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TITLE: An investigation of genetic polymorphism in association with Type 2 diabetes and metabolic syndrome.

YEAR: 2018

This work was submitted to- and approved by Robert Gordon University in partial fulfilment of the following degree:
Doctor of Philosophy, School of Pharmacy and Life Sciences.

An investigation of genetic polymorphism in association with Type 2 diabetes and metabolic syndrome.

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A thesis submitted in partial fulfilment of the

requirements of the

Robert Gordon University

for the degree of Doctor of Philosophy

May 2018

Declaration

I, Prabhakar Bhatta, hereby declare that the thesis entitled “An investigation of genetic polymorphism in association with Type 2 diabetes and metabolic syndrome” is my own original research. I confirm that this thesis has not been submitted at this University or any other institution to claim any other qualification. To the best of my knowledge, whenever required, I have cited properly the source from the Journals, books or any other unpublished articles.

Prabhakar Bhatta

Abstract

Type 2 diabetes and metabolic syndrome are the metabolic disorders which constitute a major public health problem in both developed and developing countries. Various studies have suggested the genetic susceptibility to the disorders. The main aim of the thesis was to investigate the putative association of single nucleotide polymorphisms with Type 2 diabetes (T2D), metabolic syndrome (MetS) and the major components of metabolic syndrome. This study used meta-analysis, polymerase chain reaction (PCR) based restriction fragment length polymorphism (RFLP) and Sanger sequencing methods to analyse the results. The single nucleotide polymorphism rs57829442 of peroxisome proliferator-activated receptor-γ coactivator-1 (PPARGC1A) gene and its relation to risk of type 2 diabetes has been studied in the United Kingdom population. A meta-analysis of genetic variant rs8192678 (Gly482Ser) of peroxisome proliferator-activated receptor-γ coactivator-1 (PPARGC1A) gene and its association with the components of metabolic syndrome has been studied. An association of the genetic variants rs8192678 (Gly482Ser) of the PPARGC1A gene, rs7903146 of Transcription Factor 7 Like 2 (TCF7L2) gene, rs9939609 of Fat mass and obesity-associated (FTO) gene and rs1801282 (Pro12Ala) of peroxisome proliferator-activated receptor gamma (PPARG) gene with the metabolic syndrome and its components has been studied in the Nepalese population.

The results showed that variant rs57829442 of PPARGC1A is not associated with T2D in the United Kingdom population. Further investigation with increased sample size is warranted. In the meta-analysis, the variant rs8192678 (Gly482Ser) of PPARGC1A gene was found to be significantly associated with body mass index (BMI) in Asian populations under dominant genetic model, total cholesterol (TC) in non-Asian population under recessive genetic model and with fasting plasma glucose (FPG) under a recessive model in overall and non-Asian populations. No significant association of the variants rs8192678 (Gly482Ser), rs7903146, rs9939609 and rs1801282 (Pro12 Ala) was found associated with MetS under dominant, recessive, co-dominant and additive models in the Nepalese population. However, the genotypes (AG and AA) of rs8192678 (Gly482Ser) had a statistically significant protective

effect on systolic blood pressure. The genotypes with the risk allele of rs9930609 of FTO gene was significantly associated with weight, waist circumference and diastolic blood pressure under dominant genetic model and with BMI under both dominant and recessive genetic models in the Nepalese population. To the best of our knowledge, this is the first study to report the findings in the Nepalese population.

Keywords: Type 2 diabetes, metabolic syndrome, single nucleotide polymorphism, PPARGC1A, PPARG, TCF7L2, FTO.

Acknowledgements

Fisrt of all, I would like to thank to my family members who supported and encouraged me throughout the research journey. I owe my deepest gratitute to my supervisor Dr. Rachel M Knott who constantly supported and guided me throughout the project. I would also like to express my sincere gratitute to my supervisors Dr. Giovanna Bermano and Dr Hector Williams for their support and guidance.

I would like to thank Nepal Health and Educational trust for the support and help throughout the project. I would like to thank Nepal Health and Research Council for giving opportunity to carry out the project in Nepal. I would like to thank all the participants who took part in the research project. I am grateful to all the facilitators who tirelessly helped during the research period in Nepal. I would like to thank Mr. Balram Gautam and Mr. Sunil Lekhak from Decode Genomics and Reseach Center for providing research facility.

A huge thanks to Xcelris labs for providing training and help sequencing the samples. A special thanks to Dr. Spandan Chaudhary, Dr.Prashant Bagali and Mr. Deepak Pathak for their assistance and guidance.

I would also like to thank to the Head master of J.K higher secondary school of Attaria, Kailali for providing the school clinic for my research project. A special thanks to Dr. Shiv Raj Paneru who helped drawing blood samples from the participants. My huge thanks to Andrea MacMillan from Robert Gordon University for her readiness to help at any time.

I would also like to thank all the friends and staffs of Robert Gordon University.

List of publications

Conference and proceedings:

Bhatta P, Bermano G, Williams H, Knott RM. (2014) Genetic polymorphism in Peroxisome proliferator-activated receptor-γ coactivator-1 (PPARGC1A) gene and its relation to risk of type 2 diabetes. A poster presented at a Scottish Cardiovascular forum held on February 22, 2014 in Aberdeen, UK.

Bhatta P, Bermano G, Williams H, Knott RM. (2016) Genetic polymorphism in Peroxisome proliferator-activated receptor-γ coactivator-1 (PPARGC1A) gene and its relation to risk of type 2 diabetes. A poster presented at the 13th International conference in Diabetes and Medicare Expo held during August 8-10, 2016 in Birmingham, UK.

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Abbreviations

BAT	Brown adipose tissue
BMI	Body mass index
CDKN2B	Cyclin-dependent kinase 4 inhibitor B
DBP	Diastolic blood pressure
df	Degree of freedom
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EGSIR	European Group for the study of Insulin Resistance
EXT2	Exostosin-2
FTO	Fat mass and obesity-associated protein
GCK	Glucokinase
GWAS	Genome wide association study
HC	Hip circumference
HDL	High-density lipoprotein
HHEX	Hematopoietically-expressed homeobox protein
HNF1A	Hepatocyte nuclear factor-1A
HRC	Human random control
HSF	Human splice finder
IDF	International Diabetes Federation
IGF2BP2	Insulin-like growth factor 2 mRNA-binding protein 2
LDL	Low density lipoprotein
MAF	Minor allele frequency
MetS	Metabolic syndrome
$MgCl_2$	Magnesium chloride
NCEP ATP III	National Cholesterol Education Programme, Adult Treatment Panel III
PCR	Polymerase chain reaction
PPARG	Peroxisome proliferator-activated receptor gamma
PPARGC1A	Peroxisome proliferator-activated receptor-γ coactivator-1apha
PPRE	PPAR responsive element
RFLP	Restriction fragment length polymorphism
SBP	Systolic blood pressure
SLC30A8	Solute carrier family 30 (zinc transporter), member 8
SNP	Single nucleotide polymorphism
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TC	Total cholesterol
TCF7L2	Transcription factor 7 like 2
TG	Triglyceride
VLDL	Very low-density lipoprotein
WC	Waist circumference
WHO	World health organization

Chapter 1

1.0 General Introduction:

1.1 Diabetes mellitus

Diabetes is a serious global public health problem and the prevalence of which is increasing at an alarming rate (Hex et al, 2012). Diabetes mellitus is a metabolic disorder characterized by hyperglycaemia where the body either fails to produce or utilize the hormone called insulin. According to International Diabetes Federation (2017) 38 % of people are living with undiagnosed diabetes (http://www.diabetesatlas.org). Olokoba et al (2012) noted that around 80% of adults with diabetes are living in the developing countries. Diabetes, both Type 1 and Type 2, contributes a considerable burden to the economy in the United Kingdom and the complication of the disease constitute most of the cost (Hex et al, 2012). Despite the continued effort to prevent the disease or to find a cure, the prevalence of which had continued to increase over the years.

World Health Organization (2016) reported the global prevalence of diabetes as 8.5% and the figure has doubled since 1980. The data also suggest that 442 million adults had diabetes in 2014 compared to 108 million in 1980 (WHO, 2016).

1.2 Different types of Diabetes

There are basically three types of diabetes, namely: Type 1 diabetes, Type 2 diabetes (T2D), and gestational diabetes (GD). Type 1 diabetes, previously known as juvenile diabetes, is an autoimmune condition where the body's own antibody attacks insulin producing cells. T2D is usually an adult onset diabetes and which is also known as non-insulin-dependent diabetes where the body does not produce enough insulin or the body is resistant to the insulin produced (Hex et al, 2012). Lastly, GD is manifested in females during pregnancy (Koivusalo et al., 2016). It is usually diagnosed during late or middle pregnancy and the condition is manifested when the demand of the insulin is not sufficiently supplied by the pancreatic beta cells (Buchanan et al, 2012). The women with GD have a sevenfold increased risk going on to T2D (Bellamy et al, 2009).

1.3 Complications Associated with Diabetes:

Diabetes is associated with a broad range of complications in various parts of the body. The complication due to diabetes could be classified as either short-term or long-term complications (Diabetes UK, 2017). Short term complication due to the condition is hypoglycemia diabetic ketoacidosis (DKA) and hyperosmolar hyperglycaemic state (HHS) (Diabetes UK, 2017). The long-term complications of the condition (both T1D and T2D) are: diabetic retinopathy, neuropathy, nephropathy and cardiovascular disease.

1.4 Causes of Diabetes

Type 1 diabetes (T1D) is an autoimmune disease in which the immune cells, especially T lymphocytes destroy beta cells. T1D represents 5-10% of diabetes and also known as juvenile diabetes (American Diabetes Association, 2014). The autoimmunity might be due to the complex interplay between genetic and the environmental factors. Epidemiological studies suggest that entero viral infection might trigger the onset of T1D and an earlier case study showed that an entero virus infection to the mother can cause neo-natal beta-cell autoimmunity (Otonkoshi et al, 2000). Studies suggest that there might be a causal relationship between vitamin D deficiency and T1D, which is substantiated by the fact that supplementation of vitamin D during pregnancy significantly decreased the risk of type 1 diabetes (Stene et al, 2000). It has also been reported that cow milk protein might be associated with T1D (Virtanen et al, 1993); however, further validation in required.
T1D is a multigenic disorder where, around 40 susceptibility loci have been identified so far (Concannin et al, 2009) and the genetic loci on chromosome 6, the HLA, accounts for 50 percent of the genetic susceptibility (Noble et al, 2010). Studies suggest that HLA-DR and HLA-DQ gene of HLA class II accounts for most of the genetic risk for T1D (Concannin et al, 2009). Besides HLA, most of the candidate loci for T1D suggests a defect in the immune response (Concannin et al, 2009).

In T2D, various factors are involved in the manifestation of the condition. The exact cause of the disease in not yet known; however, the following factors could be considered as a risk factor for it, such as age, insulin resistance, obesity, family history, ethnicity, physical

activity, lipid profiles, genetic and medical conditions (high blood pressure, heart disease, stroke). There is a subtle interplay between the environment and the genetic factors for the onset of the condition.

1.5 Prevalence of Type 2 Diabetes

Type 2 diabetes, predominantly contributes around 85-90 % of total diabetes cases around the globe (Hex et al, 2012). The global prevalence of T2D is 8.3%. In European populations, the highest prevalence of T2D was found in Germany (8.9%) followed by, Spain (8.1), France (6.4%) and United Kingdom (6.1%). According to public health England, 2016, 3.8 million people in England have diabetes and 90% of the cases were T2D. Public health England also noted that diabetes was more common in South Asian and African ethnic groups when compared with Caucasians and mixed ethnic groups (https://www.gov.uk/government). T2D is responsible for 5 million deaths every year globally and it has been projected to be the 7th leading cause of death in 2030 (https://www.diapedia.org).

Table 1.01. Global prevalence of Type 2 diabetes.

Region	Prevalence rate (%)
Global	8.3%
European	
United Kingdom	6.1%
France	6.4%
Italy	4.8%
Germany	8.9%
Spain	8.1%

Source: Kanavos et al, 2012

1.6 Risk Factors for Type 2 Diabetes

Much research has been conducted to find the risk factors associated with T2D. Obesity is one of major risk factors associated with T2D. Obese individuals are at a higher risk of developing Type 2 diabetes since they may also suffer from insulin resistance. It has been shown that there is a strong positive correlation between obesity and insulin sensitivity (Kahn et al, 2000). The findings also confirmed that blood pressure, triglycerides and total cholesterol were higher among diabetic participants than in healthy participants. Other studies (Barroso et al, 2005; Damasceno et al, 2013 and Wu et al, 2014) also showed similar results, where obesity, blood pressure, sedentary lifestyle, and age were the main risk factors associated with Type 2 diabetes.

1.7 Genetic Basis of Type 2 Diabetes

1.7.1 Heritability of T2D

According to Wu et al, 2014, T2D has a major genetic component that makes it hereditary. Wu et al, 2014, reported higher concordance rate of Type 2 diabetes among monozygotic (96%) than dizygotic twins. Also, 40% of first-degree relatives of Type 2 diabetes might end up developing diabetes, whereas the incidence rate stands at 6% in the general population. In an earlier study, Barroso et al, 2005, reported that the lifetime risk of developing Type 2 diabetes is 40% when only one parent had diabetes and 70% in case both parents had Type 2 diabetes. According to Barroso et al, 2005, the heredity of Type 2 diabetes is estimated to be between 20% and 80%. Therefore, the emerging evidence from various researchers confirms that Type 2 diabetes is hereditary.

1.8 Approaches to identify genetic susceptibility

1.8.1. Linkage analysis

Linkage analysis describes the way in which the genes or the markers (disease causing) close to one another tends to inherit together. Linkage analysis is a family based study where the chromosomal loci associated with the disease are transmitted to off springs more frequently than expected by chance. The genetic material between two homologous chromosomes is exchanged during meiosis and the recombinant and non-recombinant genetic material is equal in number. The chromosomal loci that are near tend to be transmitted together into the daughter cells. The loci that are in close proximity distorts the 1:1 ratio of recombinant and non-recombinant in the off springs. The proportions of recombinants between two loci are usually known as recombinant fractional and are denoted by theta (θ). It can also be interpreted as the probability of having an odd number of crossovers between two loci. Linkage analysis is a method to find the location of the marker or loci in the chromosomes that predispose to the disease in question. It assumes that: chromosome segments are inheritable; co-segregation is a result of the linked loci and the recombination event depends upon the distance between two loci. There are two methods of linkage analysis: Model-based and model free. In the model based method, the disease, genetic model is specified and recombination fraction (θ) is calculated. In model free method no known genetic model is specified and it usually relies upon the identity-by-descent between the relatives.

1.8.2 Candidate gene association studies

Candidate gene association studies are commonly used for the genetic susceptibility of complex diseases where multiple factors are associated with the condition. The variants of the candidate genes are selected based on prior knowledge of functional significance. The study is conducted in a form of case (presence of a disease in question) and control (absence of a disease in question) group. The allele frequency or the genotype frequency in the case group are compared with that of the control group and any association is analysed. Association studies are simple in design and relatively easier to execute, however; if enough

care is not taken, it might lead to erroneous results. In case-control association studies, inadequate sample size and population stratification could lead to misleading results. Population stratification might be difficult to avoid in cases where the distinct ethnicity is not known or even in the case of population admixture, however; this could be circumvented to some extent by statistical adjustment.

1.9 Candidate gene association studies and type 2 diabetes.

Except the autoimmune diabetes, very few mutations in the single gene follow the inheritance pattern according to Mendel's law. The mutation in the genes responsible for monogenic inheritance has been identified as Glucokinase (GCK) and Hepatocyte nuclear factor-1A (HNF1A) (Vaxillaire et al, 2008) and the condition is known as Maturity onset diabetes of the young (MODY). The studies also suggest that the genes responsible for the monogenic form of diabetes might also contribute for T2D (Vaxillaire et al, 2012). A Meta genome wide association study involving 34,412 cases and 59,925 controls in European populations suggest the overlapping role of HNF1A with monogenic as well as polygenic T2D (Voight et al, 2010). Type 2 diabetes is a multifactorial disorder and has been associated with many genes. The variants in transcription factor 7-like 2 (TCF7L2) has been consistently replicated to be a risk factor for T2D in various populations. TCF7L2 gene was linked as a risk factor for T2D in a linkage study in Mexican-American population which was mapped to chromosome 10 (Diggirala et al, 1999).

In order to evaluate an association of single nucleotide polymorphism in TCF7L2 gene with risk of T2D, a meta-analysis conducted with ten eligible studies in East Asian population showed a significant association of the four SNPs: rs7903146, rs12255372, rs11196205, rs290487 in this population (Luo et al, 2009).

Furthermore, a global meta-analysis conducted to evaluate the risk of T2D posed by the SNPs of TCF7L2 gene showed significant association with T2D (Cauchi et al, 2007). The SNP, rs7903146 was significantly associated with risk of T2D involving 28 studies comprising 29,195 cases and 17,202 control groups where the pooled odds ratio of 1.5 was obtained.

