprotein particles [57]. The three alleles, E2, E3, and E4, differ in their amino-acid sequences, resulting in functional differences in receptors-binding affinity. Amino-acid sequences of the E2 allele have lower binding affinity than those of the E3 and E4 alleles, causing decreased hepatic VLDL and chylomicron remnants clearance, thus reducing the uptake of postprandial lipoprotein particles [57]. E2 carriers also have an impaired conversion of the VLDL particles to LDL-C compare to E4 carriers [58], who have a

higher rate of VLDL catabolism [59], which explains in part the lower total cholesterol and LDL-C in E2 allele carriers.

Furthermore, our study highlights an association between *APOE* SNP rs445925, which is one of the selected tagSNPs within the *APOE* gene, and total cholesterol. The SNP rs445925 has not been extensively studied, however, a genome-wide association study showed a significant association between SNP rs445925 and LDL-C levels in 3,644 black and white individuals from the US and Europe [60]. In addition, previous genome-wide linkage and association studies have shown linkage disequilibrium (LD) between *APOE* SNPs rs7412 and rs445925 [61] and between 'A' allele carriers at SNP rs445925 and E2 haplotype [62], respectively, which could explain in part a similar function in cholesterol synthesis.

Besides genetic associations, our study also identified an interaction of *APOE* haplotypes (E2, E3, and E4) with intake from fat (%) on total cholesterol in the CaPS, where, among those who consumed a low-fat diet (%), individuals carrying the E2 allele had significantly lower total cholesterol concentrations than to E4 allele carriers. However, this interaction was not statistically significant after correction for multiple testing. A previous study has examined the response of *APOE* genotype to fat intake in 45 individuals using a prospective design, where after consumption of a lower-fat-cholesterol diet (34% fat, 265 mg/day) according to modified National Cholesterol Education program there was a significant reduction in total cholesterol by 14%, 9%, and 4% in E4/E4, E3/E4, and E3/E3 genotypes, respectively [63]. Another study showed that the response to a diet high in cholesterol increases total cholesterol in E3 and E4 compared to E2 allele carries in a study comprising 29 healthy men [64]. By contrast, a

cross sectional study in European Caucasians (n=996) reported that E2 allele carriers had lower total cholesterol levels, but there were no reported between interactions between saturated fatty acids and total cholesterol [65]. Given that the previous studies have given inconsistent results and have used various types of fatty acids, replication of our gene-diet interaction finding in a large well-designed randomized controlled trial is highly warranted.

Previous studies have shown that the minor allele of *LPL* SNP rs328 enhance lipolytic activity [12]. Increased activity of LPL results in enhance clearance of TAG from the circulation, and associated with higher HDL-C concentrations [66]. The *LPL* SNP rs320 (HindIII) is in LD with rs328 (S447X) and they have been shown to have similar effects on HDL-C, where minor allele was reported to increase HDL-C [24, 67]. In our study, in accordance with findings from other studies, there were associations between *LPL* SNPs, rs320 and rs328, and HDL-C concentrations, where common homozygotes of both SNPs had lower HDL-C [22-24, 26]. However, in our study, these associations were no longer statistically significant after Bonferroni correction. Furthermore, there were no significant *LPL* SNP-diet interactions with HDL-C or total cholesterol concentrations in either cohort. To date, there has only been one study that has shown an interaction between *LPL* rs328 and total fat intake on HDL-C in 8,764 individuals from the US population, where high fat intake associated with increase HDL-C in CC homozygotes and CG heterozygotes carriers [28]. One of the main reasons we did not identify a significant interaction may be our small sample size; however, we cannot rule out an effect of differences in dietary fat sources between European and the US population.

The present study has some limitations. Importantly, some lipid-related outcomes, such as LDL-C and TAG concentrations, were not measured in the PRECISE study. The PRECISE study was also conducted in two populations, a UK cohort and a Danish cohort, which used different food frequency questionnaires and this might have introduced measurement bias, even though the current results were adjusted for country in the regression analysis to avoid confounding. Another possible limitation is the use of a cross-sectional design (in both studies) to investigate genetic effects at a single point in time, whereas a longitudinal analysis design would have captured the genetic effects on lipid outcomes over a specific time period. The effect-size of the minor allele of some of the studied SNPs was relatively small, and hence a large sample size is required to detect reliably detect any interaction between SNPs and dietary factors. Despite the fact that this study was not adequately powered to detect such an interaction, it was sufficiently powered to detect the main effects (association). Significant gene-diet interactions were identified, however these did not reach the Bonferroni-corrected P value (P=0.001) and hence need to be confirmed in larger cohorts. This study is strengthened by the fact that it is the first study to investigate the role of tagSNPs at the *APOE* gene in relation to dietary factors and lipid outcomes. The fact that genetic associations from the PRECISE study were replicated in another Caucasian cohort (CaPS) confirms the validity of our findings. Additionally, CaPS was based on a cohort with a very high response rate, and is therefore closely representative of the general population.

4.6 Conclusion

Our study, carried out in two Caucasian populations, confirmed that genetic variations at the *APOE* gene locus influence plasma lipid concentrations. Thus, our

results suggest that *APOE* gene variants affect risk of dyslipidemia in individuals who carry the E4 risk allele and GG genotype at SNP rs445925. Future studies with a larger sample size examining tagSNPs at *APOE*, particularly prospectively genotyped dietary intervention studies are required to confirm the gene-diet interactions identified in our study.

Author contributions

Israa M Shatwan performed the statistical analysis and drafted the manuscript; KSV conceived and designed the nutrigenetics study; KW and MR designed and conducted the PRECISE study; PE designed and led the conduct of the Caerphilly Prospective study and YBS was involved in the design and conduct of phase V as well as obtaining funding for genetic analysis. JAL, BE, KW, MR, YBS, PE, IG, and KSV critically reviewed the manuscript. All authors contributed to and approved the final version of the manuscript.

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4.7 References

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Chapter 5

For this study, I was involved in getting the dataset from the collaborators and cleaning the dataset. I ran the entire statistical analysis using the SPSS software and wrote the first draft of the manuscript. I revised the manuscript based on the comments from all the co-authors before the manuscript was submitted to the Lipids in health and disease journal. I was also involved in drafting the responses to the comments from reviewers.

For the genetic analysis, I had to collect and prepare DNA samples from the freezer to be transferred to LGC Genomics Company to run genotyping of the selected candidate SNPs. Before running the statistical analysis, I prepared the analysis plan, which summarized the steps for the statistical methods to be used for meeting the objectives of my study.

Chapter 5 Apolipoprotein E gene polymorphism modifies fasting total cholesterol concentrations in response to replacement of dietary saturated with monounsaturated fatty acids in adults at moderate cardiovascular disease risk

Published

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5.1 Abstract

Background: Consumption of ≤10% total energy from fat as saturated fatty acids (SFA) is recommended for cardiovascular disease risk reduction in the UK; however there is no clear guidance on the optimum replacement nutrient. Lipid-associated single-nucleotide polymorphisms (SNPs) have been shown to modify the lipid responses to dietary fat interventions. Hence, we performed a retrospective analysis in 120 participants from the Dietary Intervention and VAScular function (DIVAS) study to investigate whether lipoprotein lipase (*LPL*) and apolipoprotein E (*APOE*) SNPs modify the fasting lipid response to replacement of SFA with monounsaturated (MUFA) or n-6 polyunsaturated (PUFA) fatty acids.

Methods: The DIVAS study was a randomized, single-blinded, parallel dietary intervention study performed in adults with a moderate cardiovascular risk who received one of three isoenergetic diets rich in SFA, MUFA or n-6 PUFA for 16 weeks.

Results: After the 16-week intervention, a significant diet-gene interaction was observed for changes in fasting total cholesterol (P=0.001). For the *APOE* SNP rs1064725, only TT homozygotes showed a significant reduction in total cholesterol after the MUFA diet (n=33; −0.71±1.88 mmol/l) compared to the SFA (n=38; 0.34±0.55 mmol/l) or n-6 PUFA diets (n=37; −0.08±0.73 mmol/l) (P=0.004). None of the interactions were statistically significant for the other SNPs.

Conclusions: In summary, our findings have demonstrated a greater sensitivity of the *APOE* SNP rs1064725 to dietary fat composition, with a total cholesterol lowering effect observed following substitution of SFA with MUFA but not n-6 PUFA. Further large intervention studies incorporating prospective genotyping are required to confirm or refute our findings.

5.2 Background

A high consumption of saturated fatty acids (SFA) has been linked to increased circulating concentrations of low-density lipoprotein cholesterol (LDL-C) [1], and is consequently associated with an increased cardiovascular disease (CVD) risk [2]. Therefore, dietary guidelines have focused on reducing intakes of SFA by ≤10% of total energy (TE) for CVD risk reduction [3]. It is important to consider the nutrients that replace SFA and previous findings have suggested substitution of SFA with unsaturated fatty acids may provide a greater reduction in CVD risk than refined carbohydrates [4, 5]. In particular, replacement with cis-monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (PUFA) has been shown to significantly lower fasting total and LDL-C [6, 7]. However, the inter-individual variability in fasting plasma lipid responses to dietary fat intake is high; evidence supports that this is influenced by lipid-

associated single-nucleotide polymorphisms (SNPs) such as apolipoprotein E (*APOE*) and lipoprotein lipase (*LPL*) genotypes [8-10].

Several genes are involved in the regulation of lipid transport and metabolism [11]. Among these, the most commonly studied genes with central roles in lipid metabolism are *LPL* and *APOE* [12-14]. The *LPL* SNPs, rs320 (*HindIII*) and rs328 (*S447X*), have been proposed as important genetic determinants of the inter-individual variability in fasting and postprandial triacylglycerol (TAG) concentrations and high-density lipoprotein cholesterol (HDL-C) [15-17]. Increased activity of the LPL enzyme in minor allele carriers of *LPL* SNP rs328 has been shown to be associated with lower plasma TAG and higher HDL-C levels [18]. To date, there has only been one study reporting an interaction between *LPL* rs328 and n-6 PUFA intake on fasting TAG concentrations [19]. The effect of genetic variations of *APOE* on lipid concentrations (i.e. LDL-C) [20-23] and the effect of the *APOE* polymorphisms on the circulating lipid response to dietary fat (i.e. SFA and MUFA) have been previously demonstrated; however, the findings have been inconsistent [24-26]. In addition, investigations into other SNPs of the *APOE* gene are limited.

In the Dietary Intervention and VAScular function (DIVAS) study, the isoenergetic replacement of 9.5 - 9.6 % TE from SFA with *cis* MUFA or n–6 PUFA for 16 weeks in 195 adults at moderate CVD risk resulted in significant reductions of 8.4% and 9.2%, respectively, in total cholesterol, and 11.3% and 13.6%, in LDL-C, in the fasted state [6]. To investigate whether genetic polymorphisms contributed to the observed reductions in total and LDL-C, a retrospective post hoc analysis of the DIVAS study was performed. We examined whether the two *LPL* and seven tagging SNPs

(TagSNPs) in the *APOE* gene modified the response of the fasting lipid profile to substitution of SFA with MUFA or n-6 PUFA in this study population at moderate CVD risk.

5.3. Participants and methods

5.3.1 Study participants

A detailed description of the DIVAS study design and methods has been reported elsewhere [6, 27]. Briefly, participants were recruited from Reading, UK and the surrounding area in three cohorts between November 2009 and July 2012. Participants were aged between 21 and 60 years and were all non-smoking men and women with a moderate risk of CVD. A scoring tool [27] was used to determine CVD risk based on the presence of single or multiple risk factors, including elevated fasting total cholesterol or fasting glucose, raised blood pressure, low HDL-C, being overweight or obese, and/or having a family history of premature myocardial infarction or type 2 diabetes. Eligible participants had a risk score of ≥ 2 combined points, reflecting a moderate CVD risk ($\geq$50% above the population mean). Other criteria for exclusion were the presence of abnormal fasting blood biochemistry, taking dietary supplements or the use of medications that affect lipid metabolism or hypertension, and having inflammatory disorders. The West Berkshire Local Research ethics committee (09/ H0505/56) and the University of Reading Research Ethics Committee (09/40) gave a favourable ethical opinion for conduct. The trial was registered at www.clinicaltrials.gov as NCT01478958. All participants provided written informed consent before participating. In our retrospective analysis, 120 of the 195 participants who completed the DIVAS study consented to genetic analysis, and were included in the present study.

5.3.2 Study design and diets

The DIVAS study was a randomized, single-blinded, parallel design. The participants completed 16 weeks of dietary intervention, receiving one of three isoenergetic diets based on a minimization program that matched for age, sex, body mass index (BMI), and total CVD risk score. The three intervention diets (%TE derived from SFA:MUFA:n-6 PUFA) were either rich in SFAs (17:11:4), MUFAs (9:19:4), or n-6 PUFAs (9:13:10). Given that dietary guidelines recommend limiting n-6 PUFA intake to ≤10% TE [28], SFA were replaced with 6% TE n-6 PUFA and 2% TE MUFA in the n-6-PUFA-rich diet. The total fat content of all three intervention diets was 36% TE, and intakes of protein, carbohydrates, and n-3 PUFA were unchanged. A greater SFA exchange than the target 8% TE was achieved: SFA vs MUFA was 9.5% TE and SFA vs n-6 PUFA was 9.6% TE [27].

Further details of the dietary intervention procedure and measures of compliance have been published previously [27]. In summary, these interventions were based on a flexible food-exchange model to achieve the target fatty acid intakes in free-living individuals for 16 weeks. Participants, who were randomly assigned to one of three intervention diets, replaced routinely consumed sources of exchangeable fats with study foods. The study foods included spreads, oils, dairy products, and commercially available snacks of a specific fatty acid composition. Specially formulated spreads (80% total fat) and oils (Unilever Research and Development) were used for the MUFA-rich diet (refined olive oil and olive oil/rapeseed oil blended spread) and n–6 PUFA-rich diet (safflower oil and spread). Butter (Wyke Farm) was used as both a spread and oil

replacement in the SFA-rich diet.

5.3.3 Anthropometric measurements and biochemical parameters

Clinical visits took place at the Hugh Sinclair Unit of Human Nutrition, University of Reading, during weeks 0 (baseline; V1) and 16 (after intervention; V2) as described elsewhere [6]. Alcohol and aerobic exercise were avoided 24 h before visits. Participants consumed a provided low-fat meal the evening before visits and fasted for 12 h, only drinking low-nitrate water during this time. Height and weight was recorded at the study visits at weeks 0 and 16 in order to calculate BMI. Height was recorded to the nearest 0.5 cm using a wall-mounted stadiometer and weight was measured using a digital scale (Tanita Europe) using standard settings (normal body type and 1 kg for clothing).

At weeks 0 and 16, fasting blood samples collected into a serum separator vacutainer and a K3EDTA-containing vacutainer (week 0 only) were used for the measurement of the fasting lipid profile and isolation of the buffy coat, respectively. The K3EDTA-containing vacutainer was kept on ice for 30 min before the blood tubes were centrifuged at 1700 *g* for 15 min at 20 °C (for serum) and 4 °C (for plasma). The buffy coat was stored at -20°C and serum samples stored at -80°C prior to analysis of total cholesterol, TAG, and HDL-C, and glucose (baseline only) concentrations using an autoanalyzer (reagents and analyzer: Werfen UK Ltd). Fasting LDL-C was estimated using the Friedewald formula [29]. With the use of A/A grade automated oscillometric ambulatory blood pressure (ABP) monitors (A&D Instruments Ltd.), baseline 24 h ABP was measured every 30 min from 07:00 to 21:59 and every 60 min from 22:00 to 06:59, approximately 48 h before the clinical visits.

5.3.4 SNP selection and genetic analysis

The *APOE* gene is located on chromosome 19q13.32 and comprises of four exons, which are transcribed into the 1,180 nucleotides long *APOE* mRNA. The seven tag SNPs for the *APOE* gene were chosen based on International HapMap Phase II collected in individuals of Northern and Western European ancestry (CEU) (HapMap Data release 27 Phase 2+3, Feb 09, NCBI B36 assembly, dbSNP b126). The Haploview software V3.3 (http:// www.broadinstitute.org/haploview/haploview-downloads) was used to assess the linkage disequilibrium structure between SNPs. Tagger software was used to select tag SNPs with the 'pairwise tagging only' option. Two criteria were used to filter the SNPs included in the analysis - minor allele frequency ≥5% and Hardy–Weinberg equilibrium P-value >0.01. Seven tagSNPs (rs405509 (G>T) [30, 31], rs1160985 (C>T) [32], rs769450 (G>A) [33], rs439401 (C>T) [34], rs445925 (G>A) [35], rs405697 (G>A) [36], and rs1064725 (T>G)) representing the entire common genetic variations across the *APOE* gene were selected for the study. In addition, the two commonly studied *LPL* SNPs, rs320 and rs328, were chosen. In total, nine SNPs were examined in the present study.

DNA was extracted from the buffy coat using a QIAamp DNA blood kit (QIAGEN) and stored at -20°C. The genotyping of the *LPL* and *APOE* SNPs was outsourced to LGC Genomics (http://www.lgcgroup.com/services/genotyping), which employs the competitive allele-specific PCR-KASP® assay.

5.3.5 Statistical analysis

The data are presented as mean ± standard deviation (SD) in the tables and text,

and as standard error in the figure. The normal distribution was tested for variables, and none of the variables skewed the distribution. The minor allele frequency was calculated by counting. The dominant models were a better fit for SNPs rs320, rs328, rs769450, rs439401, rs445925, rs405697, and rs1064725; thus, homozygosity for the common allele was compared with carriers of the minor allele (heterozygous and homozygous for the minor allele) in the analysis. The additive model was applied for SNPs rs405509 and rs1160985 (major allele homozygotes vs. heterozygotes vs. minor allele homozygotes). The genotype distributions of the nine SNPs at the *LPL* and *APOE* genes were in Hardy-Weinberg equilibrium (P>0.05) (**Table 5.2**). Independent t-tests were used to compare means between men and women at baseline. The baseline and over 16 weeks' associations of the selected SNPs with continuous phenotypes were evaluated by the general linear model (GLM). Moreover, potential interactions between genotype and dietary intervention on 16-week changes of lipids were analyzed by using GLM, where an interaction term was included in the model. Potential confounders associated with the outcomes were adjusted in all GLM analyses (i.e. age, sex, BMI, and ethnicity). When a significant diet x genotype interaction was found, data were split by genotype group and analyzed further by using GLM. A Bonferroni correction was applied and the significant P value was 0.0013 (0.05/9 SNPs*4 lipid outcomes). For all analyses, the statistical package SPSS version 22.0 (SPSS, Chicago, IL, USA) was used.

5.4 Results

In this retrospective analysis, 120 participants (mean age, 47± 9 years; BMI, 26.4 ± 4.0 kg/m^2) were included. **Table 5.1** illustrates the main characteristics of the study participants stratified according to sex at baseline. Women had significantly lower levels

of fasting TAG (P<0.0001), LDL-C (P=0.01), glucose (P=0.02), blood pressure (P≤0.03), and higher levels of fasting HDL-C (P<0.0001) compared to men.

The genotype distributions of both *LPL* and *APOE* polymorphisms are shown in **Table 5.2**. The participants' characteristics at the beginning of the dietary interventions (week 0) are presented in **Table 5.3** according to *LPL* and *APOE* genotypes. None of the variables (including fasting TAG, total cholesterol, LDL-C, and HDL-C) were associated with the *LPL* and *APOE* SNPs at baseline. After 16 weeks of intervention, there was also no significant association of the *LPL* and *APOE* SNPs with changes in the lipid outcomes after Bonferroni correction (**Tables 5.4-8**).

Table 5.1: Baseline characteristics of study participants in the whole group and stratified by sex

Characteristics	Whole group (N=120)	Men (N=54)	Women (N=66)	P value
Age	47±9	48± 9	46± 9	0.37
BMI	26.3± 3.9	26.7± 3.6	26.2± 4.3	0.58
Systolic blood pressure	122± 10	126± 9	120± 9	0.01
Diastolic blood pressure	75± 7	77± 7	74± 7	0.03
Total cholesterol	5.58± 1.11	5.75± 1.15	5.45± 1.06	0.16
TAG	1.28± 0.60	1.62± 0.61	1.02± 0.44	<0.0001
HDL-C	1.54± 0.35	1.36± 0.32	1.68± 0.32	<0.0001
LDL-C	3.79± 0.99	4.06± 1.01	3.57± 0.93	0.01
Glucose	5.11± 0.42	5.23± 0.44	5.03± 0.39	0.02

Data shown are represented as means ± SD, wherever appropriate. P values for the differences in the means between men and women. P values were calculated by using independent t-test. BMI; body mass index, TAG; triacylglycerol, HDL-C; high-density lipoprotein cholesterol, LDL-C; low-density lipoprotein cholesterol

Table 5.2: Genotype and minor allele frequencies of the SNPs at *LPL* and *APOE* genes in the DIVAS cohort of adults with moderate CVD risk

SNP	MAF	Common homozygous N (%)	Heterozygous N (%)	Rare homozygous N (%)
LPL				
rs320 (T>G)	0.30	56 (0.47)	54 (0.45)	9 (0.07)
rs328 (C>G)	0.13	89 (0.74)	30 (0.25)	1 (0.008)
APOE				
rs405509 (G>T)	0.47	31 (0.25)	64 (0.53)	25 (0.20)
rs769450 (G>A)	0.37	44 (0.36)	61 (0.50)	15 (0.12)
rs439401 (C>T)	0.35	50 (0.42)	52 (0.44)	16 (0.13)
rs445925 (G>A)	0.11	91 (0.77)	25 (0.21)	1 (0.008)
rs405697 (G>A)	0.25	70 (0.58)	38 (0.31)	11 (0.09)
rs1160985 (C>T)	0.45	33 (0.27)	64 (0.53)	23 (0.19)
rs1064725 (T>G)	0.05	108 (0.90)	12 (0.10)	-

MAF; minor allele frequency, LPL; lipoprotein lipase, APOE; apolipoprotein E.