The peroxisome proliferator-activated receptor-γ (PPARG) is also one of the candidate genes which has been extensively studied in many populations for the risk of T2D. The conflicting results regarding the association of one of the most studied SNP, Pro12Ala with T2D have been resolved by the meta-analysis over time in different populations (Ek et al, 2001; Lohmueller et al, 2003; Hara et al, 2003; Parikh and Groop, 2004; Ludovico et al, 2007; Gouda et al, 2010)

Peroxisome proliferator-activated receptor-γ coactivator-1 (PPARGC1A) is another gene, the SNPs of which have been thoroughly investigated. The gene is a co-activator of various nuclear receptors and has also been identified as one of the candidate genes associated with the risk of T2D. A recent study carried out in African American population showed a significant association of the SNP, rs4235308, with T2D with Odds ratio of 2.53 (Cheema et al, 2015), However; the other SNPs of this gene rs7656250, rs4235308 was found to be protective in a Haitian American population. Similarly, the SNPs; Gly482Ser and Thr528Thr were found significantly associated with T2D in a Kurdish-Iranians population (Shokouhi et al, 2015). Zhu et al, 2009 found that instead of individual association of six SNPs, a common haplotype of PPARGC1A was significantly associated with T2D in Han Chinese population. Gly482Ser of PPARGC1a along with Pro12Ala of PPARG gene have been found to be significantly associated with the risk of conversion from impaired glucose tolerance to T2D; a STOP-NIDDM double blind trial involving multicentre study with 1429 subjects (Andrulionyte et al, 2004). A similar study in the Caucasian population showed a significant association of Gly482Ser of the gene with type 2 diabetes when both initial and replication studies were combined (case: 689, control: 487) (Ek et al, 2001). The Haplotype involving four loci was significantly associated with T2D, however; no individual SNP was associated with T2D in Caucasian population (Oberkofler et al, 2004). Similar results carried out in Slovene population showed that Gly482Ser of PPARGC1A gene was the risk factor for T2D (Kunej et al, 2004). Kim et al, 2005, studied association of eight SNPs: (1789G>A, 1437C>T, 61643T>C, 61647T>C [L251S], 61719T>C, 75919G>A [G482S], 76874C>T [T612M] and 97329A>G) with T2D and the result showed that 61343T>C, 76874C>T (T612M) Variants and their haplotypes were significantly associated with T2D; however, Gly482Ser was not associated with early-onset T2D. Likewise, a case-control study in a Chinese population found a significant association of Gly482Ser with T2D specifically in men (Sun et al, 2006). It was noted that the

risk of having T2D in men was 1.85-fold higher in genotypes with the risk allele in comparison with homozygous dominant genotypes (Sun et al, 2006). Furthermore, Rai et al, 2007 found a significant association of Thr39Thr and Gly482Ser with T2D in a North Indian population. Similarly, a replicate study in a North Indian population showed a significant association of Thr39Thr and Gly482Ser variants with T2D (Bhat et al, 2007). However, no association was found in French, Japanese population, and Pima Indian, Dutch, Hans Chinese, Hispanic and non-Hispanic populations (Lacquemant et al, 2002; Hara et al, 2002, Muller et al, 2003; Stumvoll et al, 2004; Chen et al, 2004; Wang et al, 2005; Nelson et al, 2007).

Due to the conflicting results in the association of these SNPs with T2D even in the same population prompted to carry out a meta-analysis by combining all the case-control study to evaluate the overall pooled Odds ratio. The meta-analysis involving Gly482Ser polymorphism and its association with T2D suggested the modest role of this SNP (Barroso et al, 2006). It might be due to the smaller sample size (cases: 3718, controls: 4818) and the significant difference in between-study heterogeneity (Barroso et al, 2006). Keeping pace with the accumulating literature, an updated meta-analysis was carried out by Yang et al, 2011. The study involved 21 original research studies, of which 7 were from the Caucasian population, 10 from East Asian population and finally 4 from Indian populations. Due to significant between-study heterogeneity, the random effect model was used and the pooled Odds ratio [1.19 (95% CI: 1.05–1.34, p = 0.006)] of the Gly482Ser variant associated with risk of T2D was obtained which was statistically significant (Yang et al, 2011). In a subgroup meta-analysis, the Gly482Ser variant was significantly associated with T2D only in Indian populations (T2D cases: 948, controls: 1435); however, it did not reach statistical significance in East Asian and Caucasian populations (Yang et al, 2011). Similarly, the other SNP, Thr394Thr was also significantly associated with T2D in a pooled study and also in an Indian population in a subgroup analysis, but not in Caucasian and East Asian populations (Yang et al, 2011).

1.10 Genome-wide association studies (GWAS).

With the completion of the Human genome project, it has been possible to analyse genome-wide polymorphism in relation to risk of T2D. Due to the revolutionary technique, millions of SNPs could be genotyped within a short period of time and not all the SNPs is required as the

tag SNPs, a representative of SNPs in linkage disequilibrium, can be genotyped. Studies focusing on the genome-wide association (GWA) has led to a successful identification of genetic loci that are robustly associated with Type 2 diabetes (Kato, 2013). According to Kato, 2013 the GWA studies focuses on searching susceptibility variants across the entire genome without any form of bias or assumption of hypotheses. The number of loci associated with T2D has reached 70 with the increase in the number of studies in various populations (Kato, 2013) and two dozen are associated with glycaemic traits (Billings et al, 2010). However, not all the genetic polymorphisms are replicable and some are population specific.

The first case-control GWAS involving 661 subjects with T2D and 614 subjects without T2D was conducted in a French population in which 392,935 SNPs were genotyped (Sladek et al, 2007). The study discovered that 8 SNP of 5 locus: TCF7L2 (rs7903146), SLC30A8 (rs13266634), HHEX (rs1111875, rs7923837), LOC387761 (rs7480010) and EXT2 (rs3740878, rs11037909, 1113132) were significantly associated with T2D in this population (Sladek et al, 2007). The Wellcome trust case control consortium (WTCCC) study carried out in the United Kingdom populations with 1924 cases and 2938 control group, found that 10 SNPs: rs8050136 of FTO gene, rs10946398 of CDKAL1, rs5015480 of HHEX, rs10811661and rs564398 of CDKN2B, rs4402960 of IGF2BP2, rs13266634 of SLC30A8, rs7901695 of TCF7L2, rs5215 of KCNJ11 and rs1801282 of PPARG gene were significantly associated with T2D (Donnely, 2007). The association with highest odds ratio (OR) of 1.37 (95% CI 1.25-1.49)] was obtained for rs7901695 of TCF7L2 gene.

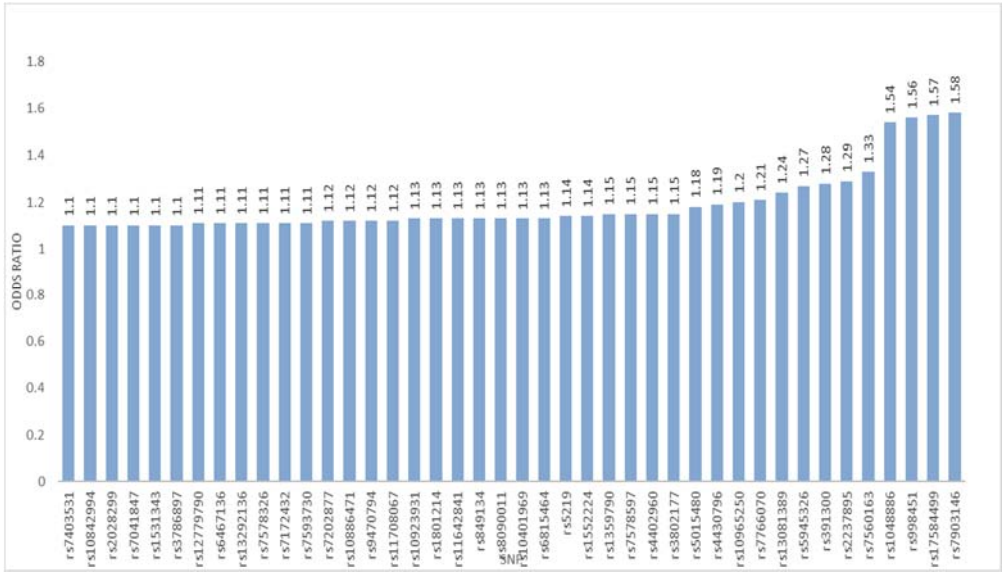

Figure 1.01: Odds ratio of the association of the SNPs with type 2 diabetes. The SNPs with an odds ratio ≥ 1.1 have only been included. Adapted from Kato et al, 2013

1.11 Association of single nucleotide polymorphisms in PPARGC1A gene with Type 2 Diabetes.

Peroxisome proliferator-activated receptor-γ coactivator-1apha (PPARGC1A) is transcriptional co-activator associated with the release of insulin by beta cells (Cheema et al, 2014). The transcriptional co-activator plays a critical role in triggering the activation of a series of nuclear receptors that end up regulating processes that impact cellular energy metabolism, thermogenesis regulation, oxidation metabolism, and glucose metabolism (Cheema, 2014). Usually, the PPARGC1A gene is responsible for co-activating peroxisome proliferator-activated receptor gamma (PPARG), which is implicated in regulatory pathways for lipid and glucose homeostasis by interacting and regulating other genes. The PPARGC1A is also linked to insulin resistance that leads to Type 2 diabetes (Cheema et al, 2014). Hence, the variants of PPARGC1A that relates to resistance to insulin as well as an impaired secretion of insulin are critical in understanding the pathogenesis of Type 2 diabetes. The human PPARGC1A is located on chromosome 4p15.1. It has been linked to Type 2 diabetes and other related phenotypes (Cheema et al, 2014).

In a recent study Zhu et al, 2017 examined PPARGC1A to determine if it is correlated with a borderline decrease in susceptibility to Type 2 diabetes Zhu et al, 2017 noted that decreased

expression of PPARGC1A in muscle cells was found in diabetic as well as non-diabetic individuals whose parents or other close relatives had diabetes.

1.12 Prediabetes, a risk factor for Type 2 diabetes and metabolic syndrome.

Prediabetes refers to an intermediate state or condition between normoglycemic and clinical diabetes. According to the American diabetes association, prediabetes is identified by HbA1C levels in a range of 5-6%, fasting plasma glucose value of 5.6-6.9 mmol/l and the glucose level 7.8- 11.1 mmol/l in an oral glucose tolerance test.

Understanding the spectrum of Type 2 diabetes is important in realizing the problem during the pre-diabetes stage. It has been estimated that about one third of the adult population (≥20 years) and around 80 million of the total population of the United States have prediabetes (Maccain, 2016). The MetS associated with pre-diabetes is an early stage of Type 2 diabetes (Grundy, 2012). MetS is sometimes synonymously referred to as a pre diabetic state (Grundy, 2012). People with MetS have 5-fold increased risk of developing diabetes and it is conceivable that the risk factor might be even larger if the MetS, impaired glucose tolerance and impaired fasting glucose is overlapped. The study suggests that obesity is commonly prevalent in both MetS and prediabetes subjects (Alexander et al, 2006). Obesity is further associated with insulin resistance (Ye, 2013), both of which are risk factors for type 2 diabetes. According to Roberts et al, 2013, metabolic syndrome is a collection of cardio-metabolic risk factors and the risk factors include insulin resistance, hypertension, and obesity.

A study by Grundy, 2012, revealed that pre-diabetes has a significant impact on microvascular disease. It has been found that even a pre-diabetes state can cause diabetic retinopathy (Tkellis et al, 2007; Tapp et al, 2008). The pre-diabetes condition creates a platform for predicting the possible macro-vascular disease, but the predictive power is mediated through metabolic syndrome. This revelation offers a promising opportunity for a better understanding of the pathway that leads to Type 2 diabetes. Such information will be critical for experts who are looking for ways to block the process that leads to diabetes. The understanding offers an opportunity for reversing the mechanism that leads to Type 2 diabetes. It is, therefore, crucial to understand the spectrum of type 2 diabetes i.e., the prediabetes, the components of metabolic syndrome and metabolic syndrome per se.

1.13 Metabolic syndrome

Metabolic syndrome (MetS) was first defined by Swedish clinician Eskil Kylin in 1920s. Kylin described MetS as a cluster of condition which includes: hypertension, hyperglycemia and gout. Over the years different terms have been coined to refer to metabolic syndrome. Reaven et al, 1988 used X syndrome, Kaplan et al, 1989 called it a deadly quartet while insulin resistance syndrome was used by DeFronzo et al, 1991. Even though, different scientists used different names, the common factor in all of them was the metabolic disorder. For it to be considered as a distinct clinical condition, a standardized unifying definition of this was needed.

In 1999, WHO consultation group proposed a working definition of metabolic syndrome (Alberti et al, 1998) which includes: Glucose intolerance, impaired glucose tolerance (Fasting glucose ≥6.1 mmol/l), impaired glucose tolerance (2-hour post glucose levels ≥7.8 mmol/l)] or type 2 diabetes and or insulin resistance with two or more of the components as shown in the table 1.02.

In 1999, the European Group for the study of Insulin Resistance (EGSIR) (Balkau et al, 1999) proposed a modified definition of metabolic syndrome and named it as insulin resistance syndrome. The insulin resistance is central to this definition and the diagnostic criteria includes insulin resistance or fasting hyperinsulinemia (greater than 75th percentile) plus two or more of the factors as shown in table1.02. In this definition, waist circumference has been proposed rather than waist hip ratio as it is easy to measure and better indicators of visceral adipose tissue accumulation (Pouliot et al, 1994).

Similarly, in 2001, National Cholesterol Education Programme, Adult Treatment Panel III (NCEP ATP III, 2001) defined metabolic syndrome as the combination of the three or more criteria as shown in the table 1.02

The NCEP ATP III criteria differed from WHO in various aspects. This criterion emphasizes central obesity (waist circumference) only rather than on overall obesity (body mass index).

The cutoff values for the blood pressure have been decreased while that of HDL-C has been increased.

Due to the debates and controversies surrounding the diagnostic criteria of metabolic syndrome, in 2005, the International Diabetes Federation (IDF) came up with a more accurate definition of metabolic syndrome. The main aim was to make it as precise as possible and a reliable tool in clinical practice and research studies. According to the IDF, 2005 as shown in the table 1.02, the definition of metabolic syndrome was: central obesity [waist circumference (WC): waist circumference: European ≥94 cm (M) or ≥80 cm (F); South Asian and Chinese ≥90 cm (M) or ≥80 cm (F); Japanese ≥85 cm (M) or ≥90 cm (F)] with ethnicity specific cut off point of WC along with two or more of criteria as shown in the table 1.02, has to be met in order to be diagnosed as metabolic syndrome.

In order to gather wider consensus in unifying the definition of metabolic syndrome, a discussion of the experts representing the various organizations took place in 2009 sponsored by IDF (Alberti et al, 2009). The criteria set forth for the clinical diagnosis of the metabolic syndrome is as followings:

In this criteria for diagnosis of metabolic syndrome, elevated waist circumference as an obligatory component was abolished. Unlike IDF, 2005 criteria, any three out of five above mentioned criteria are sufficient for the diagnosis of metabolic syndrome (Alberti et al, 2009) as shown in table 1.02.

Table 1.02: Definitions of metabolic syndrome.					
Criteria	WHO 1999	EGSIR	NCEP ATP III	IDF	NCEP ATP III modified
Author	Alberti et al, 1998	Balkau et al. 1999)	NCEP ATP III, 2001	Alberti et al, 2005	Alberti et al, 2009
Impaired glucose or insulin	Glucose intolerance, impaired glucose tolerance (Fasting glucose ≥6.1 mmol/l), impaired glucose tolerance (2h post glucose load ≥7.8 mmol/l) or type 2 diabetes and or insulin resistance	Fasting plasma glucose >6.1 mmol/l in nondiabetics	Impaired fasting glucose (≥ 6.1mmol/l)	Impaired fasting glucose (≥ 5.6mmol/l) or diabetes	Impaired fasting glucose ≥ 5.6 mmol/L or diagnosed with diabetes.
Obesity	Waist-to-hip ratio >0.9 for male or >0.85 for female and/or BMI >30 kg/m²,	Waist circumference ≥94 cm (M) or ≥80 cm (F)	Waist circumference >102 cm (M) or >88 cm (F	Waist circumference: European ≥94 cm (M) or ≥80 cm (F); South Asian and Chinese ≥90 cm (M) or ≥80 cm (F); Japanese ≥85 cm (M)or ≥90 cm (F)	Waist circumference: European ≥94 cm (M) or ≥80cm (F), United States ≥ 102 (M) or ≥88 (F), South Asian ≥90 cm (M) or ≥80cm (F), Chinese ≥85 cm (M) or ≥80cm (F), Japanese ≥85 cm (M)or ≥90 cm (F)
Elevated blood pressure	BP≥140/90mmHg or medication	BP≥140/90mmHg or medication	BP≥130/85mmHg or medication	BP≥130/85mmHg or medication	BP≥130/85mmHg or medication
Dyslipidaemia	Plasma triglycerides≥1.7mmol/l, HDL-C <0.9mmol/l for male and <1.0mmol/l for female	Triglycerides >2.0mmol/l, HDL-C <1.0mmol/l	Triglycerides≥1.69mmol/l, HDL-C <1.04mmol/l for male and <1.29mmol/l for female	Triglycerides≥1.7mmol/l, HDL-C <1.0mmol/l for male and <1.30mmol/l for female	triglycerides≥1.7mmol/l or medication, HDL-C <1.0mmol/l (M) and <1.30mmol/l (F) or on medication
Other criteria	Microalbuminuria with the excretion rate of albumin ≥20µg/min or the albumin/ creatinine ratio ≥30mg/g				

NCEP ATP III = National Cholesterol Education Programme, Adult Treatment Panel III, EGSIR= European Group for the study of Insulin Resistance, IDF = international diabetes federation

1.14 Features of metabolic syndrome

1.14.1 Impaired glucose tolerance.

Impaired glucose tolerance is one of the characteristic features of metabolic syndrome with corresponding elevated levels of fasting plasma glucose concentration. Insulin resistance is usually evident and leads to the hyperglycaemic state which could be either due to lack of production of insulin or due to defect in the beta cells but is more likely to be due to insulin being insensitive to glucose as a result of which glucose is not disposed to the target cells.

1.14.2 Obesity

Obesity is one of the components of metabolic syndrome and it has been included in every definition of metabolic syndrome. Body mass index (BMI), which is determined by weight (kilograms) divided by height squared (square meters), has been conventionally considered as a measure of obesity. BMI with 18- 24.9 kg/m^2 is considered as normal and ≥ 30 is considered as obese. Obesity is considered as one of the risk factors for metabolic syndrome and cardiovascular disease (Lakka et al, 2002; Ritchie et al, 2007). Obesity has also been considered as one of the risk factors for insulin resistance and type 2 diabetes. Even though BMI is an indicator of obesity, it does not consider the distribution of body fat (Sowers et al, 2003). BMI and waist hip ratio were included in the WHO, 1999 definition of metabolic syndrome. As these factors were not the best indicator of fat distribution, waist circumference instead has been included in the subsequent definitions (EGSIR, NCEP ATP III and IDF) of the metabolic syndrome.

1.14.3 Elevated blood pressure

Elevated blood pressure has been considered as one of the risk factors for the MetS syndrome as they are closely associated with insulin resistance and central obesity in MetS (Ferrannini et al, 1997). High blood pressure is one of the metabolic parameters linked to metabolic syndrome. In a 10.5-year follow-up longitudinal study in hypertensive subjects without cardiovascular disease, it was found that a third of the study participants had

metabolic syndrome (Schillaci et al, 2004). Different definition of metabolic syndrome has used different cut-off value of the elevated blood pressure. WHO, 1999 and EGSIR; (Balkau et al, 1999) have used elevated systolic pressure ≥140mm Hg and diastolic blood pressure ≥ 90mm Hg one of the criteria for the metabolic syndrome and these cut-off values could be classified as hypertension (Chobanian et al, 2003). In the subsequent definitions of the metabolic syndrome (NCEP ATP III, IDF), the cut-off values of the elevated blood pressure have been kept at ≥130 /85mmHg.

1.15 Prevalence of metabolic syndrome

Due to the changing definition of metabolic syndrome over time, it is difficult to measure the temporal changes and variation in the prevalence of metabolic syndrome in different populations. A study conducted on adult Americans with sample size of 3601 with participants involving ≥20 years of age group, the prevalence (unadjusted) of the overall metabolic syndrome was 34.5% using NCEP criteria (Ford et al, 2005). The prevalence of unadjusted metabolic Syndrome in overall participants was 39.0%, according to the International Diabetes Federation (IDF) criteria. In a systematic review of the prevalence of metabolic syndrome in Latin American countries under the NCEP ATP III criteria showed weighted mean prevalence of 24.9% ranging from 18·8 to 43·3%.

The prevalence of metabolic syndrome is increasing at an alarming rate in south Asian countries. It has been estimated that about a third of urban South Asian populations have metabolic syndrome (Misra et al, 2009). A cross-sectional study involving 2225 participants carried out in an Indian population showed that the prevalence of metabolic syndrome was 35.8%, according to the NCEP ATP III definition and 39.5%, based on the IDF criteria (Ravikiran et al, 2010) which is almost similar to the figures found in the USA (Ford et al, 2005). A similar study carried out in rural Pakistan with 1658 participants showed prevalence of 31%, according to ATP III criteria and 40%, according to IDF definition (Zahid et al, 2008). The prevalence of metabolic syndrome in urban Sri Lanka were 46.1% and 38.9%, according to the revised NECP ATP III Treatment Panel III and IDF criteria (Chackrewarthy et al, 2013) which is the highest among other South Asian countries.

Table 1.03: Prevalence of metabolic syndrome in various populations.

Country	Number	Age (years)	Year	Prevalence %	Diagnostic criteria	Reference
USA	3601	≥20	2005	36.0 %	NCEP ATP III	Ford et al, 2005
				39.0%	IDF	
Latin America		18-65	2011	24.9%	NCEP ATP III	Márquez-Sandoval et al, 2011
South Asia						
Nepal	14,425	≥20	2011	20.7%	NCEP	Sharma et al, 2011
				22.5%	IDF	
India	2225	≥20	2010	35.8%	NCEP ATP III	Ravikiran et al, 2010
				39.5%	IDF	
Pakistan				31.0%	NCEP ATP III	Zahid et al, 2008
				40.0%	IDF	
Sri lanka	2985	35-65	2013	46.1 %	Revised NCEP ATP III	Chackrewarthy et al, 2013
				38.9 %	IDF	
China	17,080		2016	M = 39.8% F = 45.0%	Joint Interim Statement (JIS) criteria, 2009	Xiao et al, 2016

NCEP ATP III = National Cholesterol Education Programme, Adult Treatment Panel III, EGSIR = European Group for the study of Insulin resistance, IDF = international diabetes federation, F = female, M = male

1.16 Consequences of metabolic syndrome

Because of the clustering of the metabolic factors directly associated with the risk of cardiovascular disease and type 2 diabetes, metabolic syndrome predicts the future risk of the above-mentioned diseases.

The cardiovascular mortality assessed in 3606 subjects (35-70 age group) with metabolic syndrome using WHO criteria in the Botania study carried out in Finland and Sweden (Isomma et al, 2001) showed the risk of cardiovascular mortality 3-fold higher in subjects with metabolic syndrome.

Similarly, a research based on 11 European cohorts comprising 6156 men and 5356 women without diabetes, 30 to 89 years of age showed an overall hazard ratio of cardiovascular mortality as 2.26 in men and 2.78 in women even after adjustments made with age, cholesterol levels and smoking habits (Hu et al, 2004). This study was based on the modified definition of the WHO classification of metabolic syndrome, which included hyperinsulinemia plus any two or more of the other components of metabolic syndrome.

A study from the placebo groups of the Scandinavian Simvastatin Survival Study and the Air Force/Texas Coronary Atherosclerosis Prevention Study involving 2223 subjects were assessed for cardiovascular risk using major coronary events (MCEs) as an end point. The study showed that the placebo-treated subjects were 1.5 times more likely to develop major coronary events after excluding individuals with type 2 diabetes (Girman et al, 2004). In this study the National Cholesterol Education Program (NCEP) Adult Treatment Panel-III criteria were used for the diagnosis of metabolic syndrome. The study showed that low levels of HDL-C was associated with increased risk of MCEs in both Scandinavian Simvastatin Survival Study and the Air Force/Texas Coronary Atherosclerosis Prevention Study, whereas higher levels of triglycerides, high blood pressure and obesity were major risk factors for MCEs in Air Force/Texas Coronary Atherosclerosis Prevention Study (Girman et al, 2004).

A study by Eddy et al, 2008; who used US National Health and Nutrition Evaluation Survey (NHANES) for assessing risk of CVD in people with metabolic syndrome using WHO and ATP criteria for the diagnosis of metabolic syndrome found the associated risk for CVD ranged from 1.5 to 1.6-fold. He found that some diagnostic criteria of metabolic syndrome are better predictors of the future risk of CVD, regardless of the criteria of diagnosis of metabolic

syndrome; this was firstly high glucose concentration, and then with decreasing predictive powers, hypertension, obesity, high triglycerides and low HDL-C (Eddy et al, 2008). A meta-analysis involving 21 studies assessing metabolic syndrome as a risk factor for cardiovascular disease, predicts risk ratio for cardiovascular disease as 1.74 and 1.52 for coronary heart disease (Gallasi et al, 2006).

The subjects with metabolic syndrome are at higher risk of developing type 2 diabetes. A study carried out on 5974 men in the West of Scotland Coronary prevention study to predict the incidence of diabetes in a 4.9 years of follow-up study suggests that men with 4 to 5 components of metabolic syndrome had a 24.5-fold increase in risk for type 2 diabetes. (Satter et al, 2003). In a Framingham Offspring Study with 1549 men and 1774 women aged 22 to 81years with 8 years of follow-up period showed a 6.92 relative risk of type 2 diabetes (Wilson et al, 2005). A study in the Chinese population with 541 subjects in a 5 years of follow-up period the relative risk of type 2 diabetes posed by metabolic syndrome under WHO, EGIR and IDF criteria were 2.39, 1.88, 2.05 respectively (Wang et al, 2007). Similarly, a longitudinal study in Mauritius showed strong association (odds ratio of 4.6) with the incidence of type 2 diabetes in a 5-year follow-up study (Cameron et al, 2007). A study in a community of Aboriginal Canadian found that metabolic syndrome was associated with incident diabetes under the International Diabetes Federation criteria (OR = 2.14) or National Cholesterol Education Program criteria (OR = 2.03) (Ley et al, 2009). It clearly shows that no matter what the criteria for diagnosis of metabolic syndrome is used, the existence of these traits is a potential risk factor for both CVD and type 2 diabetes.

1.17 Genetics of metabolic syndrome

1.17.1 Heritability of metabolic syndrome

The variation influenced by genetic components is usually referred to as the heritability. Metabolic syndrome is a heterogeneous disorder and multiple clusters of metabolic factors are involved in it. The genetic basis of the metabolic syndrome and its components are estimated based on familial and twin studies. A systematic review and meta-analysis conducted on twin and familial study in the heritability of body mass index, which is one of the components of metabolic syndrome, ranged from 0.47 to 0.90 conducted in 140,525 twins and from 0.24 to 0.81 in familial studies conducted on 42,968 family members (Elks et

al, 2012). Heritability of fasting plasma glucose ranged from 0.36 – 0.51 (Santos et al, 2006; Poulsen et al, 1999; Snieder et al, 2001). The heritability of fasting insulin ranged from 0.08 to 0.53 (Henkin et al, 2003; Mayer et al, 1996).

Similarly, studies carried out in different populations suggest the heritability of HDL in a range of 0.34 to 0.77 (Zakesh et al, 2012; Sung et al 2009). Likewise, the heritability of LDL and total cholesterol ranges from 0.31 to 0.77 and 0.30 -0.74 respectively. The heritability of systolic blood pressure has been found in the range of 0.20-0.70 and in the range of 0.1 -0.5 that of diastolic blood pressure (Argyropoulos et al ,2005; Snieder et al,1999; Teran-Garcia et al, 2007; Pilia et al, 2006; Elder et al, 2009; Li et al, 2012; Zarkesh et al, 2012; Rahman et al, 2009).

1.17.2 Genome wide and candidate gene association studies

With the advent of the new technology, it has been possible to sequence the whole genome of an individual known as Genome wide association studies. The information regarding the genetic variants across the whole genome could be retrieved by sequencing the entire genome of an individual and it is possible to look at if any variants that are associated with the disease or any trait in question. In this type of study usually large sample size is required to obtain statistical power. Because of the large number of variants being analysed, it opens the possibility of new loci being associated with a disease or a trait which could be of unknown function. In genome wide studies, genotyping is usually carried out using micro-array technology, which involves millions of common SNPs. The notion of capturing common variants is that it might explain the heritability of the common disease or traits in the population (Hirschhorn et al, 2005, Reich and Lander, 2001).

1.18 Genetic polymorphisms and its association with metabolic syndrome

1.18.1 Association of rs8192678 of PPARGC1A with metabolic syndrome.

The forward sequence flanking rs8192678 across the eutherian mammals as shown in figure 1.02 demonstrates that, except in humans, the major allele is highly conserved across the mammalian groups. One of the ways to argue the functionality of the SNP is to find whether it is conserved among species or not. It is conceivable that the SNPs which are in the

conserved region of the genome might have potential function as they might have been preserved through an evolutionary process to retain the function. Conservation of the SNP is also one of the ways to prioritise the SNPs as it might increase the probability of having an impact upon the phenotype in question.

```
Human                CTCAGTTCACCGGTCTTGTCT
Bonobo               CTCAGTTCACTGGTCTTGTCT
Chimpanzee           CTCAGTTCACTGGTCTTGTCT
Gorilla              CTCAGTTCACTGGTCTTGTCT
Orangutan            CTCAGTTCACTGGTCTTGTCT
Gibbon               CTCAGTTCACTGGTCTTGTCT
Vervet-AGM           CTCAGTTCACTGGTCTTGTCT
Crab-eating macaque  CTCAGTTCACTGGTCTTGTCT
Macaque              CTCAGTTCACTGGTCTTGTCT
Olive baboon         CTCAGTTCACTGGTCTTGTCT
Marmoset             CTCAGTTCACTGGTCTTGTTT
Ryukyu mouse         CTTAGTTCACTGGTCTTGTCT
Mouse                CTTAGTTCACTGGTCTTGTCT
Algerian mouse       CTTAGTTCACTGGTCTTGTCT
Rat                  CGTAGTTCACTGGTCTTGTCT
Rabbit               CTCAGTTCACTGGTCTTGTCT
```

Figure 1.02: Alignment of the rs8192678 flanking sequence in 16 eutherian mammals. The SNP (rs8192678 or Gly482Ser) and the flanking sequence represents the forward sequence of the genomic region. The nucleotide in the SNP region is the major allele. Source: www.ensemble.org

PPARGC1A gene maps on chromosome 4p.15.1. The gene consists of 13 exons and 12 introns and the SNP (rs8192678) in located in exon 8 of the gene as shown in figure 1.03. The transcription of the gene occurs on the reverse strand of the gene and the notation of the nucleotide change of the SNP (rs8192678), where the single nucleotide G (major allele) (Guanine) is replaced by A (minor allele) (Adenine), has been assigned (in our case) with respect to the reverse strand. The single nucleotide change occurs in the exonic region of the gene which, subsequently, changes the amino acid during translation. In this SNP, the amino acid residue Glycine at position 482 is replaced by Serine at the protein level. This kind of change where it alters the protein sequence is known as non-synonymous mutation. A study carried out by Choi et al, 2006 found a deleterious effect of the mutant (482Ser) on the co-activation activity of the protein. However, Nitz et al, 2007 fount that the co-activation activity of the gene was not significantly different in mutant in comparison to the wild type gene. It seems possible that the difference in the result from these two studies might be due

to the choice of the promoters, where Choi et al, 2006 has used two endogenous promoters: Tfam promoter and PPAR responsive element (PPRE) to conduct the luciferase activity in Chang human hepatocyte cell line; however, Nitz et al, 2007 has used PPARG as a promoter in a HepG2 cell lines. Choi et al, 2006 noted that under the Tfam promoter, the activity of wild type and mutant protein increased 2.5 and 10-fold respectively. It can be estimated that the activity under Tfam promoter increased 4-fold with respect to the wild type protein.

Figure 1.03, shows the schematic diagram of the exons and introns of PPARGC1A gene. The position of the SNP (rs8192678) (Gly482Ser) is indicated by arrow in exon 8 of the gene.

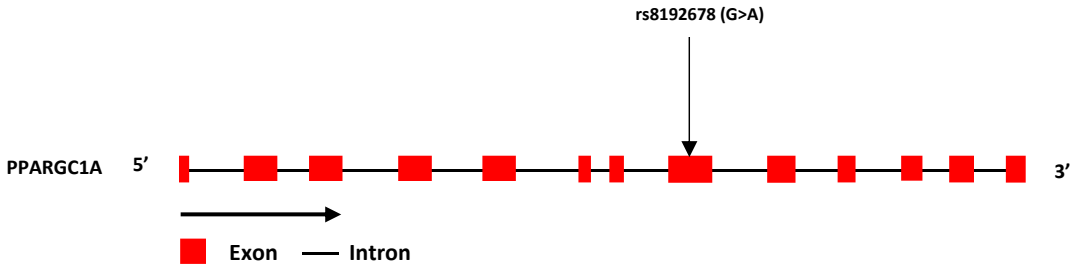

Figure 1.03: Schematic diagram of peroxisome proliferator-activated receptor-γ coactivator-1 (PPARGC1A) gene. The position of the SNP (rs8192678) is indicated by arrow. Red rectangular box indicates the coding region of the gene. Thick black line indicates the intronic region of the gene.

The global minor allele frequency (MAF) of rs8192678 is 27%. The highest MAF was found in East Asian population (44%) followed by European (36%), South Asian (29%), American (26%) and African (5%) population (Table 1.04).

Table 1.04: Global minor allele frequency of rs8192678

Geographical Area	Allele frequency of rs8192678 (G/A) of PPARGC1A gene	
	Major allele (G) frequency (%)	Minor Allele (A) frequency (%)
European	64%	36%
American	74%	26%
African	95%	5%
East Asian	56%	44%
South Asian	71%	29%
Global	73%	27%

Peroxisome proliferator-activated receptor-γ coactivator-1 (PPARGC1A) is a major regulator of energy expenditure. *In vivo* and *in vitro* model studies have suggested that PPARGC1A is involved in adaptive thermogenesis (Puigserver et al, 1999). Adaptive thermogenesis is a physiological response through which the energy is dissipated as heat while exposed to cold temperature (Cannon et al, 1998) which usually occurs in skeletal muscle and brown adipose tissue (BAT). It addition to it, the PPARGC1A is also expressed in skeletal muscle tissue in response to exercise (Barr et al, 2002). This clearly indicates the crucial role of this protein during various physiological demands. In fact, adaptive thermogenesis occurs in the mitochondria of the BAT and skeletal muscle cells (Wu et al, 1999). Interestingly, it has also been found that this gene could increase the mitochondrial density in these high energy demanding cells during cold stress or exercise (Wu et al, 1999).