At 16 weeks, after adjustment for age, sex, ethnicity and baseline BMI, a significant interaction between the *APOE* SNP rs1064725 and dietary intervention (SFA vs. MUFA vs. n-6 PUFA) on changes in fasting total cholesterol ($P_{interaction}$=0.001) was observed (**Figure 5.1)**. The 'TT' homozygotes (n=108) of SNP rs1064725 had significantly lower total cholesterol concentrations after the MUFA (n=33; −0.71±1.88

mmol/l) compared with the SFA (n=38; 0.34±0.55 mmol/l; P=0.003) and n-6 PUFA-rich diets (n=37; −0.08±0.73 mmol/l; P=0.15) ($P_{association}$=0.004) (**Figure 5.1**).

Figure 5.1 Mean (±SE) of changes in total cholesterol concentrations following three intervention diets [rich in either saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and n-6 polyunsaturated fatty acids (PUFA)] according to the *APOE* SNP rs1064725 genotype ($P_{interaction}$=0.001). A general linear model analysis was performed with adjustments for age, sex, body mass index, and ethnicity. Individuals carrying the 'TT' genotype had lower total cholesterol levels after consuming the MUFA diet compared to the SFA or n-6 PUFA diets ($P_{association}$=0.004).

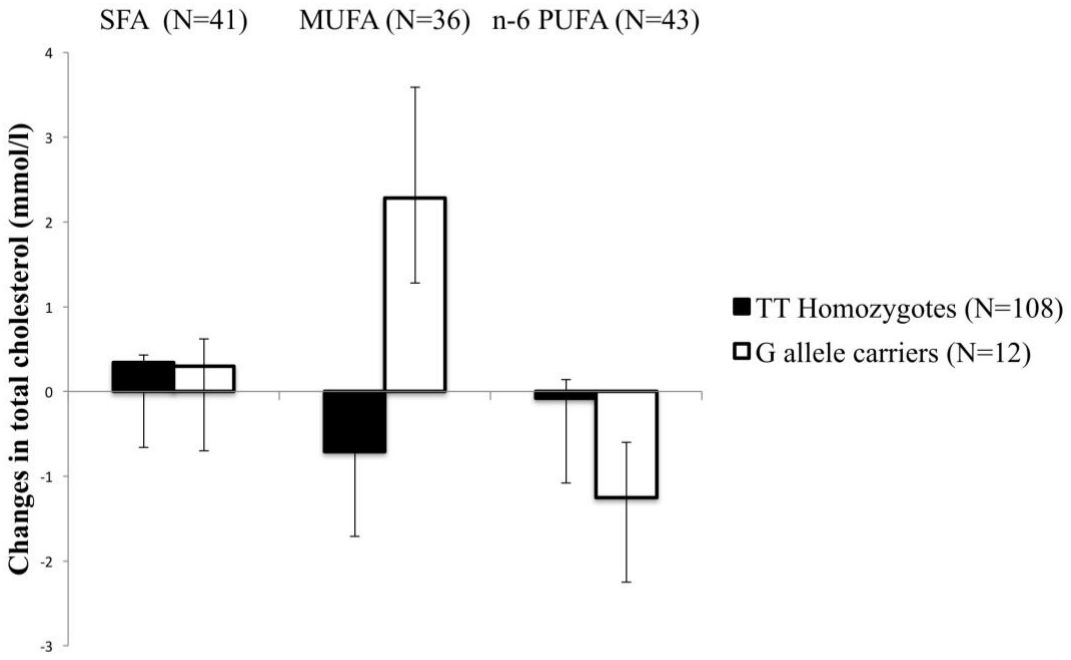

Table 5.3: Baseline characteristics of the DIVAS study participants according to the *LPL* and *APOE* genotypes

SNP	Age	Sex (M/F)	TAG (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)	Total cholesterol (mmol/l)
LPL						
rs320						
TT	46± 9	27/29	1.28± 0.63	1.54± 0.41	3.73± 0.96	5.52± 1.15
T/G	48± 9	27/36	1.27± 0.57	1.53± 0.31	3.81± 1.01	5.59± 1.07
P value			0.83	0.41	0.71	0.53
rs328						
CC	46± 9	42/47	1.29± 0.63	1.52± 0.37	3.72± 1.01	5.49± 1.17
C/G	48± 10	12/19	1.23± 0.47	1.58± 0.27	3.96± 0.88	5.79± 0.83
P value			0.51	0.93	0.42	0.49
APOE						
rs405509						
GG	47± 10	17/14	1.41± 0.63	1.42± 0.39	3.71± 1.01	5.41± 1.20
GT	47± 9	21/43	1.18 ± 0.57	1.58± 0.31	3.77± 1.00	5.58± 1.05
TT	45± 11	16/9	1.34± 0.61	1.55± 0.38	3.85± 0.94	5.67± 1.12
P value			0.57	0.24	0.81	0.70
rs769450						
GG	47± 10	24/20	1.30± 0.61	1.57± 0.37	3.88± 0.96	5.71± 1.11
G/A	46± 9	30/46	1.25± 0.59	1.51± 0.34	3.71± 1.00	5.47± 1.10
P value			0.89	0.24	0.99	0.74
rs439401						
CC	48± 9	25/25	1.37± 0.61	1.53± 0.36	3.88± 0.84	5.68± 0.95
T allele	46± 10	29/39	1.22± 0.59	1.53± 0.34	3.69± 1.07	5.47± 1.19
P value			0.43	0.95	0.54	0.54
rs445925						
GG	46± 10	40/51	1.26± 0.58	1.53± 0.35	3.72± 1.00	5.50± 1.13
A allele	49± 8	13/13	1.36± 0.67	1.56± 0.38	3.97± 1.00	5.80± 1.04
P value			0.95	0.77	0.64	0.61

rs405697						
GG	47± 9	35/35	1.32± 0.62	1.52± 0.39	3.77± 0.91	5.56± 1.01
A allele	46± 10	19/30	1.22± 0.57	1.55± 0.31	3.77± 1.08	5.56± 1.22
P value			0.77	0.91	0.95	0.95
rs1160985						
CC	46± 11	17/16	1.24± 0.62	1.55± 0.34	3.73± 0.96	5.53± 1.11
CT	47± 8	24/40	1.27± 0.61	1.57± 0.33	3.87± 0.96	5.69± 1.02
TT	46± 11	13/10	1.35± 0.51	1.38± 0.40	3.57± 1.08	5.23± 1.30
P value			0.98	0.58	0.51	0.49
rs1064725						
TT	47± 9	48/60	1.26± 0.59	1.55± 0.34	3.79± 1.00	5.59± 1.11
G allele	45± 10	6/6	1.40± 0.65	1.42± 0.46	3.60± 0.85	5.29± 1.00
P value			0.39	0.32	0.79	0.65

P values for association between genotypes and lipids levels were obtained by using general linear model adjusted for age, sex, body mass index, and ethnicity. Values are mean ± SD.

TAG; triacylglycerol, HDL-C; high-density lipoprotein cholesterol, LDL-C; low-density lipoprotein cholesterol, LPL; lipoprotein lipase, APOE; apolipoprotein E.

The dominant model was applied for all SNPs (rare homozygotes were grouped with heterozygotes and compared with common homozygotes), except SNPs rs405509 and rs1160985 where additive model was applied (common homozygotes vs. heterozygotes vs. rare homozygotes).

Table 5.4: Changes in lipid levels after dietary intervention over 16 weeks relative to baseline according to the *APOE* rs1064725 genotype

	SFA			MUFA			n-6 PUFA			$P_{interaction}$
	TT (N=38)	G allele (N=3)	$P_{association}$	TT (N=33)	G allele (N=3)	$P_{association}$	TT (N=37)	G allele (N=6)	$P_{association}$	
TAG	0.001± 1.76	0.22± 0.51	0.81	-0.01± 0.35	0.30± 0.32	0.15	-0.66± 2.05	0.04± 0.23	0.12	0.67
HDL-C	0.06± 1.70	-0.003± 0.14	0.87	0.003± 0.17	-0.06± 0.11	0.63	0.21± 2.08	0.20± 0.23	0.85	0.99
LDL-C	0.05± 1.48	2.90± 4.41	0.02	-0.55± 2.47	1.49± 2.70	0.27	-0.22± 2.24	1.99± 3.71	0.03	0.88

P values for association between genotypes and changes of means over 16 weeks with one of three diets were obtained by using general linear model adjusted for age, sex, body mass index, and ethnicity. P values for interaction between genotypes and changes of means over 16 weeks of intervention with one of three diets were obtained by using general linear model adjusted for age, sex, body mass index, and ethnicity. Values are mean ± SD.

TAG; triacylglycerol, HDL-C; high-density lipoprotein cholesterol, LDL-C; low-density lipoprotein cholesterol, SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, PUFA; polyunsaturated fatty acids.

Table 5.5: Changes in lipid levels after dietary intervention over 16 weeks according to *LPL* rs320 genotypes

	SFA			MUFA			n-6 PUFA			$P_{interaction}$
	TT (N=21)	G allele (N=20)	$P_{association}$	TT (N=11)	G allele (N=24)	$P_{association}$	TT (N=24)	G allele (N=19)	$P_{association}$	
Total cholesterol	0.37± 0.60	0.29± 0.45	0.59	0.21±2.66	-0.67± 2.07	0.21	-0.32± 1.67	-0.12± 0.64	0.92	0.34
TAG	-0.04± 2.35	0.07± 0.59	0.84	0.04± 0.50	0.01± 0.29	0.58	-0.07± 0.32	-1.19± 2.78	0.02	0.15
HDL-C	0.09± 0.15	0.01± 2.33	0.94	-0.07± 0.17	0.03± 0.15	0.04	0.02± 0.25	0.43± 2.91	0.78	0.75
LDL-C	0.29± 2.71	0.27± 0.40	0.95	-1.70± 2.85	0.16± 2.22	0.11	0.91± 2.23	-1.00± 2.50	**0.007**	**0.005**

P values for association between genotypes and changes of means over 16 weeks with one of three diets were obtained by using general linear model adjusted for age, sex, body mass index, and ethnicity. P values for interaction between genotypes and changes of means over 16 weeks of intervention with one of three diets were obtained by using general linear model adjusted for age, sex, body mass index, and ethnicity. Values are mean ± SD.

TAG; triacylglycerol, HDL-C; high-density lipoprotein cholesterol, LDL-C; low-density lipoprotein cholesterol, SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, PUFA; polyunsaturated fatty acids.

Table 5.6: Changes in lipid levels after dietary intervention over 16 weeks according to *LPL* rs328 genotype

	SFA			MUFA			n-6 PUFA			$P_{interaction}$
	CC (N=31)	G allele (N=10)	$P_{association}$	CC (N=24)	G allele (N=12)	$P_{association}$	CC (N=34)	G allele (N=9)	$P_{association}$	
Total cholesterol	0.35± 0.59	0.26± 0.25	0.63	-0.25± 2.26	-0.81± 2.28	0.46	-0.29± 1.42	-0.01± 0.65	0.86	0.55
TAG	0.06± 1.92	-0.12± 0.49	0.76	0.06± 0.35	-0.09± 0.35	0.11	-0.56± 1.72	-0.62± 2.77	0.79	0.97
HDL-C	-0.19± 1.31	0.82± 2.32	0.11	-0.04± 0.16	0.07± 0.15	0.16	0.01± 1.79	0.97± 2.44	0.53	0.45
LDL-C	0.29± 2.19	0.23± 0.23	0.59	-0.90± 2.09	0.82± 3.06	0.24	-0.10± 2.82	-0.15± 0.38	0.78	0.25

P values for association between genotypes and changes of means over 16 weeks with one of three diets were obtained by using general linear model adjusted for age, sex, body mass index, and ethnicity. P values for interaction between genotypes and changes of means over 16 weeks of intervention period with one of three diets were obtained by using general linear model adjusted for age, sex, body mass index, and ethnicity. Values are mean ± SD.

TAG; triacylglycerol, HDL-C; high-density lipoprotein cholesterol, LDL-C; low-density lipoprotein cholesterol, SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, PUFA; polyunsaturated fatty acids.

Table 5.7: Changes in lipid levels after dietary intervention over 16 weeks according to *APOE* rs405509 and rs1160985 genotypes

	SFA				MUFA				n-6 PUFA				$P_{interaction}$
***APOE* SNP rs405509**	**GG (N=12)**	**GT (N=20)**	**TT (N=9)**	**P association**	**GG (N=7)**	**GT (N=26)**	**TT (N=3)**	**P association**	**GG (N=12)**	**GT (N=18)**	**TT (N=13)**	**P association**	
Total cholesterol	0.22± 0.65	0.23± 0.45	0.67± 0.41	0.12	-0.23± 0.57	-0.51± 2.59	-0.12± 0.17	0.77	-0.001± 0.80	0.05± 0.76	-0.84± 2.00	0.24	0.55
TAG	-0.03± 0.64	0.02± 2.43	0.07± 0.44	0.94	0.19± 0.51	-0.01± 0.31	-0.03± 0.49	0.45	-0.52± 2.32	-0.50± 1.71	-0.71± 2.01	0.99	0.98
HDL-C	-0.60± 2.12	0.44± 1.64	0.07± 0.14	0.32	-0.002± 0.11	-0.01± 0.18	0.02± 0.03	0.98	0.63± 2.12	-0.31± 1.75	0.55± 2.03	0.48	0.18
LDL-C	0.20± 0.55	0.61± 1.88	-0.29± 2.91	0.60	-1.06± 1.73	0.22± 2.03	-3.88± 4.56	0.01	-1.11± 3.47	0.56± 2.12	0.40± 1.77	0.23	0.10

APOE SNP rs1160985	CC (N=11)	CT (N=19)	TT (N=11)	P association	CC (N=8)	CT (N=22)	TT (N=6)	P association	CC (N=14)	CT (N=23)	TT (N=6)	P association	$P_{interaction}$
Total cholesterol	0.64± 0.38	0.34± 0.54	-0.02± 0.46	0.01	0.05± 0.32	-0.66± 2.82	-0.20± 0.66	0.78	-0.67± 1.94	0.01± 0.81	-0.17± 0.90	0.36	0.40
TAG	0.04± 0.41	-0.001± 2.50	0.03± 0.66	0.95	0.05± 0.29	-0.05± 0.33	0.26± 0.55	0.27	-0.66± 1.93	-0.30± 1.64	-1.51± 3.07	0.52	0.62
HDL-C	0.05± 0.14	0.49± 1.68	-0.70± 2.21	0.23	-0.01± 0.18	0.002± 0.17	-0.01± 0.13	0.94	0.54± 1.94	0.06± 2.18	-0.03± 0.21	0.84	0.57
LDL-C	-0.14± 2.61	0.72± 1.92	-0.02± 0.43	0.65	-1.36± 3.21	0.21± 2.23	-1.24± 1.95	0.38	0.69± 1.81	-0.01± 2.79	-1.32± 2.72	0.25	0.44

P values for association between genotypes and changes of means over 16 weeks with one of three diets were obtained by using general linear model adjusted for age, sex, body mass index, and ethnicity. P values for interaction between genotypes and changes of means over 16 weeks of intervention period with one of three diets were obtained by using general linear model adjusted for age, sex, body mass index, and ethnicity. Values are mean ± SD.

TAG; triacylglycerol, HDL-C; high-density lipoprotein cholesterol, LDL-C; low-density lipoprotein cholesterol, SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, PUFA; polyunsaturated fatty acids.

Table 5.8: Changes in lipid levels after dietary intervention over 16 weeks according to *APOE* rs769450, rs439401, rs445925 and rs405697 genotypes

	SFA			MUFA			n-6 PUFA			$P_{interaction}$
***APOE* SNP rs769450**	**GG (N=12)**	**A allele (N=29)**	**$P_{association}$**	**GG (N=11)**	**A allele (N=25)**	**$P_{association}$**	**GG (N=21)**	**A allele (N=22)**	**$P_{association}$**	
Total cholesterol	0.56± 0.46	0.23± 0.53	0.06	-0.22± 0.74	-0.53± 2.74	0.74	-0.55± 1.66	0.06± 0.81	0.14	0.34
TAG	0.05± 0.39	0.001± 2.04	0.74	0.01± 0.37	0.02± 0.35	0.83	-0.48± 1.60	-0.65± 2.22	0.86	0.92
HDL-C	0.05± 0.13	0.05± 1.98	0.94	-0.06± 0.19	0.03± 0.14	0.26	0.72± 2.21	-0.26± 1.57	0.15	0.21
LDL-C	-0.17± 2.49	0.48±1.59	0.51	-0.77± 3.24	-0.15± 2.10	0.91	0.36± 3.34	-0.22± 1.47	0.39	0.51
***APOE* SNP rs439401**	**CC (N=19)**	**T allele (N=22)**	**$P_{association}$**	**CC (N=17)**	**T allele (N=19)**	**$P_{association}$**	**CC (N=14)**	**T allele (N=27)**	**$P_{association}$**	**$P_{interaction}$**
Total cholesterol	0.13± 0.48	0.50± 0.51	0.03	-0.23± 0.59	-0.60± 3.07	0.77	-0.06± 0.64	-0.26± 1.55	0.77	0.63
TAG	-0.42± 1.79	0.38± 1.55	0.19	-0.03± 0.39	0.06± 0.32	0.23	-0.001± 0.94	-0.87± 2.27	0.38	0.13

HDL-C	0.02± 2.47	0.08± 0.17	0.94	0.02± 0.18	-0.02± 0.15	0.91	0.001± 2.99	0.33± 1.38	0.66	0.92
LDL-C	-0.36± 2.01	0.79± 1.71	0.09	-0.37± 0.68	-0.35± 3.44	0.85	-.043± 2.98	0.33± 2.40	0.34	0.59
***APOE* SNP rs445925**	**GG (N=31)**	**A allele (N=8)**	**P association**	**GG (N=26)**	**A allele (N=9)**	**P association**	**GG (N=34)**	**A allele (N=9)**	**P association**	**P interaction**
Total cholesterol	0.35± 0.48	0.26± 0.76	0.89	-0.39± 2.68	-0.43± 0.79	0.99	-0.22± 1.42	-0.26± 0.70	0.74	0.98
TAG	0.08± 1.96	-0.22± 0.49	0.75	0.07± 0.34	-0.05± 0.34	0.47	-0.69± 2.15	-0.09± 0.16	0.65	0.68
HDL-C	0.31± 1.32	-0.84± 2.49	0.12	0.02± 0.14	-0.05± 0.22	0.65	0.26± 2.17	-0.02± 0.22	0.89	0.33
LDL-C	0.27± 2.21	0.28± 0.64	0.75	-0.53± 2.82	0.14± 1.79	0.66	-0.24± 2.29	1.21± 3.19	0.18	0.61
***APOE* SNP rs405697**	**GG (N=25)**	**A allele (N=16)**	**P association**	**GG (N=24)**	**A allele (N=12)**	**P association**	**GG (N=21)**	**A allele (N=21)**	**P association**	**P interaction**
Total cholesterol	0.26± 0.58	0.45± 0.44	0.39	-0.25± 2.24	-0.81± 2.33	0.60	-0.04± 0.73	-0.39± 1.67	0.28	0.61

TAG	-0.36± 1.53	0.60± 1.80	0.11	0.02± 0.39	-0.002± 0.27	0.91	-0.35± 1.82	-0.46± 1.53	0.87	0.20
HDL-C	0.05± 2.11	0.06± 0.17	0.97	0.03± 0.15	-0.06± 0.17	0.15	0.05± 2.41	0.36± 1.54	0.73	0.87
LDL-C	0.22± 2.46	0.36± 0.36	0.98	-0.34± 1.48	-0.40± 4.09	0.94	-0.21± 3.49	0.29± 1.37	0.52	0.86

P values for association between genotypes and changes of means over 16 weeks with one of three diets were obtained by using general linear model adjusted for age, sex, body mass index, and ethnicity. P values for interaction between genotypes and changes of means over 16 weeks of intervention period with one of three diets were obtained by using general linear model adjusted for age, sex, body mass index, and ethnicity. Values are mean ± SD. TAG; triacylglycerol, HDL-C; high-density lipoprotein cholesterol, LDL-C; low-density lipoprotein cholesterol, SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, PUFA; polyunsaturated fatty acid.

In addition, we also observed an interaction between *LPL* SNP rs320 and the dietary fat intervention (SFA vs. MUFA vs n-6 PUFA) on changes in LDL-C concentrations after 16 weeks ($P_{interaction}$=0.005) (**Table 5.5).** In the n-6 PUFA diet group, the G allele carriers (n=19) of the *LPL* SNP showed a reduction in LDL-C levels (−1.0±2.51 mmol/l) compared to the TT genotype (n=24; 0.91±2.23 mmol/l) ($P_{association}$=0.007). However, this interaction was not statistically significant after correction for multiple testing. None of the other SNPs showed a significant interaction on changes in lipid concentrations after the 16-week dietary intervention (**Tables 5.6-8**).