PPARGC1A has also been found to be expressed in the liver during prolong fasting associated with diabetic condition. During this condition the protein plays vital role in the expression of other key genes involved in hepatic gluconeogenesis, which involves the production of glucose from non-carbohydrate substrate (Rhee et al, 2003) and is a major contributor for hyperglycemia especially in people with type 2 diabetes. Reduced glucose uptake by skeletal muscle cells and failure in suppression of hepatic gluconeogenesis might lead to insulin resistance which is one of the salient features of type 2 diabetes.

Genetic variants in this gene have also been implicated in insulin resistance and Type 2 diabetes (Hara et al, 2002). The exonic variant Gly482Ser (rs8192678) showed significant differences in the levels of insulin and insulin resistance index (HOMA-IR) according to the

genotypes in non-diabetic subjects (Hara et al, 2002). The study found that the haplotype (Thr394Thr – Gly482Ser) was significantly associated with type 2 diabetes.

PPARGC1A Gly482Ser variant has also been associated with reduced insulin sensitivity in obese subjects (Fanelli et al., 2005). Moreover, several studies in various populations have shown the association of Gly482Ser variant of PPARGC1A gene with type 2 diabetes (Bhat et al., 2007, Ek et al., 2001, Barroso et al., 2006, Kunej et al., 2004, Zhang et al., 2007, Yang et al., 2011, Su et al., 2008 and Jing et al., 2012).

The PPARGC1A gene has also been associated with obesity, which is one of the components of metabolic syndrome. Abdominal fat in Qubec families (Pe´russe et al., 2001) has been assigned to the chromosomal region 4p15.1 where the PPARGC1A gene lies. Similarly, the PPARGC1A rs8192678 variant has been associated with obesity indices (BMI, Waist circumference, hip circumference, total body fat and; abdominal visceral and subcutaneous fat) in middle-aged European women (Esterbauer et al., 2002) and abdominal fat in Asian Indians (Vimaleswaran et al., 2006). A population based study involving 1,811 elderly (≥50 years) subjects, analyzing the risk of obesity associated with PPARGC1A Gly482Ser, showed that genotype with risk allele was significantly associated with obesity in physically inactive male subjects (Ridderstrale et al, 2006).

Gly482Ser variant has also been associated with blood pressure (Oberkofler et al, 2003; Cheurfa et al, 2004; Anderson et al, 2005; Sookoian et al, 2005; Vimaleswaran, 2008) which is also one of the components of metabolic syndrome. A linkage study of Dutch dyslipidemic families showed that systolic blood pressure was associated with a locus on chromosome 4p15.1-2 (Allayee et al, 2001). The gene locus PPARGC1A has been mapped to chromosome 4p15.1 (Ester bauer et al, 2002). It suggests that there could be a causal relationship between PPARGC1A gene and blood pressure. The estrogen receptor signalling pathway might partly unravel how PPARGC1A might modulates blood pressure. Studies have shown that PPARGC1A might modulate the transcriptional activity of Estrogen receptor alpha and beta, which are all expressed in smooth muscle cells (Mendelsohn et al, 1999). Animal model studies have shown that ER beta deficient mice develop hypertension with age (Zhu et al, 2002). A significant association of the Gly482Ser variant of the PPARGC1A gene with systolic and diastolic blood pressure with Ser allele has been observed in 40 to 60 years elderly men (Oberkofler et al, 2003). Similarly, Anderson et al, 2005 found that Ser allele of the variant

shows a reduced risk of hypertension in Caucasians. In contrast, Cheurfa et al, 2004 found a significant association of the minor allele of Gly482Ser with hypertension in French Caucasian men with T2D (Cherufa et al, 2004). A population based study with a sample size of 934 high school Caucasian adolescents, showed that the minor allele was statistically more frequent in hypertensive than normotensive subjects (Sookoian et al, 2005). Vimaleswaran et al, 2008 tried to resolve the controversy regarding the association of the variant with arterial blood pressure by combining all the literature available to conduct a meta-analysis and it was found that the minor allele was significantly associated with risk of systolic and diastolic blood pressure. In the study, the gene-dose effect was evident, i.e., the increasing level of blood pressure with the addition of extra minor allele in both systolic and diastolic blood pressure (Vimaleswaran, 2008).

The role of *PPARGC1A* in triglyceride metabolism in the liver has now been established where the gene is responsible for the activation of farnesoid X receptor (FXR) which decreases the level of triglycerides in the liver (Zhang et al., 2004). Furthermore, the expression of *PPARGC1A* in conjunction with its nuclear receptor hepatocyte nuclear factor 4α (*HNF4α*) in the liver increases a range of apolipoprotein which are responsible for triglyceride and very low-density lipoprotein (VLDL) metabolism (Rhee et al., 2006).

Gly482Ser (rs8192678) variant of the human *PPARGC1A* gene is one of the most studied mutations. The rare homozygote (Ser/Ser) of the PGC-1alpha has been associated with reduced clearance of non-esterified fatty acid (Franks et al., 2007). Recent studies suggest a significant association of the Gly482Ser mutation with higher levels of TC and LDL-C levels in common homozygous subjects in Iranian population (Mirzaei et al., 2012); likewise, in the control group, HDL-C levels were significantly higher in Gly482 genotypes under dominant genetic model (Shoukouhi et al., 2015). The variant has been associated with plasma HDL-C in glucose tolerant subjects in a Danish population (Ek et al, 2002). Esterbauer et al, 2002 also found that the Gly482Ser was significantly associated with HDL-C in Bavarian and Austrian populations with German ancestry. The heterozygous female showed higher levels of HDL-C in comparison to rare homozygous females (Esterbauer et al, 2002). The level of fasting plasma HDL-C was significantly different among the three genotypes of Gly482Ser in French-Canadians with lowest found in Gly/Gly genotype group and it remained statistically significant even after adjusting for age, sex and BMI (Vohl et al, 2005). The levels of

triglycerides in pre-obese and an obese group were significantly higher in common homozygotes under a dominant genetic model in a Mexican population (Vazquez-de Mercado et al, 2015). The variant of interest i.e. Gly482Ser, is associated with various components of metabolic syndrome and it is imperative to understand the role of this variant in the Nepalese population.

1.18.2 Peroxisome proliferator-activated receptor gamma (PPAR-γ or PPARG) (Pro12Ala) (rs1801282)

Peroxisome proliferator-activated receptor gamma (PPAR-γ or PPARG) maps to chromosome 3. From the data in figure 1.04, it is apparent that the SNP is highly conversed across 18 eutherian mammals. As mentioned earlier, the highly conserved region might have potential function.

```
Human                TCCTATTGACCCAGAAAGCGA
Bonobo               TCCTATTGACCCAGAAAGCGA
Chimpanzee           TCCTATTGACCCAGAAAGCGA
Gorilla              TCCTGTTGACCCAGAAAGCGA
Orangutan            TCCTATTGACCCAGAAAGCGA
Gibbon               TCCTATTGACCCAGAAAGCGA
Vervet-AGM           TCCTATTGACCCAGAAAGCGA
Crab-eating macaque  TCCTATTGACCCAGAAAGCGA
Macaque              TCCTATTGACCCAGAAAGCGA
Olive baboon         TCCTATTGACCCAGAAAGCGA
Marmoset             TCCAATTGACCCAGAAAGCGA
Mouse Lemur          TCCTATTGACCCAGAGAGTGA
Prairie vole         GCCTGTTGACCCAGAGCCTGG
Ryukyu mouse         TCCTGTTGACCCAGAGCAAGG
Mouse                TCCTGTTGACCCAGAGCATGG
Algerian mouse       TCCTGTTGACCCAGAGCATGG
Shrew mouse          TCCTGTTGACCCAGAGCATGG
Rat                  TCCTGTTGACCCAGAGCATGG
```

Figure 1.04: Alignment of the rs1801282 flanking sequence in 18 eutherian mammals. The SNP and the flanking sequence represents the forward sequence of the genomic region. The nucleotide in the SNP region is the major allele. The polymorphism is shown in red colour. Source: www.ensemble.org.

The exons and introns of the PPARG gene is shown in figure 1.05. The gene has eight exons and seven introns and the SNP (rs1801282) (Pro12Ala) is located at the second exon.

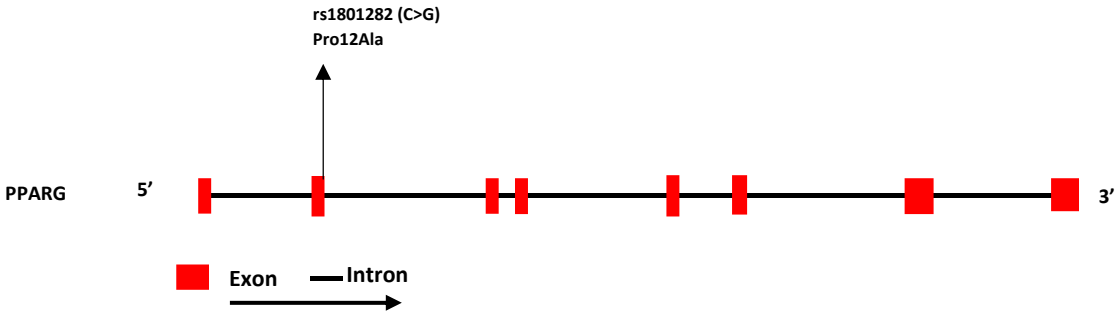

Figure 1.05: Schematic diagram of peroxisome proliferator-activated receptor-γ (PPARG) gene on chromosome 3, 3p25.2. The position of the SNP is indicated by arrow. Red rectangular box indicates the coding region of the gene. Thick black line indicates the intronic region of the gene.

Table 1.10 shows the minor allele frequency of rs1801282 and it is similar in European, American and South Asian population (12%). The lower minor allele frequency was found in African populations (1 %) and East Asian population (3%) population.

Table 1.05: Global minor allele frequency of rs1801282

Geographical Area	Allele frequency of rs1801282 (C/G) of PPARG gene	
	C allele Frequency (%) (Major allele)	G allele Frequency (%) (Minor allele)
European	88%	12%
American	88 %	12 %
African	99 %	1 %
East Asian	97 %	3 %
South Asian	88 %	12 %
Global	93 %	7 %

Peroxisome proliferator-activated receptor gamma (PPARG) is a member of the nuclear hormone receptor family and was initially characterized by its role in adipogenesis (Tontonoz et al, 1995). At low levels, it has also been found in other cell types such as skeletal muscle cells (Loviscach et al, 2000), and epithelial cells of gastrointestinal tract liver and pancreatic

beta cells. However, aside from adipocytes, it has been found to be highly expressed in immune cells, especially in macrophages (Cunnard et al, 2004). The role of this gene is not only limited to adipogenesis but has also been realized in triglyceride metabolism (Gunan et al, 2002) and Fatty acid trapping in adipocyte cells (Schoonjans et al, 1996). One of the most extensively studied SNPs in the exonic region of the gene is proline amino acid being replaced by alanine at codon 12 (Pro12Ala). The Pro12 Ala has been found to be partially impairing the transcriptional activity in inducing adipogenesis and improved insulin sensitivity (Masugi et al, 2000).

A study conducted in French population by Meirhaeghe et al, 2005 with sample size of 1155 found that the haplotype GTGC constructed from SNPs: P3 −681C>G, P2 −689C>T, Pro12Ala, and 1431C>T showed significant association (Odds ratio = 2.37) with MetS in a whole sample as well as in a sample stratified by gender. However, the individual SNPs were not associated with MetS in this population (Meirhaeghe et al, 2005).

Obesity is one of the components of metabolic syndrome and many studies have found association of the variant with obesity. A number of authors have claimed an association of Pro12Ala polymorphism with obesity indicators such as, BMI (Robitaille et al, 2003; Rhee et al, 2006; Deeb et al, 1998; Beamer et al, 1998), body weight (Rhee et al, 2006) waist circumference (Robitaille et al, 2003 and Tellechea et al, 2009) and fat mass (Robitaille et al, 2003 and Rhee et al, 2006).

In contrast to the reports stating improved insulin sensitivity of the minor allele of the Pro12Ala, a recent study suggested its association with insulin resistance which was conducted in Chinese populations with a sample size of 792. Similarly, rather than the single SNP (rs1801282) (Pro12Ala), the haplotypes in the promoter region were significantly associated with metabolic syndrome.

Some studies have also reported the gender specific association of the SNP. A recent study in a Taiwanese population suggested a significant association of the minor allele of Pro12Ala with overweight, BMI and total cholesterol in females (Hsiao et al, 2015). Similarly, the gender specific association of the SNP with BMI and obesity was also reported in Spanish (Sanches et al, 2002) and Tunisian male populations (Ben Ali et al, 2009). The SNP has also

been significantly associated with obesity in Indian (Prakash et al, 2012; Bhatta et al, 2012) and Iranian population (Mirzaei et al, 2009).

Frederiksen et al, 2002 studied the effect of Pro12 Ala of PPARG gene in the Danish population. He noted that, Ala/Ala rare homozygotes showed significantly decreased diastolic pressure and serum triglycerides in comparison to Pro/Pro wild type homozygotes. A similar tendency was shown by insulin resistance as measured by HOMA-IRand the statistical significance did not change even after adjusting by age, gender and BMI (Frederiksen et al, 2002). In contrary, a significant association of the variant with hypertension was noted in the East Asian population in a recent meta-analysis carried out by Wang and Lu, 2012.

1.18.3 Transcription Factor 7-Like 2 (TCF7L2) (rs7903146 C>T)

Transcription factor 7 like 2 (TCF7L2) gene has been mapped to human chromosome 10. The protein encoded by the gene acts as a transcription factor and plays vital role during various developmental stages. Studies suggest that it is an effector of Wnt signalling pathway (Jin and Liu, 2008). The TCF7L2 gene contains 19 exons and the SNP rs7903146 is located at intron 4 as shown in figure 1.06.

Comparative study of the variant in eighteen eutherians as shown in figure 1.06, suggest that it is highly conserved across the group except in Humans and Rat indicating the specialized function of this evolved protein in those groups.

```
Human                TTT--TTAGATACTATAT-------AATTT
Bonobo               TTT--TTAGATATTATAT-------AATTT
Chimpanzee           TTT--TTAGATATTATAT-------AATTT
Gorilla              TTT--TTAGATATTATAT-------AATTT
Orangutan            TTT--ATAGATATTATAT-------AATTT
Gibbon               TTT--ATAGATATTATAT-------AATTT
Vervet-AGM           TTT--ATAGATATTATAT-------AATTT
Crab-eating macaque  TTT--ATAGATATTATAT-------AATTT
Macaque              TTT--ATAGATATTATAT-------AATTT
Olive baboon         TTT--ATAGATATTATAT-------AATTT
Marmoset             TTT--ATAGATATTATAT-------AATTT
Mouse Lemur          TTT--ATAGACATTATGC------------
Prairie vole         ---TCACGGACATTAGATGATCCAG-----
Ryukyu mouse         -----TTGGACATTCTGCGATCTAA-----
Mouse                -----TTGGACATTCTGCGATCTAA-----
Algerian mouse       -----TTGGACATTCTGCGATCTAA-----
Shrew mouse          -----TCGGACATTCTGCAATCTAA-----
Rat                  -----TCAGACACTCGGTGGTCTAA-----
```

Figure 1.06: Alignment of the rs7903146 flanking sequence in 18 eutherian mammals. The SNP and the flanking sequence represents the forward sequence of the genomic region. The nucleotide in the SNP region is the major allele. Source: www.ensemble.org

Table 1.06: Global minor allele frequency of rs7903146

Geographical Area	Allele frequency of rs7903146 (C/T) of TCF7L2 gene	
	C allele Frequency (%) (Major allele)	T allele Frequency (%) (Minor allele)
European	68%	32%
American	77%	23%
African	74%	26%
East Asian	98%	2%
South Asian	70%	30%

Table 1.06, shows the global minor allele frequency of rs7903146. The highest minor allele frequency (MAF) has been found in European populations and lowest was found in East Asian populations. This clearly indicates the ethic specific MAF of the SNP.

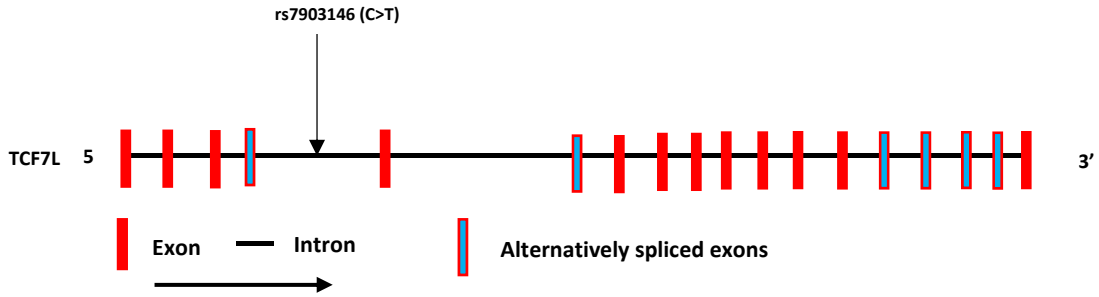

Figure 1.07: Schematic diagram of transcription factor 7 like 2 (TCF7L2) gene mapped on chromosome 10:112998590. The position of the SNP is indicated by arrow. Red rectangular box indicates the exonic region of the gene. Thick black line indicates the intronic region of the gene. Adapted from Ip et al, 2012.

The gene has been found to be expressed in various cell types such as adipose, pancreatic beta, lung, brain, liver, kidney and heart cells, but in low levels in skeletal muscle cells (Cauchi et al, 2006; Elbein et al, 2007).

The SNP (rs7903146) has been associated with type 2 diabetes in a genome wide association study (Grant et al, 2006). As far as Asian population is concerned, in a GWAS meta-analysis the SNP is significantly associated with T2D in Indian populations (Phani et al, 2016). It has also been associated with gestational diabetes in both Asian and Caucasian populations under dominant, recessive, allelic and heterozygote genetic model in a meta-analysis (Hou et al, 2017).

The SNP has been significantly associated with metabolic syndrome studied in a Malmö Preventive Project involving 16,143 non-diabetic subjects with mean follow-up of 23 years (Sjogren et al, 2008). In this study the component of metabolic syndrome does not follow any common definitions or criteria, but rather a modified definition has been used and the SNP is significantly associated with at least three of the four components of MetS even after correction for multiple testing in a follow-up study (Sjogren et al, 2008). The risk allele of rs7903146 has also been significantly associated with waist circumference in Emirati population (Saadi et al, 2008). A study in European population showed that the minor allele under the dominant genetic model is significantly associated with MetS, elevated glucose, higher VLDL and triglyceride, lower LDL and HOMA-B (Warodomwichit et al, 2009). The minor allele carriers of the SNP have been significantly associated with increased risk of

metabolic syndrome, higher insulin concentration, impaired insulin sensitivity, raised abdominal obesity, higher BMI and systolic blood pressure in European female subjects when compared with homozygous dominant genotypes (Philips et al, 2012). Similarly, in a mixed European population with a sample size of 450, the risk allele has been significantly associated with elevated blood pressure (systolic and diastolic blood pressure) under a dominant genetic model (Delgado-Lista, 2011).

Even though the minor allele of the SNP is not directly associated with metabolic syndrome itself, except few studies (Philips et al, 2012; Warodomwichit et al, 2009), however; it has been associated with the key components of metabolic syndrome. In an Interventional study, the baseline study shows that the minor allele is associated with lower levels of HDL and elevated levels of triglycerides, total cholesterol, fasting glucose and HOMA- IR (Calanna et al, 2012). Similarly, another Interventional study, the risk allele has been associated with elevated fasting glucose and BMI at baseline (Corella et al, 2013).

Due to the ethnicity specific risk associated with the SNP, it is imperative to understand the association of this SNP in the Nepalese population.

1.18.4 Fat mass and obesity-associated protein (FTO) (rs9939609 T>A).

Fat mass and obesity-associated protein is encoded by the gene FTO which is also known as alpha-ketoglutarate-dependent dioxygenase FTO. The gene is located on Human chromosome 16. The SNP rs9939609 is located in intron 1 of the FTO gene as shown in figure 1.09. The phylogenetic study of the SNP as shown in figure 1.08 suggests that this SNP (rs9939609) is conserved in majority of the mammals except the Humans, which might indicate its specialized function. The minor allele of the variant of the SNP (rs9939609) has been associated with obesity indices in Humans suggesting the involvement of the gene in energy metabolism, which has now been established using animal model studies (Fredriksson et al, 2008).

```
Human                CTGTGAATTTTGTGATGCACT
Bonobo               CTGTGAATTTAGTGATGCACT
Chimpanzee           CTGTGAATTTAGTGATGCACT
Gorilla              CTGTGAATTTAGTGATGCACT
Orangutan            CTGTGAATTTAGTGATGCACT
Gibbon               CTGTGAATTTAGTGATGCACT
Vervet-AGM           CTGTGAATTC-ATGATGCACT
Crab-eating macaque  CTGTGAATTC-ATGATGCACT
Macaque              CTGTGGATTC-ATGATGCACT
Olive baboon         CTGTGAATTC-ATGATGCACT
Marmoset             CTATGAATTCAGTGATGCACT
Dog                  CTGTGAATGCAGTGATCCACT
Cat                  CTGTGAATTCAGTGATCCACT
Horse                CTGTGAATTCAATGATCCACT
```

Figure 1.08: Alignment of the rs9939609 flanking sequence in 14 eutherian mammals. The SNP and the flanking sequence represents the forward sequence of the genomic region. The nucleotide in the SNP region is the major allele. Source: www.ensemble.org. The polymorphism is shown in red colour.

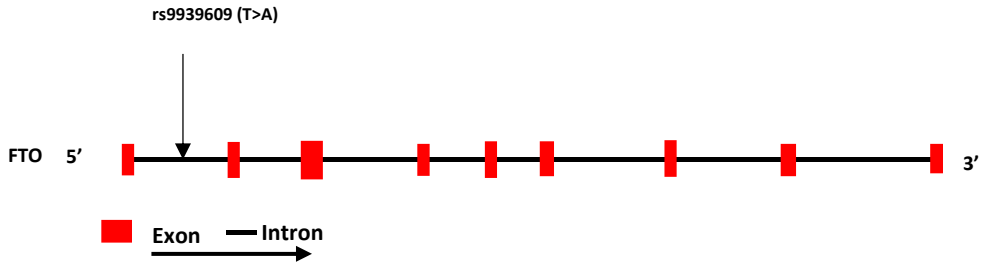

Figure 1.09: Schematic diagram of FTO gene showing exons and introns. The position of the SNP is indicated by arrow. Red rectangular box indicates the coding region of the gene. Thick black line indicates the intronic region of the gene.

Table 1.07 shows the global minor allele frequency of the SNP (rs9939609). The global MAF was reported to be 34%. The highest MAF was found in the African population (49%) while the lowest MAF was reported in East Asian population (17%). In South Asian population, the MAF was found to be 29%. These are the average MAF found in that region which might be different depending upon the ethnicity.

Table 1.07: Global minor allele frequency of rs9939609

Geographical Area	Allele frequency of rs9939609 (T/A) of FTO gene	
	T (major) allele frequency	A (minor) allele frequency
European	59 %	41 %
American	74 %	26 %
African	51 %	49 %
East Asian	83 %	17 %
South Asian	71 %	29 %
Global	66 %	34 %

Source: www.ensemble.org

Several studies have revealed that the SNP (rs9939609) has been associated with metabolic syndrome. The risk allele of rs9939609 has been significantly associated with metabolic syndrome in a Malmö Preventive Project involving 16,143 non-diabetics with mean age 49 years (Sjogren et al, 2008). The SNP has also been associated with MetS in a multi-ethnic study group (Canadians from South Asian and Chinese origin; Oji-Cree and Inuit originally from Greenland) with a sample size of 2121. The SNP has been associated with MetS under dominant genetic model either classified by IDA or NCEP/ATPIII definition (Al-Atter et al, 2008). This association might be due to population stratification, however; individual populations, i.e South Asians and Green land insuits under IDF and NCEP/ATPIII definitions respectively, were strongly associated with MetS (Al-Atter et al, 2008).

In a study comprising of 17,037 white individuals of European descent showed that the SNP is significantly associated with MetS along with its' key defining components: Waist circumference, fasting glucose, fasting HDL-C, Fasting triglycerides (Freathy et al, 2008). The SNP of interest, i.e. rs9939609 has also been significantly associated with weight, waist circumference and BMI in a female Filipino population (Marvelle et al, 2008).

A Dutch cohort study TRAILS (Tracking Adolescents' Individual Lives Survey) involving 1216 adolescent subjects showed that the SNP was significantly associated with weight, BMI, the sum of skinfold thicknesses, fasting glucose and metabolic syndrome (Liem et al, 2010)

Similarly, a case-control study conducted with sample size 1677 (case = 1096, control = 581) in a Japanese population showed that the risk allele is significantly associated with the MetS under additive genetic model (Hotta et al, 2011) . Furthermore, the SNP of interest along with other SNPs: rs8050136, rs1558902 and rs1421085 and 1121980 was also significantly associated with impaired fasting glucose, dyslipedimia and hypertension in the same study (Hotta et al, 2011).

The SNP (rs9939609) of the FTO was significantly associated with obesity in both the genders and with MetS in men under the dominant genetic model in a study carried out in 1967 adult Turks. The other commonly studied SNP rs1421085 was also significantly associated with metabolic syndrome in men in the same study (Guclu-Geyik et al, 2016).

Wang et al, 2010 studied an association of 41 SNPs with metabolic syndrome in the Chinese population with a total sample size of 236 with a number of case and control 108 and 128

respectively (Wang et al, 2010). Out of 41 SNPs studied, only rs8047395 was significantly associated with metabolic syndrome under a recessive genetic model with Odds ratio of 1.64. In this study the SNP of interest, i.e. rs9939609 has not been associated with metabolic syndrome in the Chinese population (Wang et al, 2010). Another study carried out in the Chinese populations by Cheung et al, 2011 in a cross-sectional and longitudinal study showed rs8047395 significantly associated with central obesity but not with the MetS in a 12 year longitudinal study. However, in this study the SNP of interest was not included and I would have been interesting to know if it has any association with MetS.

Similarly, Cruz et al (2010) could not find any association of the SNP of interest in the FTO gene, neither with T2D nor with metabolic syndrome in a Mexican population. Furthermore, Steemburgo et al, 2012 studied an association of two SNPs of FTO: rs8047395 and rs9939609 with metabolic traits as a predictor of metabolic syndrome in type 2 diabetes subjects with a sample size of 236 in a cross-sectional study in southern Brazil. It was found that one of the SNPs, rs8047395, was significantly associated with waist circumference, BMI and microalbuminuria while other, rs9939609, the SNP of interest, was not significantly associated with the above-mentioned traits.

Similarly, Ranjith et al (2011) also could not find any association of rs9939609 with MetS in young (≤ 45 years) South African, Asian Indians with Acute myocardial infraction classified by the presence and absence of metabolic syndrome.

1.19 Overall Aim

The main aim of the thesis if to analyse the association of the single nucleotide polymorphisms with Type 2 diabetes, metabolic syndrome and the components of metabolic syndrome.

The specific aim of the thesis is as follows:

a. To analyse an association of the SNP (rs57829442) of PPARGC1A with Type 2 diabetes in the United Kingdom population.

b. Analyse an association of the SNP (Gly482Ser) (rs8192678) with various components of metabolic syndrome (MetS) to unravel the overall and sub group effect of the polymorphism on various components of metabolic syndrome using various genetic models. The meta-analysis sought to clarify the controversy regarding an association of the SNP with the components of metabolic syndrome.

c. Analyse the prevalence of single nucleotide polymorphisms of the rs8192678 (Gly482Ser) of PPARGC1A, rs7903146 of TCf7L2 gene, rs9939609 of FTO and rs1801282 (Pro12Ala) of PPARGC1A genes in the Nepalese population.
d. Investigate the risk of the allele and genotypes of above mentioned SNPs with MetS and its components in the Nepalese population.
e. Analyse the strength of association between genotypes and components of MetS using various statistical models in the same population.

Chapter 2

Genetic polymorphism in intronic variant (rs57829442) of Peroxisome proliferator-activated receptor-γ coactivator-1 (PPARGC1A) gene and its relation to risk of Type 2 diabetes.

2.1 Introduction

An intronic variant rs57829442 lies on chromosome 4 of peroxisome proliferator-activated receptor-γ coactivator-1 (PPARGC1A) gene at chromosomal position 23803015. The Single Nucleotide Polymorphism (SNP) is located in intron 10 of the gene as shown in figure 2.01.

The position of the SNP (C/G) is on the reverse strand of the gene and the ancestral allele is C (https://www.ncbi.nlm.nih.gov).

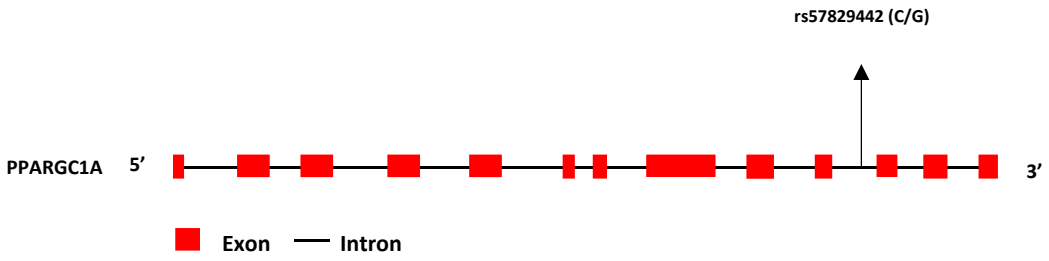

Figure 2.01: Schematic diagram of peroxisome proliferator-activated receptor-γ coactivator-1(PPARGC1A) gene. The position of the SNP (rs57829442) is indicated by arrow. Red rectangular box indicates the coding region of the gene. Thick black line indicates the intronic region of the gene.

The selected SNP (rs57829442) has a minor allele frequency (MAF) ranging from 6- 18% with average 7% in the European population (www.international genome.org.com) as shown in table 2.01. The lowest MAF is found in African (AFR) population (i.e 6%) whereas the highest MAF is found in East Asian population (i.e. 18%). If the recorded MAF is less than 5%, they are known as rare variants and a large sample size is required for conducting candidate gene association studies on these variants.

Table 2.01: Minor allele frequencies of rs57829442 in various populations.

Geographical region	Minor allele (G) frequency
Global	11%
AFR	6%
AMR	11%
EAS	18%
EUR	7%
SAS	15%

AFR = African, AMR = American, EAS = East Asian, EUR = European, SAS = South Asian. Source: 1000 Genome project phase 3 Ensemble.

Linkage disequilibrium is the non-random association of two or more alleles in the population and the tag SNPs are in the region of high linkage disequilibrium. The SNP (rs57829442) selected for the study lies in the Haplotype block (www.hapmap.org) which is in complete linkage disequilibrium with the tag SNP (rs 141974309).

Insilico analysis using Human Splice Finder (HSF) to find the splicing efficiency due to minor allele "G" of the SNP (rs57829442) shows that the SNP creates exonic splicing enhancer site in this intronic region of the gene where splicing enhancer proteins [SF2/ASF (IgM-BRCA1)] and SF2/ASF can bind and could potentially create a branch point (Desmet et al., 2009).

In addition to it, the new branch point has been created (669bp upstream the exon 11) due to a mutation (branch point motif sequence: aagtcAc) which might affect the splicing of the gene. The branch point in the intron is an essential signal in the recognition of 3' splice site. Enhancers and silencers are regulatory elements that could be as far as 10,000 to 100,000 nucleotides away from the gene and the mutations in them could disrupt the motif for transcription factors binding andchromatin conformation (Ward and Kellis et al, 2012).

Many splice variants of the gene (PPARGC1A) have been documented (Yoshioka et al., 2009) of which, a truncated splice variant of the gene has been shown to be expressed in skeletal muscle during exercise (Zhang et al., 2009). In addition to this, a novel splice variant known as L-PGC-1alpha has been found in human liver using intron 2 as a promoter and shown to have a role in hepatic gluconeogenesis (Felder et al., 2011). The role of the SNPs in the

splicing behaviour of the gene and in turn its role in disease causation has rarely been studied.

Conservation of the SNP of interest and the region around the SNP is some of the criteria used for the selection of rs57829442 in these association studies: rs57829442 and its flanking sequence are highly conserved among species (www. Ensemble. Org). Figure 2.02 shows that the SNP flanking region is highly conserved in mammals suggesting that it could have been preserved due to natural selection and must have some functional significance. Studies suggest that around 3% of the noncoding sequence is conserved even in distantly related mammals and contains functional variations (Drake et al., 2006). Most of the studies have focused on coding variants; however, noncoding variants are gathering attention to the researchers. Even though intronic regions of the genes are removed before they are translated into proteins, their functional role in the expression and splicing behaviour of the gene cannot be underestimated.

```
Human                ACATTTGTGACTTGATT-ATGA
Chimpanzee           ACATTTGTGACTTGATT-ATGA
Gorilla              ACATTTGTGACTTGATT-ATGA
Orangutan            ACATTTGTGACTTGATT-ATGA
Gibbon               ACATTTGTGACTTGATT-ATGA
Macaque              ACATTTGTGACTTGATT-ATTA
Marmoset             ACATCTGTGACTTGATT-ATTA
Tarsier              ACACTTGTGACTTGATT-ATTA
Mouse Lemur          ACACTTGTGACTTGATT-ATTA
Bushbaby             ATATATGGGGCTTGACT-CATC
Tree Shrew           ACACTTGTGACTAGATT-ATTA
Guinea Pig           ATGTTTGGGATTCTAAT-GTCA
Mouse                GCCCTTATGACTTGGTT-AGCA
Rat                  GTCCTTATGACTTGGTT-AGTA
Squirrel             ATACTTATGTCTTGATG-ATTA
Pika                 ------GTGATCTAATT-CTTA
Rabbit               ACACTTGTGATCCGATTTCTTA
Panda                ACCCTTATGAATTGATT-CTTA
Ferret               ACCCTTGTGACTTGATT-CTTA
Dog                  ACACTTGTGACTTGATT-CTTA
Cat                  ACACCTGTGACTTGATT-ATTC
Horse                ACACTTGTAACTTTA----TTA
Microbat             ACTCCTGTGACTCAATT-ATTA
Megabat              ATTCCTGTGGCTCAATT-ATTA
Cow                  ACAATTGTGACTCCACT-ATTA
```

Figure 2.02. Output of Ensemble phylogeny data showing the conservation of SNP (rs57829442) of the PPARGC1A gene as shown in red. Only the forward sequence is shown.