5.5 Discussion

To our knowledge, this is the first study to investigate the effects of SNPs in both *LPL* and *APOE* genes on fasting serum lipid response after substituting SFA with MUFA or n-6 PUFA. Our findings, from this retrospective analysis of the DIVAS study, showed that 'TT' homozygotes (90% of study population) at *APOE* SNP rs1064725 had significantly lower total cholesterol concentrations after the 16-week replacement of SFA with MUFA in adults at moderate risk of CVD. Our findings indicate a greater sensitivity of this genotype group to dietary fat composition, particularly with respect to replacement of SFA with MUFA, which may have important public health implications.

Findings from cross-sectional studies are not adequate to prove the beneficial impact of a dietary component on disease prevention; therefore, data from chronic dietary intervention studies are preferable to detect changes in disease biomarkers over a period of time [37]. A dietary intervention study has shown a reduction of 51% in fasting total cholesterol in non-diabetic adults with mild abdominal obesity after two weeks of following a MUFA-rich diet (20% TE) compared to a SFA-rich diet (19% TE) [38]. In

support of the beneficial effect of MUFA-rich olive oil, a Mediterranean diet supplemented with extra-virgin olive oil for 4.8 years in older adults has also been shown to reduce the incidence of major CVD events [39], which suggests the potential role of MUFA and/or nutraceuticals such as polyphenols found in extra-virgin olive oil in the prevention of CVD-related outcomes [40]. Our retrospective data analysis has demonstrated a significant interaction between *APOE* SNP rs1064725 and a MUFA-rich diet on total cholesterol levels in adults at moderate CVD risk, where the MUFA-rich diet reduced fasting total cholesterol in 'TT' homozygotes compared to the SFA- and n-6 PUFA-rich diets. Our finding is in line with a previous study that also showed *APOE* genotypes to modulate changes in plasma total cholesterol and LDL-C in healthy individuals after consuming MUFA- (22% TE, virgin olive oil), SFA- (20% TE), and carbohydrate- (55% TE) rich diets for 4 weeks, where levels were higher in the *E4/E3* carriers, intermediate in *E3/E3* carriers, and lower in *E3/E2* carriers [24]. Another study showed that a MUFA-rich dietary intervention (mainly olive oil) for 12 months increased the secretion of TAG-rich lipoproteins (TRL) containing apoE and decreased the secretion of those without apoE. As a result, a MUFA-rich diet shortened the residence time of very low density lipoprotein (VLDL) particles in the circulation and increased the direct clearance of TRL from the circulation (due to the enrichment of TRLs with apoE, a ligand for receptor mediated uptake), decreasing their conversion to LDLs [41]. Hence, it can be hypothesised that a MUFA-rich diet is likely to regulate the clearance rate of TRL among 'TT' genotype carriers of the *APOE* SNP rs1064725 via effects on TRL particle apolipoprotein composition. However, the underlying mechanism of how the 'TT'

genotype acts differently from the 'G' allele on TRL metabolism in response to a MUFA-rich diet remains unclear and requires further investigation.

In our study, the common *LPL* SNP rs320 was found to modify the association between the n-6 PUFA-rich diet with changes in LDL-C levels, where the 'G' allele carriers had a tendency for a greater reduction in LDL-C concentrations compared to TT homozygotes. As far as the authors are aware, there are currently no studies to compare our findings with, except for one which showed that minor allele ('G') carriers of *LPL* SNP rs328 had lower fasting TAG concentrations when the participants had n-6 PUFA intake below 35.48% of total fat (below 35.48% of total fat median intake of LIPGENE study population) [19]. Besides *LPL*, evidence also suggests that the genetic effect of SNPs in *APOA5* and *TNFA* on lipid metabolism is modulated by n-6 PUFA [42, 43]. In mice, n-6 PUFA intake have been shown to play a role in the upregulation of genes encoding proteins involved in adipogenesis [44]. Thus, dietary n-6 PUFA may upregulate *LPL* gene expression and/or activity, leading to lower circulating lipid concentrations [19, 45]. In addition to the role of LPL in hydrolysing TRL, LPL plays a role in binding TRL (i.e. VLDL) to hepatic LDL receptors, which help to mediate the clearance of these particles [46]. This leads to a reduced conversion of VLDL to LDL, resulting in lower plasma LDL-C levels [47]. Even though the interaction between the *LPL* SNP rs320 and n-6 PUFA-rich diet on LDL-C concentrations in the current data analysis was not statistically significant after Bonferroni correction, which could be due to the small sample size, further large studies are required to explore this gene-diet interaction.

Statistically significant interactions were demonstrated in this study, however there are some limitations. The sample size was relatively small for some of the genotype

groups as the genotyping was performed retrospectively, and investigation of the lipid response according to *APOE* and *LPL* SNPs was not the main objective of the DIVAS study. Compared with cross-sectional studies, randomized clinical trials are conducted with smaller sample sizes. In our study, only 120 participants out of 195 consented to genetic analysis and hence this resulted in a small sample size for the analysis. However, we were able to identify significant gene-diet interactions on total cholesterol after Bonferroni correction. Thus, this hypothesis testing analysis has identified the need for suitably-powered dietary intervention trials using prospective genotyping to investigate the impact of dietary fat composition on plasma lipid responses according to *APOE* genotypes. A selection bias may also have existed because the participants were multi-ethnic (Asian 7% and Black 7%). However, to reduce this potential confounding effect, the analyses were adjusted for ethnicity. Furthermore, the interaction between SNP rs1064725 at *APOE* and the intervention diets on total cholesterol was still significant (P=0.003) even after excluding other ethnic groups from the analysis (data not shown). One of the main strengths of our study was that it examined the effects of three types of dietary fat (isoenergetically) consumed for a long duration (16 weeks) on lipid phenotypes in a robust randomised controlled intervention study, which addressed current dietary fat recommendations. Furthermore, we used a tagSNP approach whereby all the genetic variations in the *APOE* gene have been investigated in this study.

In conclusion, our study shows an interaction between *APOE* SNP rs1064725 and dietary fat intake on fasting total cholesterol concentrations, suggesting a greater sensitivity of the 'TT' homozygotes (90%) to dietary fat composition, with a total cholesterol lowering effect observed following substitution of SFA with MUFA but not

with n-6 PUFA. However, given that the present study was conducted in a relatively small group of individuals, further large studies using prospective genotyping are required to confirm our findings.

Author contributions: Israa M Shatwan and KSV **performed the data analysis and wrote the manuscript**. MW conducted the DIVAS study, analyzed the serum samples and critically reviewed the manuscript. KGJ and JAL designed the study and critically reviewed the manuscript. KSV conceived the genetic research. All authors approved the final draft of this article prior to submission.

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Chapter 6

For this study, I was involved in getting the dataset from the collaborators at Reading. I ran the statistical analysis using the SPSS software and wrote the first draft of the manuscript. For the genetic analysis, I had to collect and prepare DNA samples from the freezer to be transferred to LGC Genomics Company to run genotyping of the selected candidate vitamin D-related genetic variants. Before running the statistical analysis, I prepared the analysis plan, which summarized the steps for the statistical methods to be used for meeting the objectives of my study.

Chapter 6: Impact of polymorphisms in genes related to vitamin D metabolism and serum lipids on vitamin D concentrations and lipid responses to vitamin D fortified test meals.

6.1 Abstract

Deficiency in vitamin D has been associated with cardiovascular disease (CVD) risk and lipid concentrations. Genome-wide association studies have identified single-nucleotide polymorphism (SNPs) of genes involved in vitamin D metabolism, which have effects on vitamin D status. Additionally, SNPs of apolipoprotein E (*APOE*) and lipoprotein lipase (*LPL*) genes demonstrated associations with lipid concentrations. Hence, we aimed to examine the association of four vitamin D metabolism related SNPs with blood lipids and investigate whether these SNPs modified lipid response to dairy drink fortified with vitamin D in 18 men with sub-optimal vitamin D status. Also, we examined whether SNPs of *APOE* and *LPL* had an impact on lipid responses to dairy drinks fortified with Vitamin D The study was an acute, double-blind, randomised, controlled, crossover study consisting of 3 intervention arms. Men (age 49± 3 years; BMI 26.4± 0.6 kg/m^2) with sub-optimal vitamin D status were randomly assigned to one of three diets: controls diet, or fortified dairy drink with either 20µg 25- hydroxycholecalciferol (25(OH)D3) or 20µg cholecalciferol (vitamin D3). Fasting blood samples were collected for genetic and lipid profile analyses at baseline and end of intervention period (24 hour). Genotyping was performed by competitive allele-specific PCR-KASP® assay. The nicotinamide-adenine dinucleotide synthetase 1 gene (*NADSYN1*) SNP, rs12785878 (T/G), showed a significant association with high density lipoprotein cholesterol (HDL-C) (P=0.0003) after Bonferroni correction. The TT genotype had higher HDL-C levels compared to G allele carriers. None of the other genetic associations were statistically significant after correction for multiple testing. Our study provides evidence for an association between *NADSYN1* SNP

rs12785878 and HDL-C concentrations in men with sub-optimal vitamin D status, which requires confirmation in future large human intervention studies.

6.2 Introduction

Low vitamin D status is a common global health problem affecting all age groups [1]. The prevalence of vitamin deficiency is around 50% among older people worldwide [2], and between 20-30% in European adults [3]. Deficiency in vitamin D has been shown to be associated with a number of chronic conditions including cardiovascular disease [4, 5]. Several studies have suggested an association between vitamin D status and lipid levels [6-8]. In a meta-analysis of 17 cross-sectional studies, serum 25-hydroxy-vitamin D (25(OH) vitamin D; a biomarker of vitamin D status) was shown to be associated with fasted HDL-C, while an inverse association with triacylglycerol (TAG), low density lipoprotein cholesterol (LDL-C) and total cholesterol was found [6]. In a further study each 10 nmol/L increase in serum 25(OH) vitamin D was related to a significant decrease of 0.89 mg/dl of non-HDL-C, 1.08 mg/dl in total cholesterol, and 2.34 mg/dl in TAG concentrations in multi ethnic children in Canada [7]. Furthermore, supplementation of vitamin D (100,000 units) was associated with increased HDL-C in 29 children from Argentine [8].

A genome wide association study has identified a number of SNPs in genes involved with cholesterol synthesis, hydroxylation and vitamin D transport which have been shown to affect vitamin D status. These genes include *NADSYN1*, *CYP24A1* (encoding cytochrome P450, family 24, subfamily A, polypeptide 1), *GC* (encoding group-specific component vitamin D binding protein) and *CYP2R1* (encoding cytochrome P450, family 2, subfamily R, polypeptide 1) gene [9]. The functions of encoded protein of these genes are; CYP2R1 produces active vitamin D [1,25-dihydroxyvitamin D3 (1,25(OH)$_2$D$_3$)] through hydroxylation the 25-hydroxylase in the

liver and 1-alpha-hydroxylase in the kidney, CYP24A1 is transcriptionally stimulated in vitamin D target cells (liver and bones) by the action of 1,25-dihydroxyvitamin D3, its role includes inactivating the pathway from 1,25-dihydroxyvitamin D3 to calcitroic acid, GC is the vitamin D-binding protein involved with the transport of vitamin D, and NADSYN1 is involved with cholesterol synthesis [10]. Several studies have demonstrated the association of SNPs in these genes with vitamin D status in different ethnic groups [11-14]. A few studies have also reported association between these SNPs and some diseases associated with vitamin D deficiency including gestational diabetes mellitus [15] and hypertension [16].

The SNPs at *LPL* and *APOE* genes have been demonstrated to be associated with lipid concentrations [17-20]. Given that no study to date has examined whether SNPs in these genes effect lipid response to diet fortified with vitamin D, we investigated the whether *LPL* and *APOE* SNPs may modulate lipid response to vitamin D. Thus the aim of this study was to investigate whether four SNPs in genes relating to vitamin D (rs12785878 (G/T) at *NADSYN1* gene, rs6013897 (T/A) at *CYP24A1* gene, rs2282679 (A/C) at *GC* gene, rs12794714 (G/A) at *CYP2R1* gene), in addition to *LPL* and *APOE* SNPs (rs439401, rs405509, rs445925, rs1160985, rs405697, rs769450) modified the lipid responses to fortified dairy drink with either 20µg 25(OH)D3 or 20µg vitamin D3 in this study population compared with non-fortified dairy drink control in 18 men with sub-optimal vitamin D status. A secondary aim was to examine the association between vitamin D related SNPs and vitamin D status.

6.3 Participants and methods

6.3.1 Study participants

A detailed description of the study design and methods has been reported elsewhere [21].

Briefly, participants were recruited from Reading, UK and the surrounding area between May to October 2015. Participants were aged between 30 and 65 years, BMI 20-35 kg/m2 and were all non-smoking men with sub-optimal vitamin D status (plasma 25(OH) D <50 nmol/L). Criteria for exclusion were: the presence of cardiovascular, renal, gastrointestinal, respiratory and endocrine diseases, diabetes or cancer; hypertension, taking nutritional supplements; on long-term medication; having milk allergy/intolerance or lactose intolerance; outdoor workers and those who used tanning beds; overseas holidays two months before or during the study period; vigorous exercise (>3 times of 30 min aerobic exercise/week) and excessive alcohol intake (>14 units/week). The University of Reading Research Ethics Committee (approval no: 15/15) gave a favourable opinion for conduct and the trial was registered at www.clinicaltrials.gov (NCT02535910). All participants provided written informed consent.

6.3.2 Study design

The study was an acute, double-blind, randomised, controlled, crossover study consisting of three intervention arms. During the intervention period, men consumed either control, 20µg 25(OH)D3, or 20µg vitamin D3 in a random order (see details below). There was a 2-week washout period between the 3 study arms. The randomization of participants to diet order was achieved by using a web-based random letter sequence generator (https://www.randomizer.org/). The 20µg dose was used in the single meal study as it approximated to the fortification level used in the US and Canada. Detailed dietary advice including maintenance of normal diet and lifestyle, avoidance of dietary supplements and to minimize sun exposure was given to participants. Moreover, participants were asked to complete a 4-day diet diary (including 3 weekdays and 1 weekend day within the same week) to assess dietary intake. Dietplan 6.6 software was used to estimate dietary intake, before the first study visit. Fasting (12-h fast) blood

samples were collected at the beginning (baseline) and end of each intervention arm (24 hour) to investigate whether lipid concentrations (TAG, total cholesterol, LDL-C, and HDL-C) were modified in response to fortification with vitamin D.

6.3.3 Test meal protocols

Details of the test meal protocol have been described previously [21]. Briefly, participants were asked to refrain from alcohol and caffeine consumption and excessive exercise and to consume a low-fat meal (<10 g fat) as their main evening meal before each clinical visit. Also, participants were advised not to consume any fortified food or food high in vitamin D (such as egg yolk, oily fish) and they were provided with a low-nitrate water to consume the day before the study visit and during the study visit day until 24 hours (end point of experiment). The manufactured crystalline vitamin D3 and 25(OH) D3 were supplied by Dishman Netherlands BV. Vitamin D3 and 25(OH) D3 were dissolved in refined olive oil to reach a concentration of 1 μg/100 μl vitamin D3 or 25(OH) D3 stock fortified oil. Aliquots of vitamin D3 test oil (containing 20 μg vitamin D3), 25(OH) D3 test oil (containing 20 μg 25(OH) D3), and control (olive oil only) were given a random code and stored at -20 °C. On the morning of each study visit, the dairy drink was prepared using 300 ml full fat non-fortified milk.

After a 12 h overnight fast, the participants were cannulated and fasting blood sample was taken. The three study dairy drinks were control, 20μg 25(OH)D3, or 20μg vitamin D3. Participants were given a standard test breakfast that includes the study dairy drink, three slices (120 g) of toasted white bread (Hovis Ltd) with 40 g strawberry jam (Sainsbury's Supermarkets Ltd) and 15 g unsalted butter (Co-operative Ltd) (4.54 MJ energy, 125 g carbohydrate, 23 g protein and 51 g fat).

6.3.4 Anthropometric measures and biochemical analysis

Clinical visits took place at the Hugh Sinclair Unit of Human Nutrition, University of Reading, UK. Height was recorded to the nearest 0.5 cm using a wall-mounted stadiometer and weight was measured using a digital scale (Tanita Europe) using standard settings (normal body type and 1 kg for clothing). Fasting blood samples were collected into serum separator and K3EDTA-containing vacutainers. After resting at room temperature for 15 min, samples were centrifuged at 1700 g for 15 min after which the serum/plasma was aliquoted and stored at - 20 °C (for serum) and -80 °C (for 25(OH)D3 and vitamin D3) until analysis. Serum total cholesterol, HDL-C and TAG were measured using the ILAB 600 autoanalyser with standard kits and appropriate quality controls (reagents and analyser: Werfen (UK) Ltd. The LDL-C concentration was calculated using the Friedewald formula [22]. Plasma 25(OH)D3 [as sum of 25(OH) D3 and 3-epi-25(OH) D3] was analyzed by DSM Nutritional Products Ltd using a validated method [23, 24].

6.3.5 Genotyping for selected SNP

DNA samples collected in October 2016 and were outsourced to LGC Genomics Company. The genotyping was performed for the *LPL* gene SNPs (rs320 and rs328), *APOE* gene SNPs (rs439401, rs405509, rs445925, rs1160985, rs405697, rs769450, *NADSYN1* gene SNP rs12785878, *CYP24A1* gene SNP rs6013897, *GC* gene SNP rs2282679, and *CYP2R1* gene SNP rs12794714 using a KASP assay with a competitive allele-specific PCR assay®.

6.3.6 Statistical analysis

The data are presented as mean ± standard deviation (SD). Before analysis, data distribution and normality (skewness and kurtosis) were checked. The skewed data was log10

transformed for normality. The minor allele frequency was calculated by counting. The dominant models were a better fit for all SNPs except SNPs rs405509 and rs1160985 where the additive model was applied. The SNP rs328 at *LPL* and SNP rs1064725 at *APOE* were excluded from analysis as all the 18 participants were common homozygotes. The genotype distributions of the SNPs were in Hardy-Weinberg equilibrium (P>0.05) (Table 6.1). The association of the selected SNPs on continuous phenotypes was evaluated by the general linear model (GLM). Potential confounders that were associated with the outcomes were adjusted in all GLM analyses (i.e. age, BMI). A Bonferroni correction was applied and the significant P value was 0.0004 [0.05/10 SNPs*4 lipid outcomes* 3 times (before and after interventions and changes in values from baseline)], while for association with vitamin D, the significant P value was 0.001 [0.05/4 SNPs*3 diets * 3 times vitamin D levels measured (before and after interventions and changes in values from baseline)]. For all analyses, the statistical package SPSS version 22.0 (SPSS, Chicago, IL, USA) was used.

6.4 Results

6.4.1 Association between SNPs at *LPL*, *APOE*, and genes related to vitamin D with lipid levels

There was a significant association between SNP rs12785878 (T/G) at *NADSYN1* gene and HDL-C levels at baseline (P=0.0003) and after intervention with control diet (P=0.0004) (Table 7.2). The TT genotype (n=9) had higher HDL-C concentrations (1.40± 0.18 mmol/L baseline, and 1.45± 0.17 mmol/L after intervention) compared to G allele (n=9) (1.03± 0.11mmol/L baseline, and 1.12±0.11 mmol/L after intervention) (Figure 6.1). However, the change in circulating HDL-C from baseline was not significant (P<0.05).

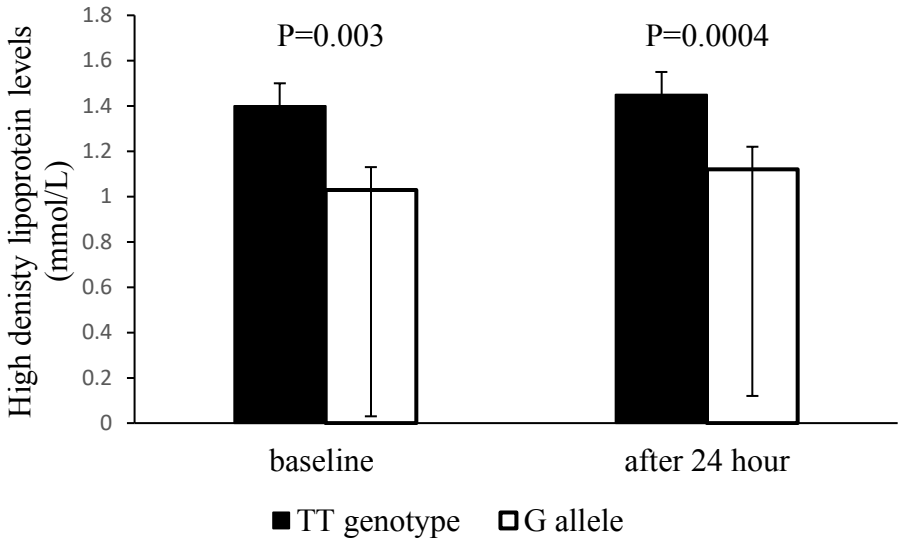

Figure 6.1 Association between SNP rs12785878 (T/G) at *NADSYN1* gene and high density lipoprotein at baseline and post-intervention in control group. The values are mean and stander errors.

There were no other significant associations between *LPL* and *APOE* SNPs and other genes related to vitamin D (*CYP24A1* gene, *GC* gene, and *CYP2R1* gene) with lipid concentrations at baseline or after interventions (P>0.0004) (Table 6.2-5).

6.4.2 Association between SNPs and vitamin D concentrations

An association was observed between SNP rs12785878 (T/G) at *NADSYN1* gene and 25(OH) vitamin D concentrations (a biomarker for vitamin D) at baseline of three diet groups (P=0.01) (Table 6.6), but did not remain statistically significant after Bonferroni correction (P>0.001). A higher 25(OH) vitamin D concentration was observed in TT genotype compared to G allele carriers. There were no other association observed between genes relating to vitamin D (*CYP24A1* gene, *GC* gene, and *CYP2R1* gene) and 25(OH)D concentrations (Table 6.6).