By using the online tool "Patch", the minor allele (G) of rs57829442 has been predicted to abolish the binding site for transcription factor c-Myb (Matys et al., 2003). The binding site for c-Myb is CAACT where the underlined nucleotide is replaced by minor allele G of rs57829442 (Matys et al., 2003).

In summary, the rationale for the selection of rs57829442 was based upon the conservation of the SNP in mammals, transcription factor binding site and potential influence in splicing behaviour of the gene.

2.2 Methods:

2.2.1 Information about study population.

The present case-control study was carried out using 183 DNA samples selected from panel 2, 3, 4, 5 and 6 from the Diabetes UK Warren 2 collection, and 167 from the control panel 1 and 2. The age and the gender of the subjects were supplied by European cell culture collections and shown in Table 2.02. The mean age of the T2D group and Human random control samples is 54.9 and 41 years respectively. The minimum age in case group was 37 and maximum 77 years; whereas minimum age was 24 and maximum 61 years in the control group.

Table 2.02: Number of male, female, sample size and age range of Type 2 diabetes (T2D) and Human random control (HRC) samples.

Population	Test group	Male	Female	Male/Female	Sample size	Age (years) mean
Warren 2 repository (Subjects with T2D)	Case	84	99	0.84	183	54.9 years (39-77)
Human random control (HRC) sample	Control	86	81	1.06	167	41 years (24-61)

T2D = Type 2 diabetes, HRC = Human random control sample.

2.2.2 Extraction of genomic DNA from U937 cell lines

DNA extraction from U937 cell line was carried out using PEQ Gold DNA minikit (Peqlab). Firstly, the cell suspension was centrifuged and the cell pellet was resuspensed in lysis buffer, Proteinase K and RNAse were added for the digestion of proteins and RNA. The DNA binding buffer was added and mixed thoroughly and the solution was loaded on the column and centrifuged. The flow-through was discarded and the column was washed with DNA wash buffer. The column was dried by centrifugation and the DNA was eluted by using Elution buffer. The quantification of DNA was carried out by measuring absorbance at 260 nm and 280 nm. The DNA ratio was calculated by taking the ratio of absorbance at 260nm and 280nm. The DNA was stored at -20°C for future use. Extracted DNA from this cell line was used as positive control in PCR based restriction fragment length polymorphism (PCR-RFLP).

2.2.3 Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) genotyping.

Specific primers were designed using Primer-Blast (Ye et al., 2012) to span the region of interest and were purchased from Sigma-Aldrich. A 100 µM stock solution was prepared with deionized water and stored at -20°C until required.

Optimization of PCR was carried out by using various concentrations of DNA (10ng, 30ng, 50ng, 70ng, and 100ng), primers (0.1µM, 0.5µM) and various annealing temperatures (53, 55, 57, 59, 61, 63, 65°C). Only one parameter was varied at each time while others were kept constant. The conditions that provided the most efficient and specific amplification with a single band and minimal primer-dimers evident was used. This resulted in the use of 10 ng DNA, 65°C annealing temperature and 0.2µM concentration of primer.

PCR with the optimized parameters was carried out using thin walled 0.2ml PCR tubes (Thermo Fischer Scientific) and thermocycler (Biometra) by adding 25µl RED Taq (Sigma-Aldrich) to a total volume of 50 µl PCR reaction mix. RED taq is a proprietary PCR reaction mix of Sigma-Aldrich which contains: [REDTaq ReadyMix PCR Reaction Mix, with MgCl2, Catalog Number R2648, 20 mM Tris-HCl, pH 8.3, with 100 mM KCl,3 mM MgCl2, 0.002 %

gelatin, 0.4 mM dNTP mix,(dATP, dCTP, dGTP, TTP), stabilizers, and 0.06 unit/mL of Taq DNA Polymerase. The amount of RED taq was used according to the protocol provided by the manufacturer.

After optimization of the PCR reaction, all the DNA samples were amplified using the amplification conditions as outlined in table 2.03.

2.2.4 Agarose gel electrophoresis and restriction digest:

The amplified PCR products were analysed using agarose gel electrophoresis. One percent of agarose was used for the visualization of the amplified PCR product and 1X of gel red (Biotium) was added for the staining of the DNA molecules. Working concentration (1X) of Tris-buffer EDTA was used as a buffer for the separation of amplified PCR product. Ten microliter of the PCR product was loaded in each well and the electrophoresis was carried out for 45 minutes at 80volts of electricity. The bands were visualized under UV light and photographs were taken using a gel documentation system (Peq gold Fusion, Peqlab, Germany).

The PCR product (5 µl) was digested by adding 5 units of 5000 units/ml of Tsp451 in a 20 µl total volume reaction including the PCR product. The restriction digestion of the PCR product was carried out by taking 5 µl of the PCR amplified product, 1 µl of Tsp451 (New England bio-labs) and 2 µl of the smart cut buffer (1X CutSmart® Buffer: 50 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, 100 µg/ml BSA, pH 7.9 @ 25°C). The reaction was incubated for 1 hour at 65°C. One and a half percent of agarose was used for agarose gel electrophoresis of the Tsp451 digested PCR product. Tris-buffer EDTA was used for the agarose gel electrophoresis.

2.2.5 Restriction fragment length polymorphism:

After the digestion of the PCR amplified product with restriction endonuclease, the genotypes of the single nucleotide polymorphism can be detected by the fragments of different length, the technique is known as PCR based restriction fragment length polymorphism. The sequence of the PCR product is 698bp in size as shown in figure 2.03. The

position of the SNP is indicated with blue and underlined nucleotide. The position of the forward primer and reverse primer is shown in red and green colour respectively.

5'**TAGGCCACACCCCTCTAGTC**TGTTCCTAAAATAAGACTAGATTTATTGGCAAAGTGAAATTTATAGCCTCTTTTATTTAGCTCT
TATTCTTCGGAGACTCATGGCTTAAAATGGCCTACATTTTAAGACTGTATTTCTTAAATTATTGTCTTTGGACCACCTGCATCAA
AATAGCTGAGGTGTGCTTGTTAAAAATGAAGGTTGTAGAACCTTATCTTACATTTTTTTTTAGTACAACTAGATAAAGCCCAAT
CATTTGCATTTTTAATCAGCACCCCAAGTAATTTTTATGTATAGTAGAGTTTAAGAACCACTCTTTTGAGGACGTCAGAGTTTGT
TCATCTCAGTATTAGAATTCTGAGTTAATGAGAAATGTAATAGTTTTGTAAGCCAGGTCAGTTTCATAACAATTTTGATTCATAAT
CAAC/GTCACAAATGTTCAAATATTTAATAGGTATTGTTCCATGATTTGAAAAAAGAATGCACTATACAGATTTCTTTTCTAACAC
ATCAAGTTCATCTGAGAGTAGAACAGAGACAGATAGGAAAGGTAGAAAAGAGTAGAGGTGAAGAGAAGAATCTATGTGAG
CAAGAAGAGATTGATACTGAAGAAATGGGAATTAATACTTGGTTCAAATGTAGTCTTTCCTTCCATTGTAGTGTATGTTTTGCT
CTAATT**GCCATGCTCAGTGGGGTTTC**3'

Figure 2.03: Sequence of the PCR product. The SNP of interest is underlined. Red coloured sequence represents forward primer and green coloured sequence represents reverse primer. The forward and reverse primers used for the amplification are shown below: The reference sequence of the PCR product was obtained from NCBI data base (https://www.ncbi.nlm.nih.gov).

The restricted products of Tsp451 digestion should yield the following: the product of common homozygote (CC) = 426bp +269bp, heterozygote (GC) = 426bp +269bp +698bp and rare homozygote (GG) = 698bp as shown in figure 2.03.

Table 2.03: Polymerase chain reaction cycle condition for the amplification of the region of interest.

Condition	Temperature(°C)	Time
Initial Denaturation	94°C	3 minutes
Number of cycles	30	
Denaturation	94°C	30 seconds
Annealing	65°C	30 seconds
Extension	72°C	45 seconds
Final extension	72°C	5 minutes

Table 2.04: The sequence of forward primer (PGC-11) and reverse primer (PGC-12). Red colour represents forward primer and green colour represents reverse primer.

Primers	Sequence
Forward primer-PGC-11	5'TAGGCCACACCCCTCTAGTC3'
Reverse primer-PGC-12	5'GAAACCCCACTGAGCATGGC3'

2.2.6 Quality control

The DNA from the U937 cell line was amplified and subjected to restriction digestion and the genotype was a heterozygote as shown in figure 2.04. The PCR product of U937 DNA was used as a positive control for every PCR-RFLP analysis. Furthermore, 40 samples of the amplified PCR product, 20 from both the Warren 2 and human random control samples, were sent to university of Dundee for sequences purposes to verify the amplification of the desired region.

2. 2.7 Statistical/ *Insilico* analysis

Allele and genotype frequency were calculated using the simple formula $p^2+q^2 = 1$, where p is major allele frequency and q is minor allele frequency and the genotype frequency was calculated by using $(p^2+q^2 = 1)^2$. To analyse whether the alleles or genotypes were in Hardy-Weinberg equilibrium, the case and control data were subjected to chi-squared test. The critical Chi-squared value was 3.84 and the p-value was set at 0.05 level. The Odds Ratio with 95% confidence interval was calculated using SPSS V 21. Clustal Omega (Mc William et al., 2013) was used for the alignment of the sequences of the samples. This program can align three or more sequences efficiencly and with greater degree of accuracy.

The DNA baser software (www.DnaBaser.com) was used for the construction of the consensus contig from the number of samples sequenced. A contig (Figure 2.06) was constructed with higher QV (confidence scores) discarding any false positive variants. The genotype of the amplified samples was identified by using BLASTn (https://blast.ncbi.nlm.nih.gov) from NCBI and they were all as expected.

2.3 Results

The optimized PCR method was used to amplifying 183 cases and 167 control group DNA samples and a representative image is shown in figure 2.04. The size of the Tsp451 digested PCR product revealed either three bands 698bp, 426bp and 269bp which is indicative of the heterozygote form (CG), or 2 bands corresponding to 426bp and 269bp indicative of the common homozygote (CC). The size of the bands was confirmed by using PCR marker as shown in figure 2.04A. The control U937 DNA was shown to be heterozygote (figure 2.04B).

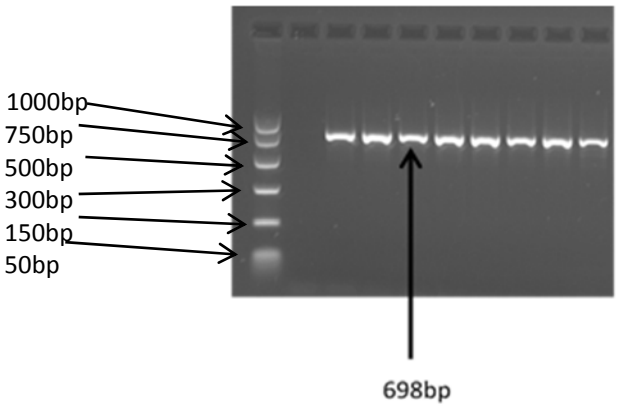

Figure 2.04A: The size of the PCR amplified product

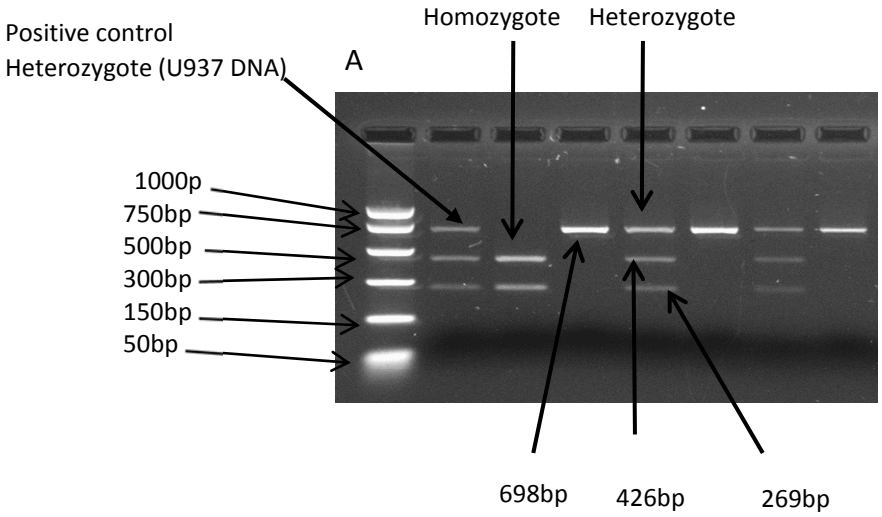

Figure 2.04B: Fragments after restriction digestion by Endonuclease Tsp451.

Chi-squared test was used to test for the deviation of genotypes from Hardy Weinberg equilibrium in both T2D and HRC samples. The result showed that the genotypes of T2D and HRC samples were both in Hardy Weinberg equilibrium. In case of T2D samples, 87.98% were common homozygotes and 12.02% were heterozygotes. Similarly, in case of HRC samples, 89.82% were common homozygotes and 10.18% were heterozygotes. Rare homozygotes were not found in any of the samples studied.

The minor allele frequency was found to be 6.1% in T2D samples and 5.1% in human random control samples. No significant difference in genotype and minor allele frequencies was observed in T2D and human random control samples. In case of T2D samples, 10

heterozygotes were found in males and 12 of them were in females. In HRC samples, 7 heterozygotes were found in males and 10 in females.

Table 2.05. The genotype and allele frequency of Type 2 diabetes and HRC samples.

Populations	Genotype frequency			Allele frequency	
	CC Number (Frequency)	CG Number (Frequency)	GG Number (Frequency)	C Number (Frequency)	G Number (Frequency)
T2D	161 (0.88)	22 (0.12)	0 (0)	344 (0.94)	22(0.06)
HRC	150 (0.90)	17 (0.10)	0(0)	317(0.95)	17(0.05)

T2D = Type 2 diabetes population, HRC = Human random control population

Under the allele counting model or additive model the OR_G (Odds ratio of G allele) was found to be 1.19 with 95% CI (0.62 to 2.29). Even though the odds ratio is greater than 1, it is not statistically significant.

The odds ratio of CG (heterozygous) versus CC (common homozygotes) was 1.21 with 95% confidence interval of 0.62-2.36. The value is not statistically significant as the confidence interval includes the value 1. This indicates that CG genotype increases the odds of type 2 diabetes by 1.21 compared to subjects with CC genotype. The odds ratio under allelic and heterozygote models are alike. The recessive model of the inheritance is not possible as no recessive homozygotes were found in any of the groups. The association of the minor allele with type 2 diabetes was also tested using Chi-squared test. The $\chi 2$ value of 0.331 was obtained which shows that the association is not statistically significant.

Table 2.06. Genotype information from the samples from Sanger sequencing.

PCR amplicons from T2D subjects.	Warren 2 sample name	Genotype	PCR amplicons from control (HRC) subjects.	HRC sample name	Genotype
P37	WR0656	CG	C21	C0208	CC
P38	WR1102	CC	C22	C0737	CC
P39	WR0057	CC	C23	C0034	CC
P40	WR2057	CC	C24	C0152	CC
P41	WR0211	CC	C25	C0855	CC
P44	WR0066	CC	C26	C0849	CC
P45	WR0511	CC	C27	C0941	CC
P47	WR0755	CC	C28	C0143	CC
P48	WR0844	CC	C30	C0100	CC
P49	WR1548	CC	C33	C0150	CC
P51	WR1270	CC	C34	C0157	CC
P52	WR1430	CC	C35	C0121	CC
P53	WR1417	CC	C37	C0938	CC
P54	WR1811	CC	C38	C0917	CC
P57	WR0626	CC	C39	C0068	CC
P58	WR0585	CC	C40	C0090	CC
P59	WR2526	CC	C43	C0045	CC
P71	WR1298	CC	C44	C0027	CC
P74	WR0907	CC	C47	C0204	CC
P75	WR1680	CC	C48	C0857	CC

T2D = Type 2 diabetes, PCR = polymerase chain reaction, HRC = Human random control samples.

2.4 DNA Sequencing

The genotype of the amplified product was verified by sequencing randomly selected 20 samples each from cases and control group. The sequence of the amplified product matched the results from PCR-RFLP analysis confirming that the genotype data generated using the PCR based RFLP are robust and reliable.

The sequencing results of the randomly selected 40 samples show that the amplified product was as predicted. The genotype of all the sequenced samples is the same as derived from RFLP as shown in table 2.06, which indicates that the methodology used was robust.

The information based upon of both the forward and reverse sequence indicates that sample P37 is heterozygote as expected and the rest of the samples were common

homozygotes. The sequencing result suggests that the information from the fragment length polymorphism is accurate and reliable. Alignment of the sequenced samples and comparing it with the reference sequence suggests the reliability of the methodology used.