Table 6.1: Genotype distribution of SNPs at *LPL, APOE, NADSYN1 CYP24A1*, *GC* and *CYP2R1* genes and Hardy Weinberg Equilibrium P values

SNP	MAF	Common homozygous N (%)	Heterozygous N (%)	Rare homozygous N (%)	HWE P value
LPL					
rs320	0.22	11 (0.61)	6 (0.33)	1 (0.05)	0.88
rs328		18			
APOE					
rs439401	0.36	9 (0.5)	5 (0.27)	4 (0.22)	0.09
rs405509	0.41	6 (0.33)	9 (0.5)	3 (0.16)	0.91
rs445925	0.11	14 (0.77)	4 (0.22)		0.59
rs1160985	0.52	3 (0.16)	11 (0.61)	4 (0.22)	0.34
rs405697	0.19	12(0.66)	5 (0.27)	1 (0.05)	0.63
rs769450	0.41	5 (0.27)	11 (0.61)	2 (0.11)	0.27
rs1160985		17			
NADSYN1 gene rs12785878 (T/G)	0.66	3 (0.16)	6 (0.33)	9 (0.5)	0.28
CYP24A1 gene rs6013897 (T/A)	0.19	13 (0.72)	3 (0.16)	2 (0.11)	0.05
GC gene rs2282679 (A/C)	0.20	10 (0.58)	7 (0.41)		0.28
*CYP2R1*gene rs12794714 (G/A)	0.36	7 (0.38)	9 (0.5)	2 (0.11)	0.72

MAF; minor allele frequency, HWE; Hardy Weinberg Equilibrium

Table 6.2: The baseline and post- intervention associations between SNPs and HDL-C (mmol/L) and HDL-C response to the three test meals

	Control diet			vitamin D3 fortified dairy drink			25(OH) D3 fortified dairy		
	Baseline	After intervention	changes in values	Baseline	After intervention	changes in values	Baseline	After intervention	changes in values
SNPs									
LPL gene									
rs320									
TT	1.24± 0.19	1.31± 0.21	0.07± 0.06	1.23± 0.26	1.28± 0.24	0.04± 0.07	1.24± 0.23	1.33± 0.25	0.08± 0.06
G allele	1.19± 0.31	1.23± 0.25	0.04± 0.08	1.13± 0.24	1.18± 0.24	0.04± 0.03	1.21± 0.27	1.24± 0.22	0.04± 0.06
P value	0.81	0.61	0.42	0.55	0.59	0.75	0.86	0.61	0.21
APOE gene									
rs405509									
GG	1.10± 0.23	1.17± 0.19	0.07± 0.08	1.13± 0.19	1.21± 0.23	0.07± 0.08	1.13± 0.23	1.21± 0.20	0.07± 0.06
GT	1.32± 0.24	1.38± 0.22	0.06± 0.06	1.26± 0.29	1.29± 0.26	0.02± 0.04	1.32± 0.25	1.39± 0.26	0.07± 0.08
TT	1.15± 0.14	1.22± 0.16	0.06± 0.05	1.08± 0.21	1.13± 0.18	0.05± 0.04	1.14± 0.14	1.18± 0.16	0.04± 0.04
P value	0.24	0.19	0.96	0.63	0.75	0.30	0.33	0.31	0.85
rs439401									
CC	1.21± 0.27	1.28± 0.25	0.07± 0.07	1.21± 0.30	1.25± 0.28	0.04± 0.04	1.24± 0.27	1.30± 0.25	0.05± 0.07
T allele	1.23± 0.21	1.29± 0.19	0.06± 0.07	1.17± 0.21	1.22± 0.21	0.05± 0.08	1.21± 0.21	1.29± 0.24	0.08± 0.06
P value	0.71	0.67	0.98	0.85	0.83	0.54	0.96	0.84	0.37
rs445925									
GG	1.22± 0.23	1.28± 0.22	0.06± 0.06	1.17± 0.26	1.21± 0.24	0.03± 0.03	1.21± 0.22	1.27± 0.22	0.05± 0.06
A allele	1.21± 0.28	1.28± 0.22	0.06± 0.08	1.26± 0.24	1.35± 0.23	0.08± 0.12	1.29± 0.31	1.40± 0.31	0.11± 0.07
P value	0.91	0.93	0.89	0.52	0.32	0.17	0.61	0.39	0.21
rs769450									
GG	1.13± 0.21	1.22± 0.18	0.09± 0.06	1.13± 0.25	1.16± 0.20	0.03± 0.05	1.17± 0.26	1.25± 0.31	0.07± 0.06
A allele	1.25± 0.24	1.31± 0.23	0.05± 0.06	1.21± 0.26	1.27± 0.25	0.05± 0.06	1.25± 0.23	1.31± 0.22	0.06± 0.07
P value	0.47	0.75	0.12	0.88	0.83	0.99	0.75	0.89	0.53
rs405697									
GG	1.20± 0.24	1.28± 0.23	0.08± 0.06	1.21± 0.27	1.27± 0.26	0.05± 0.07	1.24± 0.25	1.33± 0.25	0.08± 0.06
A allele	1.26± 0.23	1.29± 0.20	0.03± 0.05	1.14± 0.22	1.18± 0.21	0.04± 0.02	1.21± 0.23	1.23± 0.21	0.02± 0.05
P value	0.34	0.51	0.21	0.99	0.98	0.70	0.90	0.69	0.046

rs1160985									
CC	1.17± 0.17	1.25± 0.20	0.07± 0.05	1.16± 0.31	1.18± 0.24	0.01± 0.07	1.31± 0.26	1.38± 0.35	0.07± 0.08
CT	1.31± 0.23	1.35± 0.22	0.04± 0.06	1.24± 0.26	1.28± 0.25	0.03± 0.03	1.28± 0.23	1.33± 0.22	0.05± 0.06
TT	1.01± 0.15	1.11± 0.13	0.11± 0.07	1.07± 0.17	1.16± 0.24	0.09± 0.11	1.04± 0.18	1.14± 0.18	0.10± 0.05
P value	0.11	0.18	0.31	0.51	0.66	0.33	0.12	0.21	0.52
NADSYN1 gene rs12785878									
TT	1.40± 0.18	1.45± 0.17	0.04± 0.07	1.35± 0.23	1.37± 0.22	0.02± 0.04	1.37± 0.23	1.43± 0.25	0.05± 0.07
G allele	1.03± 0.11	1.12±0.11	0.08± 0.06	1.03± 0.15	1.11±0.18	0.06± 0.07	1.09± 0.14	1.16±0.14	0.07± 0.05
P value	**0.0003**	**0.0004**	0.21	0.01	0.02	0.09	0.02	0.03	0.64
CYP24A1 gene rs6013897									
TT	1.16± 0.22	1.22± 0.18	0.05± 0.06	1.11± 0.20	1.18± 0.21	0.06± 0.06	1.16± 0.20	1.22± 0.20	0.06± 0.06
A allele	1.36± 0.23	1.45± 0.22	0.09± 0.06	1.39± 0.27	1.40± 0.26	0.01± 0.05	1.41± 0.24	1.49± 0.24	0.08± 0.07
P value	0.17	0.06	0.32	0.06	0.14	0.031	0.06	0.04	0.61
GC gene rs2282679									
AA	1.14± 0.19	1.21± 0.16	0.06± 0.06	1.14± 0.19	1.19± 0.21	0.04± 0.07	1.18± 0.24	1.27± 0.25	0.09± 0.08
C allele	1.27± 0.24	1.33± 0.21	0.06± 0.08	1.17± 0.24	1.23± 0.22	0.05± 0.03	1.23± 0.18	1.27± 0.18	0.03± 0.03
P value	0.28	0.23	0.86	0.95	0.98	0.77	0.73	0.88	0.10
CYP2R1 gene rs12794714									
GG	1.13± 0.22	1.20± 0.18	0.06± 0.07	1.14± 0.17	1.21± 0.21	0.07± 0.08	1.14± 0.22	1.22± 0.19	0.07± 0.05
A allele	1.27± 0.24	1.34± 0.23	0.06± 0.06	1.22± 0.29	1.25± 0.26	0.03± 0.04	1.28± 0.24	1.34± 0.26	0.06± 0.07
P value	0.28	0.23	0.99	0.71	0.83	0.19	0.30	0.37	0.65

HDL-C; high-density lipoprotein-cholesterol. P values for association between genotypes and HDL-C levels were obtained by using general linear model adjusted for age, and body mass index. Values are mean ± SD.

Table 6.3: The baseline and post- intervention associations between SNPs and total cholesterol levels (mmol/L) and total cholesterol response to the three test meals

	Control diet			vitamin D3 fortified dairy drink			25(OH) D3 fortified dairy		
	Baseline	After intervention	changes in values	Baseline	After intervention	changes in values	Baseline	After intervention	changes in values
SNPs									
LPL gene									
rs320									
TT	5.23± 0.86	5.76± 0.93	0.53± 0.34	5.39± 1.07	5.80± 1.25	0.41± 0.41	5.69± 1.28	0.39± 0.39	1.55± 0.74
G allele	4.89± 1.01	5.19± 0.77	0.30± 0.34	4.75± 0.67	5.36± 0.87	0.61± 0.30	5.42± 0.74	0.51± 0.35	1.27± 0.37
P value	0.63	0.33	0.25	0.32	0.65	0.25	0.84	0.55	0.51
APOE gene									
rs405509									
GG	4.87± 0.84	5.26± 0.59	0.38± 0.31	5.08± 0.80	5.68± 0.98	0.60± 0.46	5.53± 0.71	0.58± 0.33	1.52± 0.83
GT	5.33± 1.04	5.84± 1.11	0.51± 0.43	5.33± 1.18	5.83± 1.26	0.49± 0.31	5.89± 1.33	0.45± 0.39	1.35± 0.57
TT	4.84± 0.64	5.21± 0.44	0.36± 0.19	4.67± 0.55	4.94± 0.91	0.27± 0.39	4.79± 0.59	0.07± 0.04	1.53± 0.49
P value	0.58	0.39	0.79	0.77	0.77	0.42	0.49	0.14	0.70
rs439401									
CC	5.13± 1.03	5.54± 1.06	0.41± 0.29	5.31± 1.18	5.78± 1.20	0.47± 0.33	5.79± 1.17	0.46± 0.31	1.32± 0.37
T allele	5.07± 0.82	5.54± 0.76	0.46± 0.42	4.98± 0.75	5.48± 1.07	0.51± 0.42	5.39± 1.02	0.40± 0.43	1.56± 0.81
P value	0.71	0.56	0.61	0.97	0.89	0.79	0.81	0.72	0.21
rs445925									
GG	5.18± 0.89	5.64± 0.94	0.45± 0.37	5.14± 1.02	5.55± 1.12	0.41± 0.33	5.56± 1.16	0.37± 0.36	1.45± 0.42
A allele	4.81± 1.04	5.21± 0.74	0.40± 0.31	5.15± 0.91	5.91± 1.19	0.74± 0.42	5.68± 0.91	0.65± 0.34	1.41± 1.20
P value	0.49	0.41	0.81	0.96	0.61	0.18	0.82	0.17	0.76
rs769450									
GG	4.58± 0.86	5.02± 0.59	0.44± 0.27	4.65± 0.69	4.98± 0.85	0.32± 0.28	4.91± 0.65	0.32± 0.38	1.24± 0.60
A allele	5.30± 0.87	5.74± 0.93	0.44± 0.39	5.33± 1.02	5.88± 1.12	0.55± 0.39	5.85± 1.13	0.47± 0.36	1.51± 0.64
P value	0.33	0.39	0.75	0.68	0.41	0.21	0.29	0.39	0.64
rs405697									
GG	5.24± 0.84	5.76± 0.89	0.51± 0.36	5.44± 0.96	6.02± 0.97	0.58± 0.35	5.96± 0.96	0.56± 0.34	1.52± 0.68
A allele	4.81± 1.04	5.11± 0.80	0.29± 0.31	4.54± 0.71	4.85± 1.02	0.31± 0.36	4.85± 1.02	0.17± 0.27	1.27± 0.51
P value	0.77	0.45	0.29	0.32	0.17	0.18	0.13	0.01	0.81

rs1160985									
CC	4.91± 0.66	5.26± 0.45	0.35± 0.20	4.91± 0.70	5.15± 1.06	0.23± 0.36	5.19± 0.76	0.35± 0.52	1.13± 0.79
CT	5.31± 0.94	5.77± 1.02	0.45± 0.41	5.24± 1.11	5.77± 1.19	0.53± 0.29	5.74± 1.26	0.36± 0.32	1.39± 0.45
TT	4.65± 0.97	5.12± 0.66	0.47± 0.34	5.03± 0.90	5.60± 1.08	0.56± 0.57	5.48± 0.90	0.68± 0.37	1.81± 0.91
P value	0.42	0.42	0.96	0.67	0.91	0.61	0.90	0.35	0.48
NADSYN1 gene rs12785878									
TT	5.37± 1.04	5.83± 1.10	0.46± 0.41	5.44± 1.16	5.89± 1.30	0.54± 0.27	5.75± 1.39	0.35± 0.37	1.21± 0.51
G allele	4.83± 0.71	5.25± 0.56	0.42± 0.31	4.84± 0.63	5.37± 0.88	0.53± 0.46	5.43± 0.72	0.52± 0.35	1.67± 0.67
P value	0.28	0.23	0.88	0.28	0.52	0.41	0.65	0.40	0.03
CYP24A1 gene rs6013897									
TT	4.90± 0.93	5.25± 0.78	0.35± 0.30	4.86± 0.91	5.34± 1.12	0.48± 0.41	5.39± 1.16	0.43± 0.36	1.47± 0.63
A allele	5.62± 0.66	6.29± 0.78	0.66± 0.41	5.87± 0.79	6.38± 0.71	0.51± 0.24	6.11± 0.71	0.44± 0.42	1.36± 0.68
P value	0.18	0.03	0.11	0.06	0.13	0.81	0.29	0.86	0.38
GC gene rs2282679									
AA	4.69± 0.89	5.19± 0.85	0.49± 0.30	4.82± 0.98	5.29± 1.26	0.46± 0.41	5.44± 1.28	0.55± 0.40	1.33± 0.78
C allele	5.44± 0.53	5.78± 0.57	0.34± 0.44	5.30± 0.56	5.85± 0.51	0.55± 0.36	5.56± 0.59	0.28± 0.30	1.53± 0.36
P value	0.11	0.22	0.37	0.53	0.59	0.96	0.93	0.18	0.91
CYP2R1 gene rs12794714									
GG	4.71± 0.77	5.13± 0.54	0.42± 0.28	4.78± 0.72	5.35± 1.11	0.56± 0.45	5.25± 0.85	0.48± 0.38	1.51± 0.78
A allele	5.35± 0.93	5.81± 1.00	0.45± 0.41	5.37± 1.07	5.81± 1.12	0.44± 0.32	5.81± 1.21	0.41± 0.37	1.40± 0.54
P value	0.18	0.15	0.92	0.26	0.49	0.42	0.36	0.73	0.61

P values for association between genotypes and total cholesterol levels were obtained by using general linear model adjusted for age, and body mass index. Values are mean ± SD.

Table 6.4: The baseline and post-intervention associations between SNPs and TAG levels (mmol/L) and TAG response to the three test meals

	Control diet			vitamin D3 fortified dairy drink			25(OH) D3 fortified dairy		
	Baseline	After intervention	changes in values	Baseline	After intervention	changes in values	Baseline	After intervention	changes in values
SNPs									
LPL gene									
rs320									
TT	1.66± 0.73	1.63± 0.62	-0.03± 0.33	1.62± 0.85	1.73± 1.02	0.11± 0.27	1.55± 0.74	1.55± 1.02	0.01± 0.19
G allele	1.21± 0.38	1.32± 0.43	0.11± 0.27	1.26± 0.53	1.57± 0.62	0.31± 0.17	1.27± 0.37	1.41± 0.62	0.14± 0.14
P value	0.22	0.31	0.51	0.39	0.94	0.17	0.51	0.91	0.25
APOE gene									
rs405509									
GG	1.55± 0.69	1.61± 0.54	0.06± 0.39	1.60± 0.81	1.78± 0.71	0.18± 0.26	1.52± 0.83	1.69± 0.76	0.16± 0.13
GT	1.38± 0.63	1.40± 0.54	0.01± 0.32	1.26± 0.41	1.45± 0.61	0.19± 0.23	1.35± 0.57	1.34± 0.51	-0.01± 0.16
TT	1.67± 0.79	1.63± 0.82	-0.04± 0.15	1.90± 1.38	2.10± 1.79	0.20± 0.41	1.53± 0.49	1.60± 0.75	0.06± 0.27
P value	0.68	0.69	0.66	0.49	0.53	0.83	0.70	0.46	0.05
rs439401									
CC	1.40± 0.53	1.40± 0.42	0.003± 0.41	1.39± 0.51	1.61± 0.54	0.22± 0.25	1.32± 0.37	1.41± 0.30	0.08± 0.18
T allele	1.57± 0.76	1.61± 0.68	0.04± 0.18	1.57± 0.95	1.72± 1.14	0.15± 0.27	1.56± 0.81	1.59± 0.84	0.03± 0.18
P value	0.41	0.41	0.83	0.61	0.83	0.14	0.21	0.83	0.12
rs445925									
GG	1.49± 0.61	1.51± 0.53	0.01± 0.34	1.45± 0.74	1.63± 0.91	0.18± 0.28	1.45± 0.42	1.49± 0.47	0.03± 0.19
A allele	1.46± 0.88	1.53± 0.76	0.07± 0.20	1.58± 0.88	1.81± 0.82	0.22± 0.18	1.41± 1.20	1.55± 1.11	0.14± 0.11
P value	0.87	0.97	0.76	0.91	0.81	0.98	0.76	0.95	0.35
rs769450									
GG	1.45± 0.67	1.50± 0.66	0.05± 0.18	1.63± 1.06	1.86± 1.35	0.23± 0.31	1.24± 0.60	1.37± 0.68	0.13± 0.21
A allele	1.50± 0.66	1.51± 0.55	0.1± 0.35	1.42± 0.63	1.60± 0.67	0.17± 0.24	1.51± 0.64	1.55± 0.62	0.03± 0.16
P value	0.83	0.99	0.64	0.46	0.78	0.47	0.64	0.59	0.98

rs405697									
GG	1.59± 0.59	1.61± 0.46	0.02± 0.37	1.53± 0.59	1.74± 0.61	0.21± 0.25	1.52± 0.68	1.59± 0.62	0.06± 0.18
A allele	1.27± 0.76	1.30± 0.72	0.02± 0.15	1.38± 1.05	1.52± 1.31	0.13± 0.27	1.27± 0.51	1.32± 0.64	0.04± 0.18
P value	0.55	0.26	0.42	0.66	0.23	0.07	0.81	0.45	0.11
rs1160985									
CC	1.54± 0.92	1.51± 0.92	-0.04± 0.15	1.79± 1.46	2.03± 1.85	0.23± 0.38	1.13± 0.79	1.27± 0.93	0.14± 0.28
CT	1.33± 0.58	1.38± 0.48	0.05± 0.30	1.20± 0.43	1.41± 0.56	0.21± 0.22	1.39± 0.45	1.40± 0.41	0.01± 0.17
TT	1.86± 0.63	1.86± 0.49	-0.01± 0.47	2.01± 0.58	2.12± 0.58	0.11± 0.31	1.81± 0.91	1.94± 0.83	0.13± 0.09
P value	0.32	0.38	0.50	0.050	0.17	0.85	0.48	0.35	0.43
NADSYN1 gene rs12785878									
TT	1.20± 0.58	1.28± 0.50	0.08± 0.28	1.13± 0.31	1.23± 0.34	0.09± 0.11	1.21± 0.51	1.21± 0.41	-0.01± 0.15
G allele	1.77± 0.61	1.74± 0.55	-0.03± 0.34	1.83± 0.90	2.11± 1.03	0.28± 0.32	1.67± 0.67	1.80± 0.67	0.13± 0.18
P value	0.03	0.05	0.35	0.02	0.004	0.05	0.03	0.01	0.11
CYP24A1 gene rs6013897									
TT	1.47± 0.66	1.51± 0.60	0.03± 0.31	1.58± 0.85	1.76± 1.00	0.18± 0.27	1.47± 0.63	1.56± 0.65	0.08± 0.16
A allele	1.52± 0.68	1.51± 0.52	-0.01± 0.34	1.22± 0.23	1.43± 0.38	0.20± 0.23	1.36± 0.68	1.35± 0.56	-0.01± 0.22
P value	0.88	0.83	0.92	0.29	0.36	0.98	0.38	0.26	0.36
GC gene rs2282679									
AA	1.31± 0.68	1.48± 0.64	0.14± 0.24	1.34± 0.71	1.53± 0.75	0.19± 0.21	1.33± 0.78	1.44± 0.78	0.10± 0.16
C allele	1.62± 0.54	1.56± 0.51	-0.05± 0.31	1.71± 0.85	1.91± 1.09	0.20± 0.34	1.53± 0.36	1.58± 0.42	0.04± 0.17
P value	0.46	0.81	0.24	0.51	0.61	0.83	0.91	0.65	0.76

CYP2R1 gene rs12794714									
GG	1.40± 0.61	1.52± 0.54	0.12± 0.25	1.42± 0.77	1.61± 0.75	0.18± 0.19	1.51± 0.78	1.61± 0.76	0.10± 0.18
A allele	1.54± 0.69	1.50± 0.60	-0.04± 0.34	1.51± 0.77	1.71± 0.97	0.19± 0.30	1.40± 0.54	1.43± 0.54	0.03± 0.18
P value	0.74	0.89	0.34	0.82	0.98	0.92	0.61	0.52	0.49

TAG; triacylglycerol. P values for association between genotypes and TAG levels were obtained by using general linear model adjusted for age, and body mass index. Values are mean ± SD.