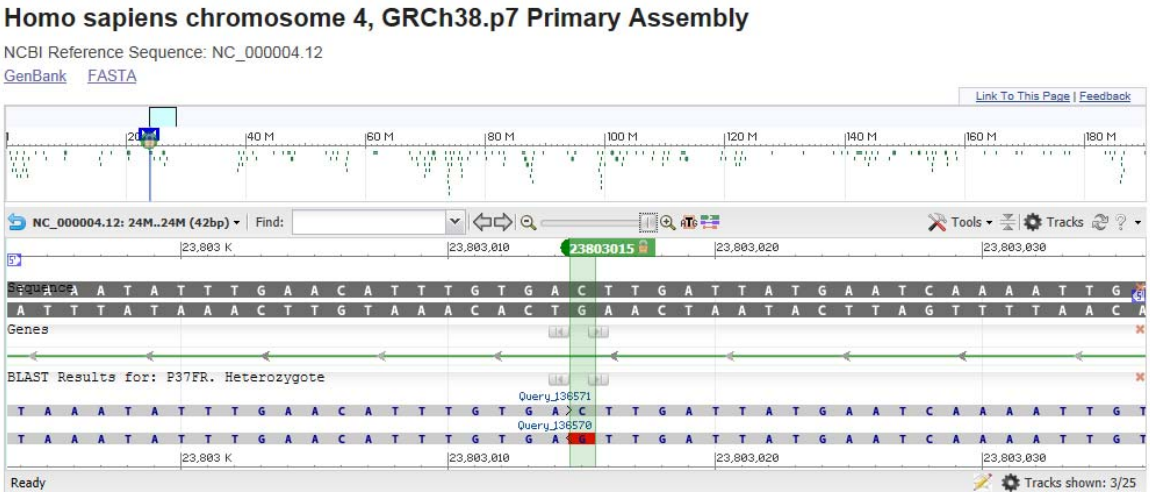

Figure 2.05: Screen image showing heterozygote at chromosome position 23803015 of sample P37. The position of the SNP is at 23803015. Both the forward and reverse sequences were analysed for heterozygote using Blastn. The red coloured base G (minor allele) was found in reverse sequence, whereas base C was found in forward sequence as shown in figure 2.05.

All the forward and reverse sequence of the amplicon aligned shows that there is a greater degree of homology between the samples. It suggests that the expected region of the genome has been amplified by the polymerase chain reaction.

There are some single nucleotide polymorphism of which some of them might be true variants while others could be due to sequencing errors.

There are around 123 reported single nucleotide polymorphism, insertions and deletions including the SNP of interest (rs57829442) in the amplified region (https://www.ncbi.nlm.nih.gov/snp). Excluding the SNP of interest, only one SNP (rs370917209) with minor allele frequency of 1.1% has been reported from 1000 genome project while the frequency of the others is unknown, they could be very rare or could be the errors during sequencing (A global reference for human genetic variation, the 1000 Genomes Project Consortium, Nature 526, 68-74 (01 October 2015) doi: 10.1038/nature15393).

A contig sequence was generated by DNA Sequence Assembler v4 (2013) to analyse the true variation ignoring the sequencing errors. A consensus contiguous sequence was needed to represent the true variation within the samples as shown in the figure 2.06.

```
  1 gaaaccccac tgagcatggc aattagagca aaacatacac tacaatggaa ggaaagacta
 61 catttgaacc aagtattaat tcccatttct tcagtatcaa tctcttcttg ctcacataga
121 ttcttctctt cacctctact cttttctacc tttcctatct gtctctgttc tactctcaga
181 tgaacttgat gtgttagaaa agaaatctgt atagtgcatt ctttttttcaa atcatggaac
241 aatacctatt aaatatttga acatttgtga cttgattatg aatcaaaatt gttatgaaac
301 tgacctggct tacaaaacta ttacatttct cattaactca gaattctaat actgagatga
361 acaaactctg acgtcctcaa aagagtggtt cttaaactct actatacata aaaattactt
421 ggggtgctga ttaaaaatgc aaatgattgg gctttatcta gttgtactaa aaaaaaatgt
481 aagataaggt tctacaacct tcatttttaa caagcacacc tcagctattt tgatgcaggt
541 ggtccaaaga caataattta agaaatacag tcttaaaatg taggccattt taagccatga
601 gtctccgaag aataagagct aaataaaaga ggctataaat ttcactttgc caa
```

Figure 2.06: A contig sequence generated using DNA baser v 4.36.0.

Contig is a contiguous segment of the genome in which the order of the bases is assigned according to high confidence scores. When the contig sequence is compared with the known genomic sequences, any changes in the sequence in the contig can be detected. It is usually helpful in identifying mutations with high level of accuracy. A total of 40 samples with 20 each from T2D and human random control were sequenced. Even though there were many base changes in the individual samples, it would require a contig sequence which could be used for the identification of the mutation with greater degree of accuracy and confidence. The DNA baser (DNA Sequence Assembler v4 (2013), Heracle BioSoft, www.DnaBaser.com) uses QV (confidence scores) to construct a contig sequence. The contig sequence is constructed by the software using algorithm where the sequences with threshold criteria are included. The sequence was then blasted using nucleotide blast (https://blast.ncbi.nlm.nih.gov) to find any changes in bases with respect to the GRCh38 genome assembly. The change in contig sequence were then compared with individual sequenced (both forward and reverse) samples to confirm the change in the nucleotide bases. The contig sequence in figure 2.06 is 658bp and 96.8% similar with the reference sequence having 15 mismatches and 6 gaps.

Table 2.07: Chromosomal position and base change in novel single nucleotide polymorphism.

GRCh38.P7 assembly Chromosome coordinate	Samples	Reference base	Contig base	Polymorphism	genotype
23802753	P41F, P45F,C34F	A	C	SNP	AC
23802770		G	T	SNP	
23802768		T	INDEL	SNP	
23802773		C	G	SNP	
23802774		A	G	SNP	
23802776		A	T	SNP	
23802780	C48F	T	A	SNP	TA
23802781	P54F, C22F, C25F, C26F, C27F, P75F	A	C	SNP	AC
23802783	P41F, P45F, C24F, C25F, C26F, C43F, C47F,P75F	A	C	SNP	AC
23802784	C48F(C>A)	C	N	SNP	CA
23802786	P59F, C22F, C24F, C25F	A	C	SNP	AC
23802793	P51R, P53R, P54R, C23R	A	G	SNP	AG
23802797	P59R, C23R, C24R, C25F, C25R (Homozygous recessive) C43F, C43R (indel), C47R	A	G	SNP	AG (C25 is GG)
23803359		A	G	SNP	
23803382		A	C	SNP	
23803383		A	T	SNP	

SNP = single nucleotide polymorphism, Indel = insertion-deletion

The contig was compared with reference sequence GRCh38.P7 primary and chromosome coordinates were mapped. The novel single nucleotide polymorphisms have been identified at chromosome coordinates: 23802753, 23802780, 23802781, 23802783, 23802784, 23802786, 23802793, and 23802797. Most of the samples in which novel SNPs were identified were heterozygotes except C25 which was rare homozygote.

“F” and “R” are the forward and reverse primers used for the sequencing

In the chromosomal position 23802793 and 23802797, the SNPs lie in the forward sequence and the other SNPs lie in the reverse sequence.

2.5 Discussion

The present case-control study was conducted to determine if there was any association of the intronic variant rs57 829442 with type 2 diabetes (T2D) in the United Kingdom (UK) population. The study suggests that there is no significant association between rs57829442 variant of the PPARGC1A gene and risk of type 2 diabetes within the population studied. A complex disease like T2D might be influenced by many genes and each having a small contribution to the overall risk. The study lacks gene environment interaction as no metabolic data were known about the subjects. PPARGC1A gene is one of the most studied candidate genes for type 2 diabetes (Franks et al., 2011) and single nucleotide polymorphisms in the gene have been associated with type 2 diabetes and related phenotypes as evident from meta-analysis (Barroso et al., 2006) but the genome wide association studies carried out in the UK population could not show this association (Zeggini et al 2007).

A very recent study carried out by Park et al., 2017 shows that the intronic variants (rs10517030, rs10517032 and rs10212638) of the PPARGC1A gene were positively associated under a dominant genetic model with the prevalence of type 2 diabetes in the Korean population. It was conducted in 8842 Korean adults of aged 40 -60 years. The study also showed that the interaction between rs10517030 and energy intake could pose the risk of T2D in the population studied. In contrary, the genome wide association studies in relation to risk of T2D in Korean population did not show any association with the PPARGC1A gene (Lee et al., 2008).

Even though the genome-wide association studies have identified variants associated with complex disease such as T2D, the variants might have small effect size and could only account for a minor contribution from heritability (Manolio et al., 2009). One of the major challenges in genome-wide association studies to analyse disease risk model is that the large number of variants are genotyped for a small number of subjects. There is also a problem of multicollinearity if the SNP are in linkage disequilibrium among themselves (Choi et al., 2016) to which multiple regression is very sensitive and unstable (Wang et al., 2005).

In the present study there are also some limitations, such as the case and control groups are not exactly age matched where the mean age of cases is higher than controls and the overall

sample size is relatively smaller. This would suggest that some of the younger subjects of the control group might possibly manifest type 2 diabetes later in their life and distort the odd ratio. Even though the present study did not show any association with T2D, there is also a possibility that some of the control subjects in their later life might develop type 2 diabetes and this might possibly increase the allele frequencies of the cases and skew the results toward the positive association. Larger sample size with age matched group is required to analyse any association of the SNP with type 2 diabetes.

The Sanger sequencing of the 698bp region of the intronic region of the gene also presented an opportunity to explore other variation within that region. Although many variants were found in the individual case and control samples, a consensus sequence was constructed that represents the high-quality variants with a greater degree of confidence, and showed that the region harbours some novel genetic variants.

From the present study, with the best of our knowledge, we have also identified some novel polymorphic regions of the genome (Table 2.07). Before submitting the novel SNPs identified to the human genome data bases, the polymorphism found in those genomic regions must be further validated using combination of RFLP and Sanger sequencing methods as these novel SNPs identified might be due to sequencing errors.

In conclusion, the present case-control study showed that the SNP (rs57829442) is not associated with the risk of type 2 diabetes. Further investigation by using a larger sample size is warranted. The sequenced region of the amplicon shows some novel single nucleotide polymorphisms which needs further validation.

Chapter 3

Association of the rs8192678 (Gly482Ser) variant of peroxisome proliferator activator receptor gamma coactivator 1 (*PPARGC1A*) gene with the components of metabolic syndrome: a meta-analysis.

3.1 Introduction

Due to the lack of clinical and anthropological data available for the Warren 2 repository DNA samples, it was not possible to analyse an association of the SNPs with those variables. As already described in the introduction section of this thesis, the SNPs of this gene has been associated with various components of metabolic syndrome (MetS). An association of the most studied SNP, i.e. Gly482Ser of this gene with various components of metabolic syndrome using different genetic model has been looked at in this chapter. This will help in resolving the controversies regarding the association of the SNP with various components of MetS which has already been described in the introduction section. A meta-analysis conducted in this chapter will seek to unravel the association of the SNP with the components of metabolic syndrome, of which some have not been conducted before, for example, an association of the SNP (rs8192678) (Gly482Ser) with lipid levels, while others (BMI, fasting blood glucose, systolic and diastolic blood pressure) will be an update on existing meta-analysis.

Peroxisome proliferator activator receptor gamma coactivator 1 (PPARGC1A), an activator of many nuclear receptors, is involved in hepatic gluconeogenesis (Yoon et al, 2001), mitochondrial fatty acid oxidation (Vega et al, 2000), adaptive thermogenesis (Puigserver et al, 1998) and mitochondrial biogenesis (Wu et al, 1999) suggesting its vital role in energy metabolism.

Some variants of this gene have already been associated with obesity and related traits. The minor allele of this gene has been associated with higher body fat mass as studied in Korean children (Ha et al, 2015). The obesity related markers such as higher body weight, BMI, body fat mass and body fat ratio were present in the minor allele carriers of Gly482Ser genotypes (Vazquez-de Mercado et al., 2015). Similarly, abdominal obesity along with hyperinsulinemia

and insulin resistance was associated with the minor allele of the gene (Weng et al, 2010). The minor allele of this variant has also been associated with excess weight gain in male subjects with type 1 diabetes receiving diabetes therapy (Deeb et al, 2009) although the minor allele did not show any association with metabolic syndrome in Danish population (Ambye et al, 2005). On the other hand, the risk allele of Gly482Ser variant failed to show an association with body fat mass in Indian population (Vimaleswaran et al, 2006).

The minor allele of the Gly482 has also been associated with hypertension in young individuals (Sookoian et al, 2005) and the meta-analysis carried out by Vimaleswaran, 2008 also suggests an association of minor allele with elevated blood pressure in young adults. It is also associated with high blood pressure in middle aged men (Oberkofler et al, 2003), in men with type 2 diabetes (Cheurfa et al, 2004) while reduced risk of hypertension was observed in Danish Caucasians with the minor allele (Anderson et al, 2005).

An emerging body of literature suggests that PPARGC1A plays a vital role, directly or indirectly, in lipid metabolism. PPARGC1A is responsible for the activation of farnesoid X receptor (FXR) which decreases the level of triglycerides in the liver (Zhang et al., 2004). It also regulates the activity of the human hepatic lipase gene (LIPC) which is involved in plasma lipid metabolism (Rufibach et al., 2006). Furthermore, the expression of PPARGC1A in conjunction with its nuclear receptor hepatocyte nuclear factor 4α (*HNF4α*) in the liver increases a range of apolipoprotein which are responsible for triglyceride metabolism and very low-density lipoprotein (VLDL) metabolism (Rhee et al., 2006).

It has been noted the role of this gene in glucose transport, especially in skeletal muscle (Benton et al., 2010) and pancreatic beta-cells (Oropeza et al., 2015), acting as a co-activator for cholesterol 7-α-hydroxylase (*CYP7A1*) gene, an enzyme essential for cholesterol metabolism (Shin et al., 2003). Studies carried out in liver specific heterozygous Ppargc1a (notation of gene in mice) showed impaired hepatic fatty acid oxidation and lipid accumulation (Jennifer et al., 2009) indicating the essential role of the gene in lipid metabolism. The heterozygous mutant mice in the fed state showed higher circulating serum triglycerides, which indicates the upregulation of the genes involved in lipoprotein synthesis.

The SNP has been associated with reduced clearance of non-esterified fatty acid (Franks et al., 2007). Recent studies suggest a significant association of the Gly482Ser mutation with

higher levels of TC and LDL-C levels in homozygous dominants, in participants from an Iranian population (Mirzaei et al., 2012); likewise, in the control group, the mean HDL-C level was significantly higher in Gly482 genotypes under the dominant genetic model (Shoukouhi et al., 2015). The levels of triglycerides in the pre-obese and obese group were significantly higher in homozygous dominant genotypes under a dominant genetic model in a Mexican population (Vazquez-de Mercado et al., 2015). The other studies such as: Andrulionyte et al, 2005; Hiu et al, 2007; Zhang et al, 2007; Goyenechea et al, 2008 and Niktin et al., 2010 failed to show any significant difference in the levels of serum lipids in different genotype groups. Due to the conflicting results in association of Gly482Ser with components of metabolic syndrome, the present meta-analysis has been conducted to seek to address the inconsistencies and to determine if further insights into the role of this variant in association with biochemical and metabolic criteria can be derived.

3.1. 1 Aim

The main aim of the meta-analysis was to analyse an association of the SNP (Gly482Ser) (rs8192678) with various components of metabolic syndrome. The study also seeks to unravel the overall and sub group effect of the polymorphism on various components of metabolic syndrome using various genetic models. The current meta-analysis intends to clarify the controversy regarding an association of the SNP with the components of metabolic syndrome.

3.2. Methods

3.2.1. Search strategy

An electronic search of the relevant published articles was conducted using web of science ((http://apps.webofknowledge.com), PubMed ((http://www.ncbi.nlm.nih.gov/pubmed), EMBASE and Cochrane library. All the research articles before 06/11/2016 have been searched by two individuals PB (PhD student) and RK (principal supervisor) independently using the keywords, "Peroxisome proliferator activator receptor gamma coactivator 1", PPARGC1A, PGC-1, PGC-1alpha, PGC1alpha alone or in conjunction with “polymorphism”, “Gly482Ser” and “rs8192678”.

3.2.2 Eligibility criteria

Articles have been included if they are: original research articles; published in the English language; conducted on humans; studies must include genotype data related to rs8192678 (Gly482Ser) with its effect on components of metabolic syndrome; case-control study, cross-sectional study and intervention studies may be included. In case of intervention studies, only the data before the intervention had been carried out have been included. Articles that included data and corresponding biological data, including, total cholesterol, triglycerides, HDL-C, and LDL-C and the corresponding BMI, fasting plasma glucose concentration and blood pressure were selected.

3.2.3 Exclusion criteria

Articles that do not contain full genotype data for individuals with corresponding biological data have been excluded. The reported data relating to the participants under the age of 18 or those taking statins were excluded from the meta-analysis. Study data presented in the format of the interquartile range are also excluded from the study as it was not possible to combine this with other data formats.

3.2.4 Data extraction

The following information was obtained from the selected peer reviewed articles: authors (articles recorded as first author et al., and year of publication.) ethnicity, age, gender ratio, body mass index, sample size, the mean value of the lipid levels (HDL-C, LDL-C, TG, TC), fasting plasma glucose (FPG), systolic blood pressure (SBP) and diastolic blood pressure (DBP) of the corresponding genotypes along with standard deviation. The measurement units of plasma lipids in mg/dl were converted into mmol/l, whenever required. The following conversion factor was used: for HDL-C, LDL-C and TC (1mmol/L = 38.67mg/dl), Triglyceride (1 mmol/L = 88.57mg/L) and for glucose (1mmol/L = 18.01 mg/dl). If more than one data sets were found within a single published article, it was treated as a discrete data under the same author with appropriate qualification of distinction of the data. If the sample size varies with each lipid variable as in the case of Vohl et al, 2005, the lowest possible sample size will be considered. When the metabolic data in three genotype groups were available, the mean and standard deviation of the two genotypes were combined to analyse the data under either the dominant or the recessive genetic models. The two data sets from

Okauchi et al, 2008 representing male and female subjects with or without type 2 diabetes have been combined into one single group.