Table 6.5: The baseline and post-intervention associations between SNPs and LDL-C (mmol/L) and LDL-C response to the three tests meals

	Control diet			vitamin D3 fortified dairy drink			25(OH) D3 fortified dairy		
	Baseline	After intervention	changes in values	Baseline	After intervention	changes in values	Baseline	After intervention	changes in values
SNPs									
LPL gene									
rs320									
TT	3.23± 0.57	3.70± 0.67	0.46± 0.26	3.41± 0.83	3.73± 0.94	0.32± 0.31	3.35± 0.86	3.65± 1.00	0.30± 0.31
G allele	3.14± 0.75	3.35± 0.60	0.21± 0.22	3.04± 0.57	3.46± 0.77	0.41± 0.26	3.13± 0.44	3.53± 0.57	0.40± 0.29
P value	0.91	0.45	0.09	0.54	0.83	0.38	0.76	0.98	0.46
APOE gene									
rs405509									
GG	3.06± 0.69	3.35± 0.56	0.28± 0.19	3.22± 0.61	3.65± 0.73	0.43± 0.36	3.11± 0.38	3.55± 0.39	0.43± 0.28
GT	3.38± 0.65	3.82± 0.74	0.43± 0.34	3.49± 0.87	3.87± 0.95	0.37± 0.22	3.49± 0.90	3.88± 1.03	0.39± 0.28
TT	2.92± 0.42	3.24± 0.22	0.31± 0.19	2.72± 0.20	2.85± 0.49	0.13± 0.29	2.87± 0.51	2.87± 0.42	-0.001± 0.09
P value	0.54	0.24	0.55	0.45	0.41	0.39	0.42	0.31	0.12
rs439401									
CC	3.27± 0.73	3.62± 0.79	0.34± 0.24	3.46± 0.91	3.78± 0.98	0.32± 0.23	3.47± 0.387	3.84± 0.96	0.37± 0.25
T allele	3.12± 0.54	3.51± 0.52	0.38± 0.32	3.08± 0.53	3.47± 0.76	0.38± 0.34	3.06± 0.49	3.37± 0.67	0.31± 0.35
P value	0.85	0.75	0.45	0.77	0.97	0.43	0.52	0.52	0.79
rs445925									
GG	3.28± 0.57	3.66± 0.63	0.38± 0.31	3.30± 0.77	3.60± 0.88	0.29± 0.24	3.32± 0.78	3.61± 0.93	0.29± 0.30
A allele	2.92± 0.82	3.22± 0.67	0.30± 0.17	3.17± 0.73	3.73± 0.94	0.56± 0.36	3.08± 0.45	3.57± 0.51	0.49± 0.28
P value	0.31	0.27	0.63	0.82	0.74	0.14	0.64	0.95	0.23
rs769450									
GG	2.78± 0.66	3.11± 0.48	0.33± 0.19	2.77± 0.49	2.96± 0.66	0.19± 0.23	2.84± 0.41	3.03± 0.49	0.18± 0.31
A allele	3.36± 0.56	3.74± 0.64	0.37± 0.31	3.46± 0.75	3.88± 0.81	0.42± 0.29	3.42± 0.76	3.83± 0.85	0.40± 0.28
P value	0.22	0.27	0.76	0.41	0.22	0.18	0.41	0.24	0.22
rs405697									
GG	3.31± 0.61	3.74± 0.68	0.42± 0.28	3.52± 0.75	3.95± 0.77	0.43± 0.25	3.45± 0.73	3.91± 0.75	0.44± 0.26

A allele	2.97± 0.66	3.22± 0.46	0.25± 0.24	2.77± 0.41	2.98± 0.70	0.21± 0.32	2.88± 0.56	3.01± 0.72	0.12± 0.26
P value	0.71	0.41	0.49	0.24	0.14	0.25	0.36	0.11	0.03
rs1160985									
CC	3.03± 0.50	3.32± 0.28	0.28± 0.21	2.92± 0.51	3.04± 0.78	0.11± 0.27	3.01± 0.47	3.23± 0.59	0.21± 0.41
CT	3.40± 0.60	3.78± 0.68	0.38± 0.34	3.45± 0.82	3.85± 0.91	0.40± 0.21	3.45± 0.84	3.76± 0.99	0.31± 0.25
TT	2.78± 0.69	3.16± 0.59	0.37± 0.13	3.04± 0.65	3.46± 0.76	0.41± 0.45	2.93± 0.30	3.45± 0.45	0.52± 0.32
P value	0.21	0.19	0.98	0.51	0.64	0.58	0.40	0.76	0.43
NADSYN1 gene rs12785878									
TT	2.98± 0.55	3.34± 0.48	0.35± 0.23	2.97± 0.48	3.31± 0.64	0.33± 0.35	3.05± 0.34	3.44± 0.46	0.38± 0.32
G allele	3.42± 0.65	3.79± 0.74	0.37± 0.33	3.57± 0.86	3.95± 0.97	0.38± 0.22	3.47± 0.94	3.77± 1.11	0.29± 0.28
P value	0.21	0.15	0.98	0.09	0.13	0.99	0.23	0.42	0.57
CYP24A1 gene rs6013897									
TT	3.06± 0.67	3.34± 0.57	0.28± 0.21	3.02± 0.66	3.36± 0.83	0.33± 0.33	3.13± 0.75	3.46± 0.91	0.32± 0.31
A allele	3.56± 0.30	4.14± 0.49	0.57± 0.34	3.91± 0.57	4.33± 0.53	0.41± 0.13	3.62± 0.52	3.99± 0.51	0.37± 0.28
P value	0.15	0.01	0.05	0.01	0.03	0.89	0.22	0.24	0.74
GC gene rs2282679									
AA	2.96± 0.69	3.32± 0.66	0.36± 0.24	3.06± 0.72	3.39± 0.92	0.32± 0.32	3.10± 0.83	3.51± 0.99	0.41± 0.31
C allele	3.42± 0.36	3.73± 0.51	0.31± 0.31	3.34± 0.55	3.75± 0.61	0.41± 0.27	3.33± 0.37	3.56± 0.51	0.23± 0.29
P value	0.17	0.25	0.52	0.66	0.71	0.97	0.64	0.94	0.24
CYP2R1 gene rs12794714									
GG	2.93± 0.65	3.24± 0.50	0.31± 0.20	2.99± 0.55	3.41± 0.82	0.41± 0.37	2.94± 0.42	3.29± 0.58	0.35± 0.32
A allele	3.37± 0.58	3.77± 0.67	0.40± 0.32	3.45± 0.82	3.77± 0.91	0.32± 0.23	3.47± 0.81	3.81± 0.94	0.33± 0.29
P value	0.18	0.10	0.56	0.22	0.45	0.43	0.15	0.24	0.88

LDL-C; low-density lipoprotein cholesterol. P values for association between genotypes and low density lipoprotein levels were obtained by using general linear model adjusted for age, and body mass index. Values are mean ± SD.

Table 6.6: The baseline and post-intervention associations of SNPs at *NADSYN1 CYP24A1*, *GC*, and *CYP2R1* genes with 25(OH)D levels and 25(OH)D response to three test meals

	25(OH)D (nmol/L)								
	Control diet			vitamin D3 fortified dairy drink			25(OH) D3 fortified dairy drink		
SNP	Baseline	After intervention	changes in values	Baseline	After intervention	changes in values	Baseline	After intervention	changes in values
NADSYN1 gene rs12785878									
TT	41.80± 20.32	45.98± 23.47	4.18± 5.51	44.70± 16.24	50.22± 17.71	5.52± 5.01	43.65± 17.84	52.13± 16.54	8.48± 3.70
G allele	21.97± 8.03	21.69± 7.60	-0.27± 1.78	22.32± 9.89	26.92± 10.91	4.61± 2.91	22.53± 9.44	31.11± 11.66	8.58± 3.79
P value	0.01	0.01	0.81	0.01	0.01	0.65	0.01	0.01	0.79
CYP24A1 gene rs6013897									
TT	31.84± 20.05	32.68± 22.39	0.84± 3.01	32.36± 18.20	38.11± 20.01	5.74± 4.24	31.44± 17.81	40.27± 17.31	8.83± 2.97
A allele	32.01± 13.83	36.84± 18.99	4.82± 6.92	36.49± 16.66	39.77± 16.48	3.28± 2.91	37.39± 18.23	45.14± 19.88	7.75± 5.35
P value	0.58	0.86	0.17	0.91	0.71	0.22	0.96	0.97	0.42
GC gene rs2282679									
AA	32.83± 23.68	34.26± 26.10	1.43± 2.98	35.79± 21.06	40.41± 23.51	4.62± 4.71	33.85± 21.16	41.68± 21.42	7.83± 3.61
C allele	30.03± 9.11	31.75± 14.44	1.72± 6.17	27.98± 10.28	33.79± 9.15	5.81± 3.31	30.56± 13.43	39.08± 11.49	8.51± 2.91
P value	0.62	0.62	0.79	0.28	0.41	0.73	0.39	0.25	0.84
CYP2R1 gene rs12794714									

GG	23.74± 8.62	24.41± 8.84	0.67± 1.33	26.06± 10.98	31.35± 11.14	5.29± 2.71	24.26± 9	34.25± 11.52	9.99± 3.27
A allele	37.07± 20.93	39.83± 24.56	2.76± 5.73	38.25± 19.46	43.17± 21.35	4.92± 4.76	38.71± 19.71	46.32± 19.61	7.61± 3.69
P value	0.13	0.15	0.43	0.25	0.34	0.82	0.12	0.18	0.15

25(OH)D; 25-hydroxy-vitamin D, P values for association between genotypes and vitamin D levels were obtained by using general linear model adjusted for age, and body mass index. Values are mean ± SD.

6.5 Discussion

In this study, we investigated whether SNPs in *LPL*, *APOE* and vitamin D related genes modified lipid responses to fortified dairy drink (20µg 25(OH)D3, or 20µg vitamin D3). A significant association between SNP rs12785878 (T/G) at *NADSYN1* gene and circulating HDL-C was observed at baseline of control diet, where individuals carrying the TT genotype had higher HDL-C concentrations compared to G allele carriers. Our result suggested a favorable effect of TT genotype in SNP rs12785878 (T/G) at *NADSYN1* gene on HDL-C levels, however, due to the small sample size replication of our result in larger groups are required for robust finding.

Evidence from cross-sectional and intervention studies reported association between vitamin D levels and lipid concentrations (i.e TAG, HDL-C, and total cholesterol) [6, 7]. In a cohort of 3240 middle age and elderly European adults increased 25-hydroxyvitamin D was associated with higher fasted HDL-C [25]. The results of the current study were in line with previously reported finding from a cross-sectional study conducted in 323 non-diabetic African [26], which reported that GG genotype of SNP rs12785878 (T/G) in *NADSYN1* gene was associated with dyslipidemia (defined as higher total cholesterol, TAG, and LDL-C or lower HDL-C concentrations). However, another study in 36 Egyptian men (controls and patients with coronary artery disease) did not find SNP rs12785878 in *NADSYN1* as a risk marker for coronary artery disease, which could be due to a small sample size [27]. A proposed function of vitamin D is related to increased activity of LPL enzyme, which is associated with higher HDL-C levels [28]. However, the underlying mechanism of how the TT genotype could differentially influence HDL-C concentrations compared to G allele remains unclear and requires further investigation.

Furthermore, our study highlights an association between the SNP rs12785878 (T/G) at

NADSYN1 and circulating 25(OH) vitamin D, where the TT homozygotes had higher 25(OH) vitamin D concentrations compared to G allele carriers. A similar association was also demonstrated in previous studies [26, 29]. *NADSYN1* gene is located close to the gene dehydrocholesterol reductase (*DHCR7*), which encodes for dehydrocholesterol reductase enzyme, that converts 7-dehydrocholesterol into cholesterol, essential for vitamin D synthesis [9, 30]. Thus, it could be speculated that the TT genotype could enhance the activity of dehydrocholesterol reductase enzyme, thus increasing vitamin D synthesis. However, this association was not statistically significant in our study possibly due to the small sample size.

In this study, we examined whether lipid response to 20μg 25(OH)D3 or 20μg vitamin D3 were modulated by SNPs at *LPL* or *APOE*, or vitamin D related genes. There was significant association between SNP rs12785878 (T/G) at *NADSYN1* gene and HDL-C levels after the control diet, but the changes from baseline was not significant. However we did not observe any significant effects of the other SNPs on lipid responses after test meals of varying vitamin D composition.

This study has some strengths and limitations. A crossover study design was considered a strength as it allowed for comparison within- and between-groups which minimised the influence of cofounding factor as each participant acted as his/her matched control [31]. Overall, although a significant association was achieved between SNP rs12785878 (T/G) at *NADSYN1* and HDL-C, our sample size was relatively small, which is the main limitation and further investigation is required to confirm our findings. Another limitation is that the participants were retrospectively genotyped which resulted in unequal numbers in each genotype group. Ideally a prospective genotyping should be performed. This study included only men and hence may not be extrapolated to women.

In conclusion, the TT genotype of the SNP rs12785878 (T/G) at *NADSYN1* was found to be associated with higher HDL-C concentrations. Given that our study was conducted in a small group of men with sub-optimal vitamin D status, further statistically powered studies are required to replicate or refute these findings.

Author Contributions

Israa Shatwan organized the samples for genotyping, analyzed the data and wrote the paper. JG, CSBCT, and YL conducted the vitamin D trail. KGJ, JAL, JG and DIG designed the vitamin D experiments. KSV conceived the genetics study and wrote the manuscript.

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Chapter 7 Discussion and conclusion

7.1 Discussion

The field of nutrigenetics contributes to the understanding of the interactive effects of genes and diet on lipid outcomes. The knowledge of nutrigenetics will help to develop and implement better dietary guidelines and health strategies based on the genotype of individuals to improve healthcare and reduce the burden of chronic diseases such as cardiovascular disease (CVD) [1, 2]. However, multiple challenges are being faced in nutrigenetics research as many genes and environmental factors are involved in the development of chronic diseases [3]. This thesis has set out to examine the interaction between candidate genes involved in lipid metabolism and dietary factors on lipid outcomes. Two lipid metabolism-related genes, lipoprotein lipase (*LPL*) and apolipoprotein E (*APOE*), were considered as candidates for this study, given that the single nucleotide polymorphisms (SNPs) of the *APOE* [4, 5] and *LPL* [6-8] genes have been extensively studied and shown to be significantly associated with variation in lipid concentrations.

In this thesis, we investigated the association of common SNPs at the *LPL* gene and seven tagging SNPs (tagSNPs) at *APOE* with various lipid outcomes. Also, we the examined interaction between these SNPs and dietary factors (fat, carbohydrates, and protein) on lipid concentrations. Given that one of the aims of this thesis was to replicate findings obtained from one study in another independent cohort, various studies were used. A total of six different study designs from two populations [Chennai Urban Rural Epidemiological Study (CURES) Asian Indian, and European Caucasian from the UK and Denmark] were used to test our study objectives. The studies used were a postprandial study (n=261), a case-control study (Asian India, n=1,845), three cross-sectional studies [Prevention of Cancer by Intervention with Selenium (PRECISE study; UK, n=468; Denmark, n=192), and the Caerphilly prospective study (CaPS; UK, n=1,238)], a 16-week dietary intervention Dietary

Intervention and VAScular function (DIVAS) study (n=120) and a randomized, controlled, 3-way crossover, double-blind vitamin D trial (n=18). The statistical analysis for examining the associations and interactions was carried out applied using the general linear regression model using SPSS version 22. Potential cofounders were adjusted in the analyses such as age, sex, body mass index and ethnicity (wherever appropriate). A summary of findings obtained in this project are discussed below.

7.1.1 Findings relating to *LPL* gene

The main function of the LPL enzyme is hydrolysis of triacylglycerol (TAG) from chylomicrons and very low density lipoprotein (VLDL) [9]. Several SNPs at the *LPL* gene have been shown to be associated with TAG and high density lipoprotein cholesterol (HDL-C) [10, 11]. In my PhD work, I found that there was a tendency for the G minor allele at *LPL* SNP rs328 (C/G) to be positively associated with HDL-C compared with CC common homozygotes in the Asian Indian and Caucasian (postprandial and PRECISE cohorts) populations; however, this association was not statistically significant after Bonferroni correction in the PRECISE study. The *LPL* SNP rs320 (T/G) was also associated with HDL-C in the PRECISE study, where the G minor allele carriers had higher HDL-C. In the Asian Indian population, *LPL* SNP rs285 showed a significant association with TAG levels, while the association between rs328 and TAG in the CaPS (Caucasian population) was not statistically significant after Bonferroni correction (see Appendix Table 9.4). Given that postprandial TAG concentrations are identified as an independent risk factor for CVD [12], we examined association between two *LPL* SNPs (rs320 and rs328) and postprandial TAG levels. We found a significant association between SNP rs328 (C/G) and postprandial TAG in the postprandial study, where CC homozygotes had 12% higher TAG area under the curve. The findings from this thesis have demonstrated evidence for the association between the

LPL gene SNPs and HDL-C and TAG concentrations in the Asian Indian and Caucasian populations, although these need confirmations in further RCTs.

A novel finding of the thesis is the potential interaction between *LPL* SNP rs1121923 (C/T) and dietary intake of fat (energy %) as a determinant of HDL-C concentration in an Asian Indian population after adjustment for age, sex, BMI, and type 2 diabetes. Carriers of the T allele of the *LPL* SNP rs1121923 were associated with a greater increase in HDL-C when they consumed a high fat diet compared to the CC homozygotes. There are no previous studies that have reported an interaction between SNP rs1121923 at *LPL* and fat intake on lipids. However, a study in Caucasian Americans found that another *LPL* SNP rs328 (C/G) was found to interact with fat intake (energy %) on HDL-C levels, where, among those who consumed a high fat diet, the CC homozygotes and CG heterozygotes had significantly higher HDL-C concentrations compared to the GG homozygotes [13]. Although these two studies (our Asian Indian study and the study in Caucasian Americans [13]) were conducted in two different ethnic groups, both of them reported the protective effect of T allele at SNP rs1121923 and CC homozygotes at SNP rs328 of *LPL* gene in those who consumed a high fat. However, in the PRECISE and CaPS studies, we did not observe the interaction between *LPL* SNPs rs328 and rs320 and fat intake on HDL-C. This could be due to the fact that the sample size (n=660 and 1238, respectively) of these two cohorts was small and hence the study did not have the statistical power to detect the small interaction effect sizes. Furthermore, the DIVAS study found a tendency for a lower serum low density lipoprotein cholesterol (LDL-C) concentrations in G allele carriers of the SNP rs320 (T/G) at *LPL* than in TT homozygotes in response to n-6 polyunsaturated fatty acid (PUFA); however, this effect was not statistically significant after Bonferroni correction. Previous results from cross sectional studies support the interaction between *LPL* SNPs and PUFA intake on TAG and HDL-C [14, 15]. The role of n-6 PUFA is to upregulate *LPL* gene expression, leading to

lower circulating concentrations of TAG and LDL-C [16, 17]. The LPL plays a role in binding TAG- rich lipoprotein (TRL) (i.e. VLDL) to LDL receptors, which helps to mediate clearance of these particles [18]. This action reduces the conversion of VLDL to LDL-C, which explains the reduction in LDL-C levels [19]. No other significant interactions were observed between *LPL* SNPs and dietary factors in other studies investigated in this thesis.

7.1.2 Findings related to *APOE* gene

The APOE protein plays a key role in the transport and metabolism of cholesterol and TRL particles by serving as a receptor-binding ligand that mediates the clearance of dietary derived chylomicrons, and hepatically derived VLDL and their remnants from the circulation [4]. Thus, the effects of seven tagSNPs (rs405509, rs769450, rs439401, rs445925, rs405697, rs1160985, and rs1064725) in the *APOE* gene along with the common haplotype (rs7412 and rs429358; E3, E2, E4) on lipid concentrations in the PRECISE (Caucasian participants), CaPS (Caucasian participants), DIVAS (majority Caucasian participants), and postprandial studies were examined. The *APOE* haplotypes (E2, E3, and E4) and *APOE* SNP rs445925 were associated with total cholesterol in the PRECISE study and CaPS study. Around 7% of the variance in the total cholesterol levels have been shown to be determined by the *APOE* gene polymorphism [20]. Similarly, Suwalak et al. and El-Lebedy et al. [21, 22] found a significant association between *APOE* haplotypes (E2, E3, and E4) and total cholesterol. In addition, a genome-wide association scan has reported a significant association between *APOE* SNP rs445925 and LDL-C levels [23]. The mechanism explaining the differences in total cholesterol levels in the *APOE* alleles, is related to differences in APOE protein binding affinity to receptors such as LDL receptors. The E2 allele has reduced binding affinity, causing lower efficiency of the clearance of VLDL and chylomicron remnants from the blood to the liver, therefore slowing the uptake of postprandial lipoprotein particles compared with carriers of the E3 and E4 alleles [24]. Impaired conversion of the intestinal VLDL particles to

LDL-C in E2 [25] compared with E4, which have a higher rate of catabolism of VLDL particles [26], also, could explains the lower total cholesterol in E2 allele carriers. None of the other SNPs (rs405509, rs769450, rs439401, rs1160985, rs405697 and rs1064725) in the *APOE* gene showed an association with lipid concentrations in studies examined in my PhD work (PRECISE, Caerphilly, and DIVAS). Even in the postprandial cohort, the *APOE* SNPs did not show any association with fasting lipid concentrations and postprandial TAG (see Appendix Table 9.2 and 9.3) after sequential meal challenge [breakfast (0 min; 49 g fat) and lunch (330 min; 29 g fat)], which could be due to a small sample as analysis was performed retrospectively. In contrast, a previous postprandial study in 51 healthy European Caucasian men showed that the TT homozygotes of the *APOE* SNP rs405509 (G/T) had higher postprandial TAG in response to a fatty meal rich in vitamin A (1g of fat/ kg body weight and 60,000 IU vitamin A) compared to G allele carriers [27]. From my PhD work, I have demonstrated and confirmed the association between *APOE* haplotypes (E2, E3, and E4) and *APOE* SNP rs445925 and total cholesterol in the Caucasian population.

In terms of the *APOE* gene-diet interaction analysis, in the DIVAS study, isoenergetically substituting a high saturated fatty acid (SFA) diet with high monounsaturated fatty acid (MUFA) diet had a significant effect on reducing total cholesterol concentrations in the TT homozygotes of the *APOE* SNP rs1064725 (T/G). A previous study showed that a MUFA-rich dietary intervention (mainly olive oil) for 12 months increased the secretion of TRL containing APOE and decreased the secretion of those without APOE. As a result, a MUFA-rich diet shortened the residence time of VLDL particles in the circulation and increased the direct clearance of TRL from the circulation by APOE, a ligand for receptor mediated uptake, which eventually decreased their conversion to LDLs [28]. In the CaPS study, a tendency for an interaction between *APOE* haplotypes (E2, E3, and E4) and fat energy intake (%) on total cholesterol was observed, where, among those who consumed a

low fat diet (%), individuals carrying the E2 allele had significantly lower total cholesterol concentrations compared to E4 allele carriers. However, this interaction was not considered statistically significant after correction for multiple testing. The PRECISE study, also failed to show a significant interaction between *APOE* SNPs and dietary factors on lipid concentrations. Given that PRECISE and CaPS studies were relatively small and CaPS was only in men, future replication of these findings is warranted.

7.1.3 Findings from vitamin D intervention trial

In this thesis, given that vitamin D status has been shown to be associated with CVD related traits and lipid-related outcomes [29, 30], we examined the association between vitamin D related SNPs (rs12785878 (G/T) at nicotinamide-adenine dinucleotide synthetase 1 (*NADSYN1*) gene, rs6013897 (T/A) at encoding cytochrome P450, family 24, subfamily A, polypeptide 1 (*CYP24A1*) gene, rs2282679 (A/C) at encoding group-specific component vitamin D binding protein *(GC*) gene, rs12794714 (G/A) at encoding cytochrome P450, family 2, subfamily R, polypeptide 1 (*CYP2R1)* gene) and lipid outcomes using a crossover study in 18 Caucasian men with sup-optimal vitamin D status. The study was an acute, double-blind, randomised, controlled, crossover study consisting of 3 intervention arms. Participants were randomly assigned to one of three diets: a control diet, or fortified diary drink with either 20µg 25(OH)D3 or 20µg vitamin D3. In this study, the TT genotype of the SNP rs12785878 (T/G) at *NADSYN1* gene showed an association with higher HDL-C levels compared to G allele carriers. This is in line with a previous study in 323 non-diabetic Africans, where TT genotype of the SNP rs12785878 was associated with decreased risk of dyslipidemia [31]. In the present study, we also found that TT homozygotes of the SNP rs12785878 had higher vitamin D levels, but not after Bonferroni correction. It was found that the fortified diary drink with either 20µg 25(OH)D3 or 20µg vitamin D3 did not affect lipid levels. From this study, I conclude that the TT genotype of the SNP rs12785878 (T/G)

at *NADSYN1* may have a favorable effect on HDL-C levels, however, the finding requires a replication in a similar independent study.

7.1.4 Limitations and strengths

A major limitation was that some of these studies had a small sample size and hence did not have sufficient statistical power to detect an interaction effect. However, despite this limitation, we found significant interactions of the SNPs at *LPL* and *APOE* with dietary factors on lipid outcomes in Asian Indian and Caucasian populations. In this PhD project, the cross-sectional study design was predominantly used (PRECISE, Caerphilly, and CURES) which examined the genetic effects at a single point in time compared to a longitudinal study design, which captures the genetic effects that vary during a specific time period. Thus, it is difficult to identify the causal relationship between a risk factor and disease outcome. Also, there were some factors that could have introduced bias in the studies that were used, such as type 2 diabetes participants in CURES (selection bias), the use of two different food frequency questionnaires in the PRECISE cohort as this study was conducted in two different countries (UK and Denmark) (measurement bias), and use of multi-ethnic (Asian 7% and Black 7%) participants in the DIVAS study (selection bias); therefore, to reduce these potential bias, the analyses were adjusted for these factors (diabetes status, country and ethnicity, wherever appropriate) in the statistical analysis. The data was limited in some of the studies, for example, lipid outcomes were limited by HDL-C and total cholesterol in the PRECISE cohort, and dietary information was not available for phase 5 of the CaPS study and I was not able to run the SNP-diet interaction analysis. The main strengths of this thesis was that consistent associations were found between the *LPL* SNP rs328 and HDL-C and between *APOE* haplotypes (E2, E3, and E4) and the *APOE* SNP rs445925 and total cholesterol. Also, in this thesis, I used different study designs (cross sectional, intervention, postprandial, crossover) to confirm the observed findings. The use of the tagging approach

helped me to select seven tagSNPs representing the entire common genetic variations across the *APOE* gene. Furthermore, my study was the first to report a gene-diet interaction on lipid outcomes in an Asian Indian population.

7.2 Conclusion

In conclusion, my thesis work has confirmed significant interactions between *LPL* and *APOE* SNPs and dietary factors (in particular, fat intake) on lipid outcomes. Given that reduced intake of fat, especially SFA, is recommended for CVD prevention [32], my gene-diet interaction findings will have significant public health implications where people with the risk alleles of the *LPL* and *APOE* genes could be advised to consume a low fat diet. These findings add to the evidence base for possible associations between increased fat intake and elevated lipids which may be used to inform personalised dietary guidance Also, findings obtained in this thesis contributes to a better understanding of the interaction between genetic and dietary factors in relation to the progression of CVD. Replication of these findings in other studies with a larger sample size and with participants of different ethnic origin is required to confirm or refute the study findings. Additionally, prospective genotyping should be performed to prevent imbalance in the different genotype groups which could confound the results and randomized control trials should be carried out to identify the cause and the effect. This thesis addressed only lipid outcomes as one of the risk factors of CVD and hence, other risk factors need to be taken into consideration in order to implement dietary strategies for the prevention of CVD. Furthermore, a genetic risk scoring approach also provides evidence for the genetic effect on phenotypes. A recent study showed that the genetics risk score (comprising 6 SNPs) was associated with lipid levels and coronary artery disease in a Chinese population [33].

In conclusion, my study has demonstrated a consistent association between *LPL* rs328

and HDL-C, and *APOE* haplotypes (E2, E3, and E4) and *APOE* SNP rs445925 and total cholesterol. However, the gene-diet interactions require a replication in an independent larger cohort.

Table 7.1 Summary of the results and interpretations from this PhD project

Chapters	Study design	SNPs analyzed	Results	Interpretation
Chapter 3 Impact of Lipoprotein Lipase gene polymorphism, *S447X*, on postprandial triacylglycerol and glucose response to sequential meal ingestion	Postprandial study	*LPL* SNPs (rs320 and rs328) seven *APOE* tagSNPs (rs405509, rs769450, rs439401, rs445925, rs405697, rs1160985, and rs1064725)	Significant association was observed between *LPL* SNP rs328 and fasting HDL-C and postprandial TAG. No association with lipid was found for the *APOE* SNPs.	The CC homozygotes of the SNP rs328 had lower fasting HDL-C concentrations and higher postprandial TAG area under the curve compared to the G allele carriers.
Chapter 4 High fat diet modifies the association of *LPL* gene polymorphism with HDL-C in an Asian Indian population	Case-control study	*LPL* SNPs (rs1121923, rs328, rs4922115 and rs285) *APOE* SNPs were not available in the dataset	Signification association was observed between SNPs rs328 and rs285 with HDL-C and serum TAG. The interaction between SNP rs1121923 and fat intake (energy %) on HDL-C was also significant	The CC homozygotes at SNP rs328 had lower fasting HDL-C relative to the G allele carriers. The CC homozygotes of the SNP rs285 had higher TAG concentrations than T allele carriers. Among those who consumed a high fat diet, the T allele carriers of the SNP rs1121923 had significantly higher HDL-C compared to the CC homozygotes.
Chapter 5 Apolipoprotein E and lipoprotein lipase gene polymorphisms, dietary factors and blood lipids	Cross sectional study	*LPL* SNPs (rs320 and rs328) seven *APOE* tagSNPs (rs405509, rs769450, rs439401, rs445925, rs405697, rs1160985, and	A statistically significant association of the *APOE* haplotype (rs7412 and rs429358; E2, E3, and E4) and *APOE* tagSNP rs445925 with total cholesterol was observed in the PRECISE	Carriers of the E2 allele had lower total cholesterol concentration than those with the E3 and E4 alleles. The 'A' allele carriers at SNP rs445925 had lower levels of total cholesterol compared to GG homozygotes.

		rs1064725) one *APOE* haplotype (rs7412 and rs429358),	study. This association was further replicated in the CaPS study	
Chapter 6 Apolipoprotein E gene polymorphism modifies fasting total cholesterol concentrations in response to replacement of dietary saturated with monounsaturated fatty acids in adults at moderate cardiovascular disease risk	Intervention study	*LPL* SNPs (rs320 and rs328) seven *APOE* tagSNPs (rs405509, rs769450, rs439401, rs445925, rs405697, rs1160985, and rs1064725)	After the intervention, a significant diet-SNP rs1064725 interaction on change in total cholesterol was observed.	The TT homozygotes of the *APOE* SNP rs1064725 showed a significant reduction in total cholesterol after the MUFA diet compared to the SFA and n-6 PUFA diet.
Chapter 7 Impact of polymorphisms in genes related to vitamin D metabolism and serum lipids on vitamin D concentrations and lipid responses to vitamin D fortified test meals	Crossover study	*LPL* SNPs (rs320 and rs328) seven *APOE* tagSNPs (rs405509, rs769450, rs439401, rs445925, rs405697, rs1160985, and rs1064725) Four vitamin D-related SNPs *NADSYN1* SNP rs12785878, *CYP24A1* SNP rs6013897, *GC* SNP rs2282679, *CYP2R1* SNP rs12794714	The SNP rs12785878 at *NADSYN1* showed a significant association with HDL-C at baseline. None of the SNPs showed an effect on changes in lipids after the intervention of fortified diary drinks with either 25(OH)D3 or vitamin D3.	The TT genotype at SNP rs12785878 had higher HDL-C levels compared to G allele carriers.

7.3 Future prospects

Although remarkable improvements have been achieved in epidemiological studies in the field of nutrigenetics, future research should focus on understanding the metabolic pathways underlying gene-diet interactions [34-36]. Therefore, the science that identifies the connection between compounds in food and diet, and genetic susceptibility is needed [37]. Food scientists and nutritionists have described a new discipline called "Foodomics", which is defined as the application of new methodologies, or "omics", to improve individual health [36, 38]. This field has helped to identify the interactions of bioactive compounds from the diet at a molecular and cellular level to provide evidence on their health benefits, and to understand variations and differential response to nutrition interventions [36]. For instance, several studies have investigated the therapeutic activities of dietary polyphenols with respect to their ability to control intracellular signalling and biochemical mechanisms for the prevention of cancer [39, 40].

Another approach is nutrigenomics, which investigates the effect of diet and its bioactive components on gene expression. This field of research will help in understanding how diet interacts with the metabolic pathways, which may have a role in diet-related diseases [41]. While nutrigenetics investigates gene-diet interaction or in other words, explores how the genes (at the levels of SNPs) causes the disease in response to a particular diet [42, 43]. The knowledge from these two fields will help in designing optimal diets that allow health maintenance and disease prevention in an individual [41, 44]. However, at present, a large gap exists between nutrition recommendations and individual eating behaviour. Therefore, the implementation of a personalised approach could be more widely-accepted by the public [45]. Personalised nutrition advice has been found to be more effective in improving dietary behaviours compared to population-based 'one size fits all' advice [46].

A recent study has also reported that gene-based personalised nutrition was more effective in reducing SFA intake than standard dietary advice in *APOE* gene carriers [47]. Besides personalised nutrition, Precision nutrition is another approach, which is aimed to develop more comprehensive nutritional recommendations based on the interaction between internal and external parameters of an individual's environment throughout life. Precision nutrition takes into account the genetic factors, dietary habits, food behavior, physical activity, the microbiota and the metabolome [48].

In summary, clear guidance from nutrigenetics studies is required for the implementation of personalised nutrition and foodomics, which can only be achieved by using large and well powered studies, examining various ethnic groups, considering the variety in dietary patterns globally, and conducting additional testing for other modifiable factors such as physical activity.

7.4 References

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Chapter 8 Appendices

8.1 Appendix A: Genotype and minor allele frequencies of the SNPs at *APOE* genes in postprandial study

SNP	MAF	Common homozygous N (%)	Heterozygous N (%)	Rare homozygous N (%)	HWE P value
rs405509 (G>T)	0.46	47 (0.28)	85 (0.51)	34 (0.20)	0.69
rs769450 (G>A)	0.40	53 (0.31)	92 (0.55)	21 (0.12)	0.05
rs439401 (C>T)	0.39	62 (0.37)	77 (0.46)	26 (0.15)	0.79
rs445925 (G>A)	0.12	125 (0.75)	38 (0.23)	2 (0.01)	0.63
rs405697 (G>A)	0.28	87 (0.51)	68 (0.40)	14 (0.08)	0.88
rs1160985 (C>T)	0.42	49 (0.29)	94 (0.56)	24 (0.14)	0.05
rs1064725 (T>G)	0.05	149 (0.89)	17 (0.10)	-	0.48

MAF; minor allele frequency, HWE; Hardy Weinberg Equilibrium

8.2 Appendix B: Baseline and postprandial characteristics of the participants according to *APOE* SNP rs405509

Participants characteristics	GG	GT	TT	P value
TC (mmol/l)	5.62± 0.96	5.58± 1.05	5.81± 0.88	0.47
TAG (mmol/l)	1.66± 0.73	1.48± 0.75	1.48± 0.65	0.68
HDL-C (mmol/l)	1.35± 0.42	1.37± 0.43	1.34± 0.42	0.43
LDL-C (mmol/l)	3.51± 0.98	3.53± 1.01	3.79± 0.85	0.20
TAG AUC (mmol/l × 480 min)	1164.46± 501.37	1063.48± 493.24	1114.72± 527.02	0.93
TAG IAUC (mmol/l × 480 min)	358.96± 230.39	327.35± 200.28	321.32± 233.20	0.75

Abbreviations: TC, total cholesterol; TAG, triacylglycerol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; AUC, area under the curve; IAUC, incremental area under the curve. P values were obtained by using a general linear model adjusted for age, sex, BMI.

8.3 Appendix C: Baseline and postprandial characteristics of the participants according to *APOE* SNPs rs439401, rs769450, rs445925, rs405697, rs1160985, and rs1064725

	rs439401		
Participants characteristics	CC	T allele	P value
TC (mmol/l)	5.79± 1.02	5.53± 0.97	0.34
TAG (mmol/l)	1.59± 0.72	1.48± 0.73	0.39
HDL-C (mmol/l)	1.38± 0.42	1.34± 0.43	0.47
LDL-C (mmol/l)	3.68± 1.01	3.51± 0.94	0.64
TAG AUC (mmol/l × 480 min)	1137.58± 499.21	1081.99± 507.15	0.61
TAG IAUC (mmol/l × 480 min)	359.28± 233.62	322.01± 204.52	0.26
	rs769450		
	GG	A allele	
TC (mmol/l)	5.69± 0.97	5.60± 1.00	0.43
TAG (mmol/l)	1.44± 0.63	1.57± 0.77	0.44
HDL-C (mmol/l)	1.42±0.50	1.33± 0.38	0.39
LDL-C (mmol/l)	3.59± 1.01	3.57± 0.95	0.66
TAG AUC (mmol/l × 480 min)	1078.26± 535.27	1116.99± 485.51	0.94
TAG IAUC (mmol/l × 480 min)	311.81± 218.52	347.76± 213.94	0.47
	rs445925		
	GG	A allele	
TC (mmol/l)	5.65± 0.95	5.62± 1.17	0.41
TAG (mmol/l)	1.54± 0.76	1.55± 0.68	0.61
HDL-C (mmol/l)	1.32± 0.39	1.48± 0.51	0.07
LDL-C (mmol/l)	3.64± 0.88	3.39± 1.27	0.04
TAG AUC (mmol/l × 480 min)	1089.13± 490.48	1186.13± 578.20	0.08
TAG IAUC (mmol/l × 480 min)	329.93± 211.65	362.44± 271.47	0.21
	rs405697		
	GG	A allele	
TC (mmol/l)	5.65± 0.99	5.61± 1.00	0.72
TAG (mmol/l)	1.53± 0.71	1.56± 0.78	0.95
HDL-C (mmol/l)	1.41± 0.44	1.29± 0.40	0.05
LDL-C (mmol/l)	3.56± 0.98	3.60± 0.96	0.83
TAG AUC (mmol/l × 480 min)	1086.61± 493.85	1125.83± 505.76	0.76
TAG IAUC (mmol/l × 480 min)	331.65± 222.34	340.16± 206.45	0.99
	rs1160985		
	CC	T allele	
TC (mmol/l)	5.65± 0.97	5.63± 1.00	0.76
TAG (mmol/l)	1.39± 0.62	1.58± 0.76	0.33
HDL-C (mmol/l)	1.45± 0.51	1.31± 0.38	0.18
LDL-C (mmol/l)	3.56± 1.03	3.60± 0.95	0.94
TAG AUC (mmol/l × 480 min)	1052.45± 532.46	1125.55± 484.90	0.97
TAG IAUC (mmol/l × 480 min)	312.56± 223.84	346.91± 210.43	0.67
	rs1064725		
	TT	G allele	
TC (mmol/l)	5.69± 0.98	5.17± 1.00	0.35
TAG (mmol/l)	1.59± 0.76	1.24± 0.54	0.49
HDL-C (mmol/l)	1.33± 0.43	1.50± 0.32	0.64

LDL-C (mmol/l)	3.65± 0.97	3.17± 0.89	0.58
TAG AUC (mmol/l × 480 min)	1126.06± 501.45	906.14± 455.51	0.51
TAG IAUC (mmol/l × 480 min)	341.57± 217.13	288.72± 203.65	0.82

Abbreviations: TC, total cholesterol; TAG, triacylglycerol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; AUC, area under the curve; IAUC, incremental area under the curve. P values were obtained by using a general linear model adjusted for age, sex, BMI.

8.4 Appendix D: Association of the *APOE* and *LPL* SNPs with triacylglycerol in the Caerphilly prospective study

SNP	Triacylglycerol (mmol/L)
LPL	
rs320	
TT	1.82± 0.90
T/G	1.74± 0.89
P	0.055
rs328	
CC	1.82± 0.91
C/G	1.65± 0.87
P	**0.004**
APOE	
rs405509	
GG	1.77± 0.91
GT	1.82± 0.91
TT	1.71± 0.87
P	0.47
rs769450	
GG	1.77± 0.93
A allele	1.79± 0.88
P	0.73
rs439401	
CC	1.82± 0.89
T allele	1.76± 0.90
P	0.11
rs445925	
GG	1.76± 0.86
A allele	1.86± 1.01
P	0.043
rs405697	
GG	1.80± 0.90
A allele	1.77± 0.89
P	0.64
rs1160985	
CC	1.73± 0.87
CT	1.82± 0.91
TT	1.77± 0.91
	0.40
rs1064725	
TT	1.79± 0.91
G allele	1.53± 0.65
P	0.09
(rs7412- rs429358) E2, E3, and E4	
E3	1.79± 0.85
E4	1.82± 0.90
E2	1.88± 0.99
P	0.025

Values are given as mean ± SD. P values for differences between genotypes were obtained

using linear regression model adjusted for age, and BMI. Adjusted P value after correction for multiple testing was 0.001. MAF; minor allele frequency.

8.5 Appendix E: Interaction between *APOE* and *LPL* SNPs and dietary factors on triacylglycerol in the Caerphilly prospective study

Interaction between rs320 at *LPLdietary factors on Triacylglycerol**		
Interaction between SNP rs320* fat energy % intake	Interaction between SNP rs320* protein energy % intake	Interaction between SNP rs320* carbohydrate energy % intake
-0.01 (0.01) 0.28	-0.01 (0.02) 0.60	0.01 (0.01) 0.21
Interaction between rs328 at *LPL* *dietary factors on Triacylglycerol		
Interaction between SNP rs328* fat energy % intake	Interaction between SNP rs328* protein energy % intake	Interaction between SNP rs328* carbohydrate energy % intake
-0.02 (0.01) 0.02	-0.03 (0.03) 0.32	0.02 (0.01) 0.01
Interaction between rs405509 at *APOE* *dietary factors on Triacylglycerol		
Interaction between SNP rs405509* fat energy % intake	Interaction between SNP rs405509* protein energy % intake	Interaction between SNP rs405509* carbohydrate energy % intake
0.01 (0.01) 0.61	0.03 (0.03) 0.52	-0.01 (0.01) 0.44
Interaction between rs769450 at *APOE* *dietary factors on Triacylglycerol		
Interaction between SNP rs769450* fat energy % intake	Interaction between SNP rs769450* protein energy % intake	Interaction between SNP rs769450* carbohydrate energy % intake
-0.003 (0.01) 0.71	-0.01 (0.02) 0.77	0.004 (0.01) 0.63
Interaction between rs439401 at *APOE* *dietary factors on Triacylglycerol		
Interaction between SNP rs439401* fat energy % intake	Interaction between SNP rs439401* protein energy % intake	Interaction between SNP rs439401* carbohydrate energy % intake
0.002 (0.01) 0.81	-0.03 (0.02) 0.12	0.003 (0.01) 0.70
Interaction between rs445925 at *APOE* *dietary factors on Triacylglycerol		
Interaction between SNP rs445925* fat energy % intake	Interaction between SNP rs445925* protein energy % intake	Interaction between SNP rs445925* carbohydrate energy % intake
$-2.01*10^{-5}$ (0.01) 0.99	-0.03 (0.02) 0.17	0.004 (0.01) 0.66
Interaction between rs405697 at *APOE* *dietary factors on Triacylglycerol		
Interaction between SNP rs405697* fat energy % intake	Interaction between SNP rs405697* protein energy % intake	Interaction between SNP rs405697* carbohydrate energy % intake
0.01 (0.01) 0.30	-0.02 (0.02) 0.42	-0.01 (0.01) 0.50
Interaction between rs1160985 at *APOE* *dietary factors on Triacylglycerol		

Interaction between SNP rs1160985* fat energy % intake	Interaction between SNP rs1160985* protein energy % intake	Interaction between SNP rs1160985* carbohydrate energy % intake
-0.01 (0.01) 0.61	-0.01 (0.02) 0.95	0.01 (0.01) 0.61
Interaction between rs1064725 at *APOE* *dietary factors on Triacylglycerol		
Interaction between SNP rs1064725* fat energy % intake	Interaction between SNP rs1064725* protein energy % intake	Interaction between SNP rs1064725* carbohydrate energy % intake
-0.01 (0.03) 0.67	0.06 (0.09) 0.45	0.01 (0.03) 0.80
Interaction between *APOE* (E2,E3, and E4)*dietary factors on Triacylglycerol		
Interaction between SNP *APOE* (E2, E3, and E4)* fat energy % intake	Interaction between SNP *APOE* (E2, E3, and E4)* protein energy % intake	Interaction between SNP *APOE* (E2, E3, and E4)* carbohydrate energy % intake
-0.004 (0.01) 0.92	-0.03 (0.03) 0.22	0.01 (0.01) 0.66

Values represented β regression coefficients (± S.E), and $P_{interaction}$. P values were obtained by using a general linear model adjusted for age, sex, BMI, country and total calorie whenever appropriate.

8.6 Appendix F: Genotype, and major and minor allele frequencies of the SNPs at *LPL* and *APOE* genes of all SNPs studied in various cohorts

SNP	Common homozygous N (%)	Heterozygous N (%)	Rare homozygous N (%)	Major allele frequency	Minor allele frequency	Chi square	Hardy Weinberg Equilibrium P value
Postprandial study							
rs320	131 (0.56)	100 (0.43)	0	0.78	0.22	2.69	0.10
rs328	213 (0.81)	48 (0.18)	0	0.91	0.09	2.67	0.10
CURES							
rs4922115	864 (0.68)	347 (0.27)	50 (0.39)	0.82	0.17	4.02	0.05
rs1121923	1147 (0.87)	157 (0.12)	7 (0.005)	0.93	0.06	0.4	0.52
rs328	877 (0.72)	303 (0.25)	24 (0.02)	0.85	0.14	0.13	0.71
rs285	758 (0.41)	845(0.45)	252 (0.13)	0.63	0.36	0.45	0.50
PRESICE study							
rs320	354 (0.53)	271 (0.40)	39 (0.05)	0.74	0.26	1.8	0.17
rs328	522 (0.80)	127 (0.19)	3 (0.005)	0.89	0.10	2.6	0.11
rs405509	183 (27.6%)	330 (49.8%)	149 (22.5%)	0.52	0.47	0.0001	0.99
rs769450	228 (34.5%)	339 (51.4%)	92 (13.8%)	0.60	0.39	3.66	0.06
rs439401	291 (44.2%)	290 (44.1%)	76 (11.5%)	0.66	0.33	0.08	0.77
rs445925	506 (77.3%)	142 (21.7%)	6 (0.9%)	0.88	0.11	1.33	0.24
rs405697	365 (54.9%)	257 (38.7%)	42 (6.3%)	0.74	0.25	0.13	0.71
rs1160985	200 (30.2%)	344 (52%)	117 (17.7%)	0.56	0.43	2.18	0.13
rs1064725	606 (91.4%)	56 (8.4%)	1 (0.1%)	0.95	0.04	0.06	0.81
Caerphilly prospective study							
rs320	721 (0.53)	536 (0.39)	86 (0.06)	0.73	0.26	1.05	0.31
rs328	1068 (0.79)	266 (0.19)	9 (0.006)	0.89	0.10	3.01	0.08
rs405509	381 (0.28)	675 (0.50)	287 (0.21)	0.53	0.46	0.13	0.71
rs769450	452(0.33)	672(0.50)	219(0.16)	0.58	0.41	1.35	0.24
rs439401	560 (0.41)	615 (0.45)	168 (0.12)	0.64	0.35	0.0018	0.96
rs445925	1056 (0.78)	271 (0.20)	16 (0.01)	0.88	0.11	0.08	0.77

rs405697	728 (0.54)	513 (0.38)	102 (0.07)	0.73	0.26	0.77	0.38
rs1160985	394 (0.29)	688 (0.51)	261 (0.19)	0.54	0.45	1.61	0.20
rs1064725	1076 (0.96)	35 (0.03)	0	0.98	0.01	0.28	0.59
DIVAS study							
rs320	57 (0.46)	57 (0.46)	9 (0.07)	0.69	0.30	1.07	0.30
rs328	93 (0.75)	30 (0.24)	1 (0.008)	0.87	0.12	0.72	0.39
rs405509	31 (0.25)	65 (0.52)	28 (0.22)	0.51	0.48	0.29	0.59
rs769450	47 (0.37)	62 (0.5)	15 (0.12)	0.62	0.37	0.63	0.42
rs439401	50 (0.40)	53 (0.43)	19 (0.15)	0.62	0.37	0.61	0.43
rs445925	95 (0.78)	25 (0.20)	1 (0.008)	0.88	0.11	0.21	0.64
rs405697	71 (0.57)	38 (0.30)	14 (0.11)	0.73	0.26	5.58	0.01
rs1160985	36 (0.29)	65 (0.52)	23 (0.18)	0.55	0.44	0.44	0.51
rs1064725	111 (0.89)	13 (0.05)	0	0.94	0.05	0.37	0.54
Vitamin D study							
rs320	11 (0.61)	6 (0.33)	1 (0.05)	0.77	0.22	0.02	0.88
rs328	18					NS	
rs405509	9 (0.5)	5 (0.27)	4 (0.22)	0.63	0.36	0.014	0.91
rs769450	5 (0.27)	11 (0.61)	2 (0.11)	0.58	0.41	1.19	0.27
rs439401	6 (0.33)	9 (0.5)	3 (0.16)	0.58	0.41	2.85	0.09
rs445925	14 (0.77)	4 (0.22)	0	0.88	0.11	0.28	0.59
rs405697	3 (0.16)	11 (0.61)	4 (0.22)	0.47	0.52	0.23	0.63
rs1160985	12(0.66)	5 (0.27)	1 (0.05)	0.80	0.19	0.91	0.34
rs1064725	17 (0.94)	0	0			NS	
rs12785878	3 (0.16)	6 (0.33)	9 (0.5)	0.33	0.66	1.12	0.28
rs6013897	13 (0.72)	3 (0.16)	2 (0.11)	0.80	0.19	3.94	0.05
rs2282679	10 (0.58)	7 (0.41)	0	0.79	0.20	1.14	0.28
rs12794714	7 (0.38)	9 (0.5)	2 (0.11)	0.63	0.36	0.12	0.72

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RESEARCH ARTICLE

Interaction between *TCF7L2* polymorphism and dietary fat intake on high density lipoprotein cholesterol

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Abstract

Recent evidence suggests that lifestyle factors influence the association between the Melanocortin 4 receptor *(MC4R)* and Transcription Factor 7-Like 2 *(TCF7L2)* gene variants and cardio-metabolic traits in several populations; however, the available research is limited among the Asian Indian population. Hence, the present study examined whether the association between the *MC4R* single nucleotide polymorphism (SNP) (rs17782313) and two SNPs of the *TCF7L2* gene (rs12255372 and rs7903146) and cardio-metabolic traits is modified by dietary factors and physical activity. This cross sectional study included a random sample of normal glucose tolerant (NGT) (n = 821) and participants with type 2 diabetes (T2D) (n = 861) recruited from the urban part of the Chennai Urban Rural Epidemiology Study (CURES). A validated food frequency questionnaire (FFQ) was used for dietary assessment and self-reported physical activity measures were collected. The threshold for significance was set at P = 0.00023 based on Bonferroni correction for multiple testing [(0.05/210 (3 SNPs x 14 outcomes x 5 lifestyle factors)]. After Bonferroni correction, there was a significant interaction between the *TCF7L2* rs12255372 SNP and fat intake (g/day) ($P_{interaction}$ = 0.0001) on high-density lipoprotein cholesterol (HDL-C), where the 'T' allele carriers in the lowest tertile of total fat intake had higher HDL-C (P = 0.008) and those in the highest tertile (P = 0.017) had lower HDL-C compared to the GG homozygotes. In a secondary analysis of SNPs with the subtypes of fat, there was also a significant interaction between the SNP rs12255372 and polyunsaturated fatty acids (PUFA, g/day) ($P_{interaction}$ <0.0001) on HDL-C, where the minor allele carriers had higher HDL-C in the lowest PUFA tertile (P = 0.024) and those in the highest PUFA tertile had lower HDL-C (P = 0.028) than GG homozygotes. In addition, a significant interaction was also seen between *TCF7L2* SNP rs12255372 and fibre intake (g/day) on HDL-C ($P_{interaction}$<0.0001). None of the other interactions between the SNPs and

Abbreviations: *MC4R*, Melanocortin 4 receptor; *TCF7L2*, Transcription factor 7 like 2; CURES, Chennai Urban Rural Epidemiological Study; NGT, Normal glucose tolerance; T2D, Type 2 diabetes.

lifestyle factors were statistically significant after correction for multiple testing. Our findings indicate that the association between *TCF7L2* SNP rs12255372 and HDL-C may be modified by dietary fat intake in this Asian Indian population.

Introduction

Genetic variants, unhealthy dietary intake, physical inactivity and their multiple interactions are considered to be contributory factors to the development of obesity and type 2 diabetes (T2D) [1–3]. After China, India has the highest number of people with T2D in the world and according to the Indian Council of Medical Research–INdia DIABetes (ICMR–INDIAB) study, T2D cases have reached 62.4 million and 77.2 million people are pre-diabetic [4]. Furthermore, obesity and T2D are risk factors for non-communicable diseases (NCDs) such as cardiovascular disease (CVD) and it is estimated that India will have the highest rate of CVD mortality in the world [5]. Asian Indians have different biochemical characteristics from other populations from birth, often referred to as the 'Asian Indian Phenotype' which consists of increased visceral fat and waist circumference, hyperinsulinemia and insulin resistance [6].

The association between several genes and metabolic diseases has been identified by the candidate gene approach and genome-wide scans; to date, besides the *FTO* (Fat mass and obesity associated) gene, the strongest obesity risk loci known so far [1, 3, 7, 8], two commonly studied candidates for obesity and T2D have been the Melanocortin 4 Receptor (*MC4R)* and Transcription Factor 7-Like 2 (*TCF7L2*) genes. Strong association between the *MC4R* gene and risk of obesity was identified by a genome-wide association (GWAS) study [9] whereas the association between the Transcription Factor 7-Like 2 (*TCF7L2*) gene and risk of T2D was identified by a genome wide linkage study [10]. *MC4R* is expressed in the hypothalamus within the brain and therefore it is suggested that it contributes to body weight regulation by its effect on food intake and energy homeostasis [11]. A strong association was identified between *MC4R* rs17782313 genetic variant and risk of obesity in a European population [12] which was then replicated in other populations [13–15], including Asian Indians [16]. The *TCF7L2* gene is involved in the Wnt signalling pathway where it affects the expression of pro-glucagon and consequently blood glucose regulation [10]. In addition, the effect of pro-glucagon on Glucagon-like peptide 1 (GLP-1) also influences blood glucose regulation with insulin [17]. Decreased insulin secretion and increased glucose production in the liver is suggested to be the result of over expression of the *TCF7L2* gene [18]. A couple of studies [19, 20] have shown strong associations between the two *TCF7L2* single nucleotide polymorphisms (SNPs) (rs7903146, rs12255372) and risk of T2D among Asian Indians living in India, in addition to a meta-analysis [17].

In recent years, several studies have examined whether the association between the genetic variants of *MC4R* and *TCF7L2* genes and cardio-metabolic traits is modified by lifestyle factors such as diet and physical activity in various populations [21–25], however there are no studies to date among Asian Indians [26]. Whilst most studies in European populations found no significant interactions between the *MC4R* SNP rs17782313 and dietary factors on obesity traits [11, 22, 23], a prospective cohort study reported significant interactions between the SNP and fat and protein intake on body mass index (BMI) and risk of T2D [27]. Similarly some studies identified significant interactions between *TCF7L2* SNPs and fibre and fat intake on T2D[28–30]; however there were discrepancies between the studies which could be due to differences in sample size, study design, dietary assessment and genetic heterogeneity. The objectives of

this paper were to determine whether the *MC4R* SNP rs17782313 and *TCF7L2* SNPs (rs7903146, rs12255372) were associated with cardio-metabolic traits and whether the association was modified by diet and physical activity in a sample size of up to 1,682 adults from the Chennai Urban Rural Epidemiology Study (CURES).

Materials and methods

Study participants

A random, unrelated sample of normal glucose tolerant (NGT) (n = 821) and T2D (n = 861) participants were recruited from the urban part of the cross sectional Chennai Urban Rural Epidemiology Study (CURES) which included 26,001 individuals in total as a representative sample of Chennai city. Full details of the methodology have been explained previously [31]. Briefly 26,001 adult subjects (>20 years of age) were recruited in Phase 1 of CURES using a systematic random sampling method covering the whole Chennai city. This included 1,529 'self-reported' diabetic subjects. In Phase 2, all self-reported or 'known diabetic' subjects were invited to our centre for detailed studies of whom 1382 responded (response rate: 90.4%). In Phase 3, every tenth subject from Phase 1, excluding those with self-reported diabetes, underwent an oral glucose tolerance test (OGTT). Subjects who had fasting plasma glucose <5.6 mmol/l (100mg/dl) and 2 hr plasma glucose value 7.8 mmol/l (140mg/dl) were categorized as having NGT [32]. Those who were confirmed by OGTT to have 2 hr plasma glucose value 11.1 mmol/l (200 mg/dl) were classed as 'newly detected diabetic subjects' (n = 222).For the present study, the NGT subjects were selected from Phase 3 and subjects with T2D which included 'known diabetic' and 'newly detected diabetic' subjects were selected from Phase 2 and Phase 3 of the Chennai Urban Rural Epidemiology Study. The Madras Diabetes Research Foundation Institutional Ethics Committee granted the ethical approval and informed consent was obtained from the study participants.

Anthropometric and biochemical measurements

Weight, height and waist circumference were measured by standard methods. The BMI calculation was based on the body weight (kg) divided by the square of body height (m). Roche Diagnostics (Mannheim) provided the equipments in order to be able to carry out the biochemical analyses on a Hitachi-912 Auto Analyzer (Hitachi, Mannheim, Germany)(8). Fasting plasma glucose, serum cholesterol, serum triglycerides (TG) and high-density lipoprotein cholesterol were measured by glucose oxidase-peroxidase, cholesterol oxidase-phenol-4-amino-antipyrene peroxidase, glycerol phosphatase oxidase-phenol-4-amino-antipyrene peroxidise and polyethylene glycol-pretreated enzyme methods respectively(8). The Friedewald formula was used to estimate low-density lipoprotein cholesterol concentrations [33]. Glycated haemoglobin (HbA1c) and serum insulin were determined by high-performance liquid chromatography (HPLC) on a Variant instrument (Bio-Rad, Hercules, CA, USA) and an enzyme-linked immunosorbent assay (Dako, Glostrup, Denmark) respectively [8].

Assessment of dietary intake and physical activity

A validated, interviewer administered semi-quantitative food frequency questionnaire (FFQ) [34] consisting of a list of 222 different foods was used in order to evaluate dietary intake for the previous year including macronutrient and total energy intake. Frequencies (per day, week, month, year, never) and portion sizes were estimated by the participants with the help of visual aids of measurement equipments and food sizes. Daily average food and nutrient intake was calculated by the EpiNu database system.

A validated self-report questionnaire was used to measure physical activity [5]. Individuals were divided into the vigorously active group when they both exercised and engaged in demanding work activities whereas within the moderately active group the participants either exercised or carried out heavy physical work. The remainder of the study participants were separated into the sedentary group.

SNP selection and genotyping

A strong association was identified between *MC4R* rs17782313 genetic variant and risk of obesity in a European population [12] which was then replicated in other populations (13–15), including Asian Indians [16]. Strong associations have also been found between *TCF7L2* SNPs (rs12255372 and rs7903146) and risk of T2D in a meta-analysis [17] in the Japanese population [35] as well as in Asian Indians [19, 20]. Although the *TCF7L2* SNPs (rs12255372 and rs7903 146) have showed significant linkage disequilibrium ($r^2 = 0.746$), the rs7903146 variant has been shown to have the strongest effect in Caucasian populations [36, 37]. Based on the previous studies, the above mentioned three SNPs were selected for the present study.

Phenol-chloroform method was used to extract DNA from whole blood. The methodology for genotyping *TCF7L2* rs12255372 (G/T) and rs7903146 (C/T) SNPs has been previously published [20]. Direct sequencing by an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA) helped to confirm the efficiency of the genotyping which was in 99% concordance based on random duplicates of 20% of the samples [20].

MC4R rs17782313 (T/C) SNP: PCR volume (10 μl) consisted of 1X reaction buffer, 200 mmol of dNTP, 1.5 mmol of $MgCl_2$, 1 U taq DNA polymerase and 100 ng of genomic DNA. The concentrations for primers included 15 pmol of common primer, 15 pmol of allele 1 primer and 1 pmol of allele 2 primer, equal to a 15:1 ratio of short primers to long primer. Cycles for PCR were carried out at 96˚C for 12 min, then 35 cycles at 94˚C for 30 sec, followed by 30 sec at 57˚C, 30 sec at 72˚C and finally for 10 min at 72˚C. Electrophoresis was carried out with a 3% agarose gel.

Inner primers

F: `AAGTTTAAAGCAGGAGAGATTGTATACC (C allele 222bp)`

R: `GCTTTTCTTGTCATTTCCAGCA (T allele 149bp)`

Outer primer

F: `TTACTGATTTTAAGGGCATAAGCAA`

R: `TATCATGCTGAGACAGGTTCATAAA (321bp)`

Statistical analyses

Statistical analyses were carried out by using SPSS software (version 21). BMI $\geq$ 25 kg/m^2 was categorised as obese and BMI $<$ 25 kg/m^2 as non-obese. Descriptive statistics for continuous variables are shown as means and standard deviation (SD). Fasting serum insulin and triglyceride values were log transformed to obtain normal distribution. Genotype frequencies between cases and controls were compared by Chi Square test. The difference in the means of continuous variables between the genotypes was analysed by independent sample t test. Based on an additive model of analysis, dominant models were used for all 3 single nucleotide polymorphisms (SNPs) where the common homozygous allele was compared to the combined heterozygous and rare homozygous alleles due to the low allele frequency of rare homozygotes. Association analyses between SNPs and continuous and categorical variables were carried out by linear and logistic regression models, respectively, adjusting for age, gender with the addition of BMI, when T2D was the outcome, and, adjusting for T2D, when obesity was the outcome. Linear and logistic regression models were also used for interaction analyses between

SNPs and dietary factors (continuous variables) / physical activity (categorical variable) on continuous and categorical outcomes respectively, where the interaction terms were included into the models and were adjusted for age, gender, BMI, T2D and total energy intake when appropriate. Bonferroni correction for multiple testing was calculated by multiplying 3 SNPs with 14 outcomes (T2D, obesity, BMI, waist circumference, fasting blood glucose, HbA1c, fasting insulin, systolic and diastolic blood pressure, HDL, LDL, VLDL, TG and total cholesterol) and 4 dietary factors (carbohydrate, protein, fat, fibre) and physical activity level. The P value of 0.05 was then divided by 210 (3 SNPs x 14 outcomes x 5 lifestyle factors) which set the significant p value for all results at P = 0.00023.

As a secondary analysis, given the significant SNP-fat intake interaction, individuals were grouped into tertiles based on the fatty acid subtypes [monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids] for testing the interaction between SNPs and these fatty acid subtypes on lipids. In addition, PUFA was further stratified into ALA (alpha linolenic acid) and LA (linoleic acid) for the interaction analysis.

Given that there are no previously reported effect sizes for the gene-diet interaction, we were unable to perform a prospective power calculation. However, based on the most significant interaction observed in the present study, we performed retrospective power calculations using QUANTO software, Version 1.2.4 (May 2009). We performed power calculations in the form of least detectable effects based on the assumption of significance levels and powers of 5 and 80%, respectively. At 80% power, the minimum detectable effects ranged from beta 0.02 mg/dl (HDL-C) for a SNP with MAF of 5% to beta 1.0 mg/dl for a SNP with MAF 50% in the case-control analysis. For the *TCF7L2* SNP–fat intake interaction on HDL-C (most significant interaction), the beta was 0.067, which is within the range of effect sizes for which the power was calculated.

Results

Phenotypic associations

Based on the clinical and biochemical characteristics of the individuals from the CURES study as illustrated in **Table 1,** individuals with T2D were older (P<0.0001), had higher BMI (P<0.0001), waist circumference (P<0.0001), fasting plasma glucose (P<0.0001), HbA1c (P<0.0001), fasting plasma insulin (P<0.0001), systolic and diastolic blood pressure (P<0.0001), low density and very low density lipoproteins (P<0.0001), total cholesterol (P<0.0001), TG (P<0.0001) and lower high density lipoprotein (P = 0.001) than NGT individuals.

Genetic associations

The minor allele ('T') of SNPs (rs12255372 and rs7903146) of *TCF7L2* gene showed significant susceptibility to T2D; however, in the present study after correction for multiple testing only the association between SNP rs7903146 and T2D remained statistically significant (P = 0.0001) (**S1 Table**).After Bonferroni correction, statistically significant association between the 'C' allele of the*MC4R* SNP rs17782313 and T2D was also observed (P = 0.00022) (**S2 Table**). None of the associations between the three SNPs and continuous variables remained significant after correction for multiple testing (P>0.00023) (**S1 and S2 Tables**).

TCF7L2 –dietary fat intake interactions on HDL-C

Individuals were grouped into tertiles based on their fat and subtypes of fat intake (g/day). The means for fat intake in the 1st tertile: 41 g/d; 2nd tertile: 62 g/d; 3rd tertile: 95 g/d. Means for

Table 1. Anthropometric and biochemical characteristics of T2D and NGT participants.

	NGT	T2D	P value*
N	821	861	
(men/women)	(345/476)	(398/463)	0.08
Age (yrs)	41.31±11.73	50.57±10.49	<0.0001
BMI (kg/m^2)	23.66±4.69	25.34±4.30	<0.0001
WC (cm)	83.55±11.66	90.60±9.84	<0.0001
FPG (mg/dl)	84.82±8.36	161.73±69.13	<0.0001
HbA1c (%)	5.58±0.48	8.78±2.36	<0.0001
INS (μIU/ml)	8.41±5.81	11.78±7.69	<0.0001
Log INS (μIU/ml)	6.85±1.87	9.68±1.90	<0.0001
Systolic BP (mmHg)	117.72±18.05	129.16±21.65	<0.0001
Diastolic BP (mmHg)	74.06±11.32	76.76±11.85	<0.0001
HDL (mg/dl)	43.36±9.91	41.71±9.50	0.001
LDL (mg/dl)	113.13±30.55	124.49±35.38	<0.0001
VLDL (mg/dl)	23.95±14.30	35.96±27.37	<0.0001
TC (mg/dl)	180.02±36.02	199.51±42.40	<0.0001
TG (mg/dl)	120.18±71.46	180.31±137.12	<0.0001
Log TG (mg/dl)	105.68±1.62	152.75±1.71	<0.0001
Total energy (Kcal/day)	2622.43±702.77	2468±893.75	<0.0001
Total Carbohydrate (g)	419.69±112.33	399.96±147.98	0.002
Fat (g)	68.57±24.29	64.25±27.31	0.001
Total PUFA (g)	18.22±9.09	18.75±10.04	0.25
Total MUFA (g)	20.51±7.77	18.79±8.37	<0.0001
Protein (g)	73.82±20.97	69.93±24.56	0.001
Dietary fibre (g)	31.41±9.83	31.66±12.19	0.64

Data presented as Mean±SD.

*P values are showing the differences in mean values between NGT and T2D participants.

Abbreviations: NGT, normal glucose tolerance; BMI, Body mass index; WC, waist circumference; HbA1C, glycated haemoglobin; FPG, Fasting plasma glucose; INS, Fasting plasma insulin; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; TC, Total Cholesterol; TG, triglycerides.

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PUFA intake in the 1st tertile: 9g/d; 2nd tertile: 17 g/d; 3rd tertile: 29 g/d. Means for MUFA intake in the 1st tertile: 12 g/d; 2nd tertile: 18 g/d; 3rd tertile: 29 g/d. Means for ALA intake in the 1st tertile: 0.38 g/d; 2nd tertile: 0.58 g/d; 3rd tertile: 0.89 g/d. Means for LA intake in the 1st tertile: 8 g/d; 2nd tertile: 17 g/d; 3rd tertile: 29 g/d.

The interaction between the *TCF7L2* SNP rs12255372 and fat intake (g/day) on HDL-C was statistically significant after correction for multiple testing ($P_{interaction}$ = 0.0001). The 'T' allele carriers of the *TCF7L2* SNP rs12255372 had 2.26 mg/dl higher HDL-C level in the lowest tertile of fat intake (mean: 41 g/day) than the 'GG' homozygotes (P = 0.008) and in the highest tertile of fat intake (mean: 95 g/day), HDL-C was 1.87 mg/dl lower in the risk 'T' allele carriers in comparison to the 'GG' homozygotes (p = 0.017) (Fig 1).

Stratification to fat subgroups showed significant interactions between the SNP rs12255372 and PUFA (g/day) on HDL-C ($P_{interaction}$<0.0001), where the 'T' allele carriers had 1.96 mg/dl higher HDL-C (P = 0.024) in the low PUFA tertile (mean: 9 g/day) in comparison to the 'GG' homozygotes and in the 3rd tertile (mean: 29 g/day), the HDL-C level of the 'T' allele carriers was 1.64 mg/dl lower than the 'GG' homozygotes (P = 0.028) (Fig 1). A similar interaction was also found between the SNP rs12255372 and MUFA (g/day) on HDL-C ($P_{interaction}$ = 0.0003),

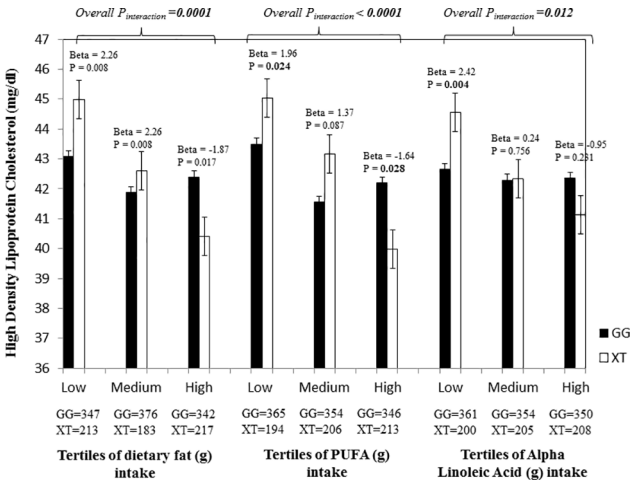

Fig 1. Interaction of the *TCF7L2* gene polymorphism (rs12255372) with fat (g) intake, PUFA intake and Alpha Linolenic Acid (g) intake on HDL-C. Individuals carrying the 'XT' genotype had 2.26 mg/dl higher HDL-C in the lowest fat tertile (P = 0.008), while those in the highest tertile had 1.87 mg/dl lower HDL-C (P = 0.017) than those who carry the 'GG' allele. Carriers of the 'XT' genotype had 1.96 mg/dl higher HDL-C in the 1st tertile of PUFA intake (g) (P = 0.024), while those in the 3rd tertile had 1.64 mg/dl lower HDL-C in comparison to the carriers of the 'GG' genotype (P = 0.028). In the 1st tertile of Alpha Linolenic acid intake (g), individuals with the 'XT' genotype had 2.42 mg/dl higher HDL-C than the 'GG' homozygotes (P = 0.004).

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where the 'T' allele carriers had 1.77 (mg/dl) higher HDL-C in the lowest MUFA tertile (mean: 12 g/day) (P = 0.03) and had 1.61 (mg/dl) higher HDL-C in the 2nd tertile (mean: 18 g/day) (P = 0.045) than the 'GG' carriers, however in the highest MUFA tertile (mean: 29 g/day) the 'T' allele carriers had 1.59 (mg/dl) decreased HDL-C (P = 0.041) than individuals with the 'GG' genotype.

PUFA was further stratified to linoleic acid (LA) and alpha linolenic acid (ALA) to investigate whether omega-3 and omega-6 fatty acids modified the association between the *TCF7L2* SNP rs12255372 and HDL-C. Significant interaction was found between the SNP and ALA on HDL-C ($P_{interaction}$ = 0.012), where the 'T' allele carriers had 2.42 (mg/dl) higher HDL-C than the 'GG' homozygotes (P = 0.004) in the lowest tertile (mean: 0.38 g/day) (Fig 1).A similar interaction was also found between the SNP rs12255372 and LA (g/day) on HDL-C ($P_{interaction}$<0.0001). S1 Table shows the interactions of *TCF7L2* SNP rs12255372 with fat, PUFA and ALA intakes on HDL-C under an additive and dominant model.

Additional gene-diet interactions

In addition to the main significant findings, there was a significant interaction between *TCF7L2* SNP rs12255372 and fibre intake (g/day) on HDL-C ($P_{interaction}$< 0.0001), where in the lowest tertile (mean: 20 g/day) individuals carrying the 'T' allele had 1.95 (mg/dl) higher HDL-C (P = 0.02), in the 2nd tertile (mean: 30 g/day), the 'T' allele carriers had 2.39 (mg/dl) higher HDL-C (P = 0.003) and in the highest tertile (mean: 44 g/day), the HDL-C level of the 'T' allele carriers was 2.37 mg/dl lower in comparison to the 'GG' homozygotes (P = 0.002). There were several other interactions which did not reach statistical significance after correction for multiple testing and these interactions are shown in the Table 2 and S2 **and** S3 **Tables**).

Gene-physical activity interactions on cardio-metabolic traits

No statistically significant interactions were observed after correction for multiple testing between the three SNPs and physical activity on obesity- and T2D- related traits ($P_{interaction}$>0.0002).

Table 2. Interactions of *TCF7L2* SNPs rs12255372 and rs7903146 with carbohydrate, fibre and protein intake on HDL-C, fasting blood glucose and diastolic blood pressure.

rs12255372	**SNP*Carbohydrate (g)**	**SNP*Fibre (g)**	**SNP*Protein (g)**	**SNP*Fat (g)**
β ± SE** (P for interaction on FPG)	0.039 ± 0.019 (0.041)	0.437 ± 0.222 (0.049)	0.132 ± 0.108 (0.222)	0.119 ± 0.095 (0.210)
β ± SE** (P for interaction on HDL-C)	0.009 ± 0.003 (0.007)	0.168 ± 0.041 (<0.0001)	0.072 ± 0.020 (0.0003)	0.067 ± 0.017 (0.00017)
β ± SE** (P for interaction on DBP)	-0.003 ± 0.004 (0.551)	-0.107 ± 0.050 (0.033)	-0.028 ± 0.024 (0.251)	-0.032 ± 0.021 (0.130)
rs7903146	**SNP*Carbohydrate (g)**	**SNP*Fibre (g)**	**SNP*Protein (g)**	**SNP*Fat (g)**
β ± SE** (P¥ for interaction on T2D)	0.002 ± 0.001 (0.011)	0.014 ± 0.010 (0.180)	0.011 ± 0.005 (0.024)	0.008 ± 0.004 (0.071)
β ± SE** (P for interaction on HDL-C)	0.006 ± 0.003 (0.057)	0.128 ± 0.040 (0.002)	0.050 ± 0.020 (0.010)	0.052 ± 0.017 (0.003)

*Interaction term

**P values are adjusted for age, gender, BMI, T2D and Total energy intake.

¥ P values are adjusted for age, gender, BMI and Total energy intake.

Abbreviations: SNP, Single nucleotide polymorphism; HDL-C, High density lipoprotein cholesterol; FPG, Fasting plasma glucose; DBP, Diastolic blood pressure.

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Interactions observed between *TCF7L2* SNP rs7903146 and physical activity on VLDL ($P_{interaction}$ = 0.012) and TG ($P_{interaction}$ = 0.014), where the risk 'T' allele carriers had higher TG and VLDL levels, did not reach statistical significance after correction for multiple testing (**S4 Table**).

Discussion

This is the first study to investigate interactions between *TCF7L2* and *MC4R* SNPs and lifestyle factors on cardio-metabolic traits among Asian Indians. The main findings suggest that total fat and PUFA intakes may modify the association between the *TCF7L2* SNP rs12255372 and HDL-C. In the lowest total fat and PUFA tertiles, participants carrying the 'XT' genotype (GT +TT) had significantly higher HDL-C, whereas in the highest tertile participants carrying the 'XT' genotype had lower HDL-C, as compared to those with the GG genotype. This finding is of public health significance given that Asian Indians tend to have low HDL-C which puts them at markedly increased risk for CVD [38, 39].

The fat intake consists of invisible, hidden and visible fat (vegetable oils, ghee, butter) in India where in the urban areas the minimum average daily intake of visible fat is approximately 22–45 g/day [40]. The recommended omega 3 PUFA intake is 0.1 gram/day, however the median of combined intake of LA and ALA is 13 g/day and the recommended ratio between LA and ALA is 5:1 to 10:1 [40]. In comparison, the results of our study suggest that total fat intake below 53 g/day, PUFA intake below 13 g/day, and ALA intake below 0.49 g/day may help maintain high levels of HDL-C in the 'T' allele carriers of the *TCF7L2* SNP rs1225 5372. The findings that MUFA intake above 22 g/day reduces HDL-C and only ALA intake below 0.5 g/day maintains high HDL-C level in risk carriers are unexpected results; however, the effect of polygenic traits cannot be ruled out.

HDL-C is generally considered to be protective against CVD due to its role in the reverse cholesterol transport; however, the recent Mendelian randomization (MR) studies [41, 42] have failed to show a causal effect of low HDL-C on cardiovascular disease risk. But, none of these MR studies have taken dietary factors into account. Furthermore, fatty acids have shown to have different modulating effect on HDL-C for which the mechanism is not fully understood [43]. On the other hand, it is argued that the total fat intake is more influential on postprandial lipoprotein abnormalities, which is a characteristic of T2D, than the type of fatty acids [44]. However, studies have also shown that different fatty acids can have differential effects on postprandial TG [45–47]. In our study, we found that those in the lowest fat intake

subgroups had higher HDL-C while those in the highest tertile groups had lower HDL-C among the risk allele carriers. A previous intervention study in the US population [30] also found significant interaction between SNP rs12255372 and fat intake where positive changes in body composition were observed in the 'T' risk allele carriers only on the low-fat diet (20% from total energy). Other studies only reported significant interactions between SNP rs7903146 and high saturated fat intake on increased metabolic syndrome risk [29] and high (n-6) PUFA intake (≥6.62% of energy intake) on increased VLDL and TG [24] among the risk 'T' allele carriers.

High omega 6 to omega 3 ratio has been shown to increase HDL in mice [43], whereas the results from this study in humans contradict those findings. It has been shown in cell and animal studies that high omega 6 PUFA intake is pro-inflammatory leading to an increased risk for CVD and diabetes [48] and in the present study reduced HDL was observed in the minor allele carriers in response to high intake of omega 6 PUFA (>21.6 g/d). According to our findings, a low omega 6 (<12.8 g/d) and a low ALA intake (<0.5 g/d) may help maintain HDL above 44 (mg/dl) in the risk allele carriers.

Though there was no significant difference in mean HDL levels in the different tertiles of total fat and PUFA intakes in the study participants (data not shown), in the presence of *TCF7L2* rs12255372 genotype, a clear interaction between the genotype and fat/ PUFA intake on HDL-C was observed. While the carriers of 'XT' genotype had increased HDL-C in the presence of a low fat/ low PUFA diet, there was a decrease in HDL-C levels in the carriers of 'XT' genotype in the presence of a high fat / high PUFA diet. It is to be noted that among those who carry the GG genotype, irrespective of the dietary fat/PUFA intake, there is no effect on HDL-C levels.

Another interesting interaction was the one between *TCF7L2* SNP rs12255372 and fibre intake on HDL-C. The results suggest that low and medium fibre intake (means: 20, 30 g/day), respectively, increase HDL-C whereas high fibre intake (mean: 44 g/day) may reduce HDL-C among risk allele carriers of the *TCF7L2* SNP rs12255372. The average fibre intake in India is 30–40 g/day [40] which is consistent with the mean intake in the medium and high tertiles and also higher compared to the mean fibre intake in the UK (~18g/day) and the US (~16g/day). A previous study investigated interaction between SNP rs7903146 and fibre intake on T2D risk [28],where the minor allele carriers had increased T2D risk with higher fibre intake (mean intake: 13.1 ± 2.2 g/4,184 kJ). High fibre intake is generally recommended to improve glycemic control in T2D individuals [49] and to reduce total and LDL-C in order to reduce CVD risk. A meta-analysis of data from clinical studies (n = 2,990) [50] indicated that high fibre diets (20–30 g/day) reduced HDL-C. Similarly, another meta-analysis of data from 24 clinical studies also suggested that medium and high carbohydrate, high fibre (≥20 g/day) diets also decreased HDL by 4% [51].However, it was argued, that the decrease in LDL-C and TG values would reduce CVD risk by 16.4% which would outweigh the increased risk of CVD by 11.9% due to decreased HDL [51]. But it is of note that these meta-analyses did not consider the genetic component and, furthermore, we also observed gene-diet interaction on other lipid outcomes such as VLDL and TG, which did not remain significant after correction for multiple testing.

The risk of coronary artery disease is increased in individuals with circulating HDL-C concentration of <40 mg/dl [52]. In our study, within the high fibre intake tertile (≥35g/day), the mean HDL-C level of the minor risk allele carriers was 39.46 mg/dL in comparison to 42.27 mg/dl of the 'GG' carriers of the *TCF7L2* SNP rs12255372. The results of our study suggest that high fibre intake (35–102 g/day) may help maintain HDL-C level above 40 mg/dl whereas below 35 g/day may lower HDL-C in the 'GG' genotype carriers of SNP rs12255372. Mechanisms on how low/high fibre intake decreases/increases HDL-C are not well understood and more research is needed to clarify the effects of dietary fibre on HDL-C metabolism [52, 53].

No studies, to date, have investigated interactions between the *TCF7L2* SNPs (rs12255372 and rs7903146) and *MC4R* SNP rs17782313 and physical activity on cardio-metabolic traits in Asian Indians. Despite the fact that the majority of people are physically inactive in India [54], no significant interactions were found after correction for multiple testing between the three polymorphisms and physical activity on cardio-metabolic traits, which could be due to a small sample size and measurement bias associated with self-reported physical activity questionnaire. However, our finding is in support of the previous study in a Spanish population [21] with a much larger sample size (n = 7,052) which also did not find a significant interaction between *MC4R* SNP rs17782313 and physical activity on obesity traits. Though the inclusion of a representative sample of Chennai for analysis and the use of a comprehensive, validated, interviewer administered semi-quantitative FFQ for dietary assessment could be considered the strengths of this study, there are some underlying limitations. The data used to calculate the measures of dietary intake and physical activity came from self-report and hence, measurement bias associated with self-reported questionnaire cannot be ruled out. Given that obesity and diabetes are multifactorial traits, several genetic and lifestyle factors are likely to contribute to the disease. While 97 loci have been shown to be associated with body weight [55], the present study examined only three common variants, given their consistent associations with obesity and diabetes, respectively, in Europeans and Asian Indians. The cross sectional study design gives only a snapshot of the prevalence and cause—effect cannot be established due to lack of follow up which is another limitation of this study. However, several outcomes and risk factors were assessed and there was no loss to follow up.

In conclusion, this study has found significant interactions between the *TCF7L2* SNP rs12255372 and dietary factors on HDL-C in this Asian Indian population. The results of this study indicate that high total fat and PUFA intakes may be associated with lower HDL-C whereas low intake is associated with higher HDL among the risk allele carriers. More research is required to better understand the interactions between the *TCF7L2* gene variant and lifestyle factors on cardio-metabolic traits. Exact mechanisms identifying the effect of different fatty acids on HDL-C and whether/how high fat- and PUFA- intake may reduce HDL-C should also be established before public health recommendations and personalised nutrition advice can be developed for this Asian Indian population in order to reduce the burden of cardiometabolic diseases.

Supporting information

S1 Table. Interactions of *TCF7L2* SNPs rs12255372 with fat, PUFA and ALA intakes on HDL-C under an additive and a dominant model of analysis.
(DOCX)

S2 Table. Associations between *TCF7L2* SNPs rs12255372, rs7903146 and obesity, T2D and related traits.
(DOCX)

S3 Table. Association and interaction between *MC4R* SNP rs17782313 and obesity, T2D and related traits.
(DOCX)

S4 Table. Interactions between *TCF7L2* SNP rs7903146 and physical activity on VLDL and TG.
(DOCX)

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