3.2.5 Statistical analysis.

Meta-analysis was performed using STATA 13.1 (StataCorp. 2013. *Stata Statistical Software: Release 13*). College Station, TX: StataCorp LP. Metan command of the STATA 13.1 was used for the present meta-analysis. All the data were analysed as mean ± Standard deviation (SD). The article with more than one subpopulation, such as populations with or without type 2 diabetes, CAD (Coronary artery disease), glucose tolerance group and obesity was treated as a separate data set under the same author.

The pooled weighted mean difference (WMD) with 95% confidence interval (CI) was used for the meta-analysis and the heterogeneity of the data was determined using the Chi-squared based Q-test and I^2 (0-100%) statistics. The Random effect model (DerSimonian and Laird et al., 1986) was used when there was significant heterogeneity within the study data and the fixed effect model (Mantel–Haenszel et al., 1959) was used when there was no significant heterogeneity. Heterogeneity refers the variation in the outcome of the study across the study. The heterogeneity is measured in Cochran's Q which follows a chi-squared distribution with k-1 degrees of freedom, where k being the number of studies.

When the heterogeneity is low, it is assumed that the studies are conducted under the similar condition and fixed effect model was considered appropriate. A random effect model was considered more appropriate in studies with higher heterogeneity where the outcome being estimated are not similar but follows some distribution. A p-value of the Q-test greater than 0.10 was considered to have no significant heterogeneity and in that case, the fixed effect model was used and is called the inverse variance method. If the p-value was less than 0.10 it was considered as having significant heterogeneity and the random effect model (Der Simonian-Laird) (Der and Laird, 1986) was used.

Subgroup analysis was then conducted and includes a comparison of Asian and non-Asian populations. All the p-values were two sided unless and otherwise stated and p-value ≤ 0.05 was considered statistically significant. The box of the forest plot represents the effect size and the horizontal line represents the 95 % confidence interval. The dashed vertical line with diamond represents overall effect.

3.3 Results

3.3.1 Characteristics of included studies.

After screening, 18 articles met all the inclusion criteria for the meta-analysis with some of the articles having more than one data set. The process of selection is outlined in Figure 3.01. In summary, a total of 27 data sets, involving 7131 subjects was extracted from 18 articles were used in the meta-analysis. A total of 18 studies were eligible for the dominant gene model while only 13 for the recessive model. In the dominant genetic model, the "GG" genotypes are compared with the combined "GA+AA" genotype groups where G is the major and A is minor allele. Similarly, in recessive genetic model the "AA" genotypes are compared with the combined "GG+GA" genotype groups.

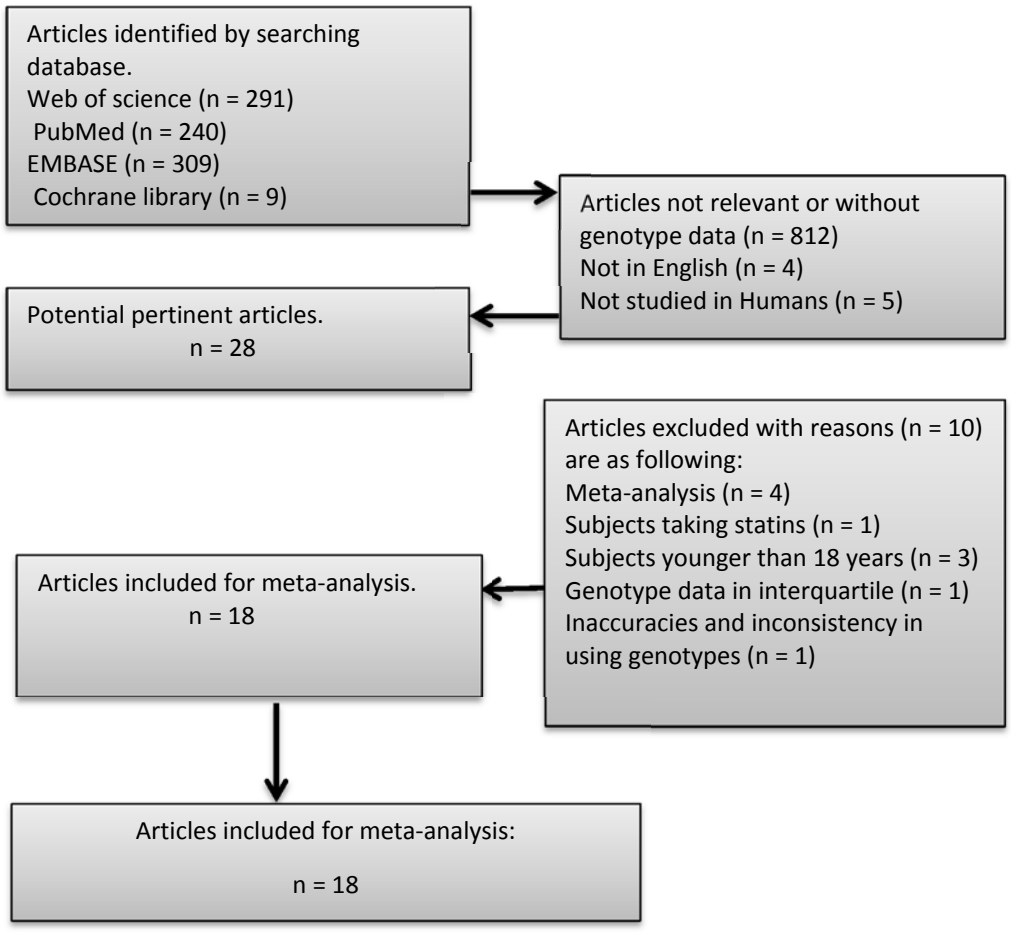

Figure 3.01. Flow chart showing the search strategy, systematic screening and selection process of the articles used in this study.

Table 3.01: Characteristics of the studies included in the meta-analysis

Number	Authors	Year of study	Number of Subjects	Sex (M/F)	Ethnicity	Groups	Data included
1	Ek 1 *et al*.	2001	198	93/105	Caucasian	Glucose tolerant subjects	BMI, HDL-C, TG, TC, FPG
2	Ek 2 *et al*.	2001	293	134/159	Caucasian	Glucose tolerant subjects	BMI, HDL-C, TG, TC, FPG
3	Andrulionyte *et al.*	2004	770	387/383	Mixed	STOP-NIDDM baseline study	BMI, HDL-C, LDL-C, TG, TC, FPG, SBP, DBP
4	Ambye *et al*.	2005	2255	1108/1147	Caucasian	Non-diabetic subjects	BMI, HDL-C, LDL-C, TG, TC, FPG, SBP, DBP
5	Vohl 1 *et al*.	2005	173	NA	North American	Non-diabetic and morbidly obese subjects	BMI, HDL-C, LDL-C, TG, TC, FPG, SBP, DBP
6	Vohl 2 *et al*.	2005	84	NA	North American	Diabetic and morbidly obese subjects	BMI, HDL-C, LDL-C, TG, TC, FPG, SBP, DBP
7	Hui 1 *et al*.	2007	96	NA	Asian	Subjects with NAFLD	BMI, HDL-C, LDL-C, TG, TC, FPG
8	Hui 2 *et al*.	2007	96	NA	Asian	Subjects without NAFLD	BMI, HDL-C, LDL-C, TG, TC, FPG
9	Zhang 1 *et al*.	2007	282	NA	Asian	Subjects without T2D	BMI, HDL-C, LDL-C, TG, TC, SBP, DBP
10	Zhang 2 *et al*.	2007	263	NA	Asian	Subjects with T2D	BMI, HDL-C, LDL-C, TG, TC, SBP, DBP
11	Goyenechea *et al*.	2008	180	93/87	Caucasian	Base line study in obese subjects	BMI, HDL-C, LDL-C, TC, FPG, SBP, DBP
12	Okauchi *et al*.	2008	155	74/81	Asian	Male and Female subjects with T2D	BMI, HDL-C, TG, TC, FPG,
13	Chae 1 *et al*.	2010	184	NA	Asian	Subjects with PCOS	BMI, HDL-C, TG, TC, FPG, SBP, DBP
14	Chae 2 *et al*.	2010	256	NA	Asian	Subjects without PCOS	BMI, HDL-C, TG, TC, FPG, SBP, DBP
15	Niktin 1 *et al*.	2010	132	NA	Caucasian	Subjects without CAD	BMI, HDL-C, LDL-C, TG, TC, SBP, DBP
16	Niktin 2 *et al*.	2010	313	NA	Caucasian	Subjects with CAD	BMI, HDL-C, LDL-C, TG, TC, SBP, DBP
17	Zhang *et al*.	2010	241	125/116	Asian	Subjects with T2D at base line study	BMI, HDL-C, LDL-C, TG, TC, FPG

18	Geloneze *et al*.	2012	55	47/8	South American	Obese subjects	BMI, HDL-C, LDL-C, TG, TC, FPG, SBP, DBP
19	Miraezi *et al*.	2012	229	NA	Middle Eastern	Overweight subjects	BMI, HDL-C, LDL-C, TG, TC, FPG
20	Nishida *et al*.	2015	112	Males only	Asian	Overweight subjects with dyslipidaemia, high BP and blood sugar	BMI, HDL-C, LDL-C, TG, TC, FPG, SBP, DBP
21	Shokouhi 1 *et al*.	2015	173	82/91	Middle Eastern	Subjects without T2D	BMI, HDL-C, LDL-C, TG, TC, FPG
22	Shokouhi 2 *et al*.	2015	173	73/100	Middle Eastern	Subjects with T2D	BMI, HDL-C, LDL-C, TG, TC, FPG
23	Vazques-Delmercado 1 *et al*.	2015	153	NA	Central American	Normal weight subjects	BMI, HDL-C, LDL-C, TG, TC, FPG
24	Vazques-Delmercado 2 *et al*.	2015	144	NA	Central American	Pre-obese subjects	BMI, HDL-C, LDL-C, TG, TC, FPG
25	Vazques-Delmercado 3 *et al*.	2015	78	NA	Central American	Obese subjects	BMI, HDL-C, LDL-C, TG, TC, FPG
26	Tai *et al*.	2016	177	56/62	Asian	Subjects with severe obesity (Class 3)	BMI, HDL-C, LDL-C, TG, TC
27	Tobina *et al*.	2016	119	49/70	Asian	Elderly healthy subjects	BMI, HDL-C, LDL-C, TG, TC

NA = not available; BMI = body mass index; HDL-C = high density lipoprotein; LDL-C = low density lipoprotein; TG = triglyceride; TC = total cholesterol; FPG = Fasting plasma glucose; SBP = systolic blood pressure; DBP = diastolic blood pressure; T2D = type 2 diabetes; PCOS = polycystic ovary syndrome; NAFLD = non-alcoholic fatty liver disease; CAD = coronary artery disease.

Table 3.01, contains all the information regarding the meta-analysis. It includes the sample size, number of article and the number of data sets with in the article.

3.3.2 Associations between PPARGC1A Gly482Ser polymorphism and components of metabolic syndrome.

The weighted mean difference (WMD was calculated for the 27 data sets and are presented in table 3.02. Under the dominant genetic model, 27 data sets were used for BMI and HDL-C: similarly, 22 data sets for LDL-C and 26 for TG and TC were analysed. Under the recessive model, 19 data sets for BMI, HDL; 14 for LDL, 18 for TG and 19 for TC, 13 for SBP, 23 for FPG were used for the analysis. . Under the recessive model, 19 data sets for BMI, HDL; 14 for LDL, 18 for TG and 19 for TC, 23 for FPG, 13 for SBP and DBP were used for the analysis.

Table 3.02, shows the results of the summary of the meta-analysis. Two different genetic models (dominant and recessive) were used for the study and according to the heterogeneity of the outcome, fixed or random effect model was used.

3.3.3 Association between PPARGC1A Gly482Ser polymorphism and BMI under dominant model.

In the overall studied population for BMI, the genotype "GG" had a higher BMI when compared to the "GA+AA" genotype (WMD = 0.13, 95%CI = -0.03 to 0.30, p-Value = 0.10) (Figure 3.02) although this did not reach statistical significance. The heterogeneity was not statistically significant for this outcome (Het.Chi-squared = 18.64 (df = 26), Het.p-Value = 0.85; I^2 = 0.0%).

In non-Asian, BMI was higher in "GG" genotype group in comparison to "GG + GA" genotype group, but no statistically significant difference was observed (WMD = 0.04, 95% CI = 0.16 to 0.24, p-Value = 0.68) (Figure 3.02). However, BMI was found significantly higher in "GG" genotype in comparison to "GA+AA" genotype group (under the dominant genetic model) in Asian populations (WMD = 0.32, 95% CI = 0.04 to 0.60, p-value = 0.02) (Figure 3.02). In the non-Asian (Het.Chi-squared = 8.38 (df = 15), Het.p-Value = 0.91; I^2 = 0.00%) and Asian (Het.Chi-squared = 7.69) (df = 10), Het.p-Value = 0.66; I^2 = 0.00%) populations, no significant heterogeneity was observed.

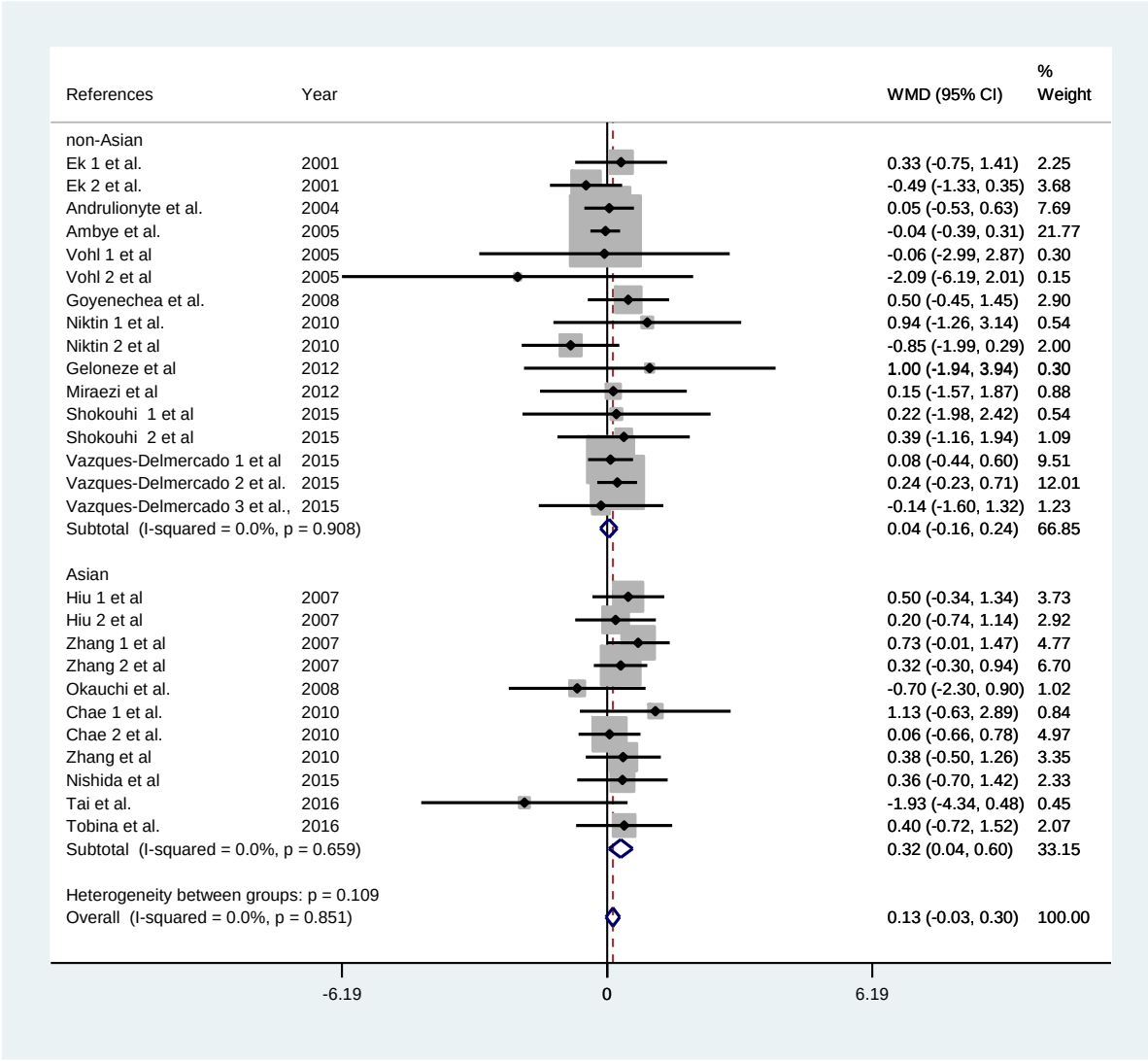

Figure 3.02: Forest plot showing BMI levels between two genotype groups (dominant genetic model: GG versus GA+AA) of the Gly482Ser (rs8192678) polymorphism under continuous fixed effect model.

In the Asian population, BMI is significantly higher even after exclusion of Tai et al, 2016 and Nishida et al, 2016 in "GG" genotype group in comparison to "GG + GA" genotype group (dominant genetic model) (WMD = 0.35, 95% CI = 0.06 to 0.65, p-Value = 0.02) (Figure 3.03) with no significant heterogeneity (Het.Chi-squared = 4.29 (df = 8), Het.p-Value = 0.83; I2 = 0.00%).

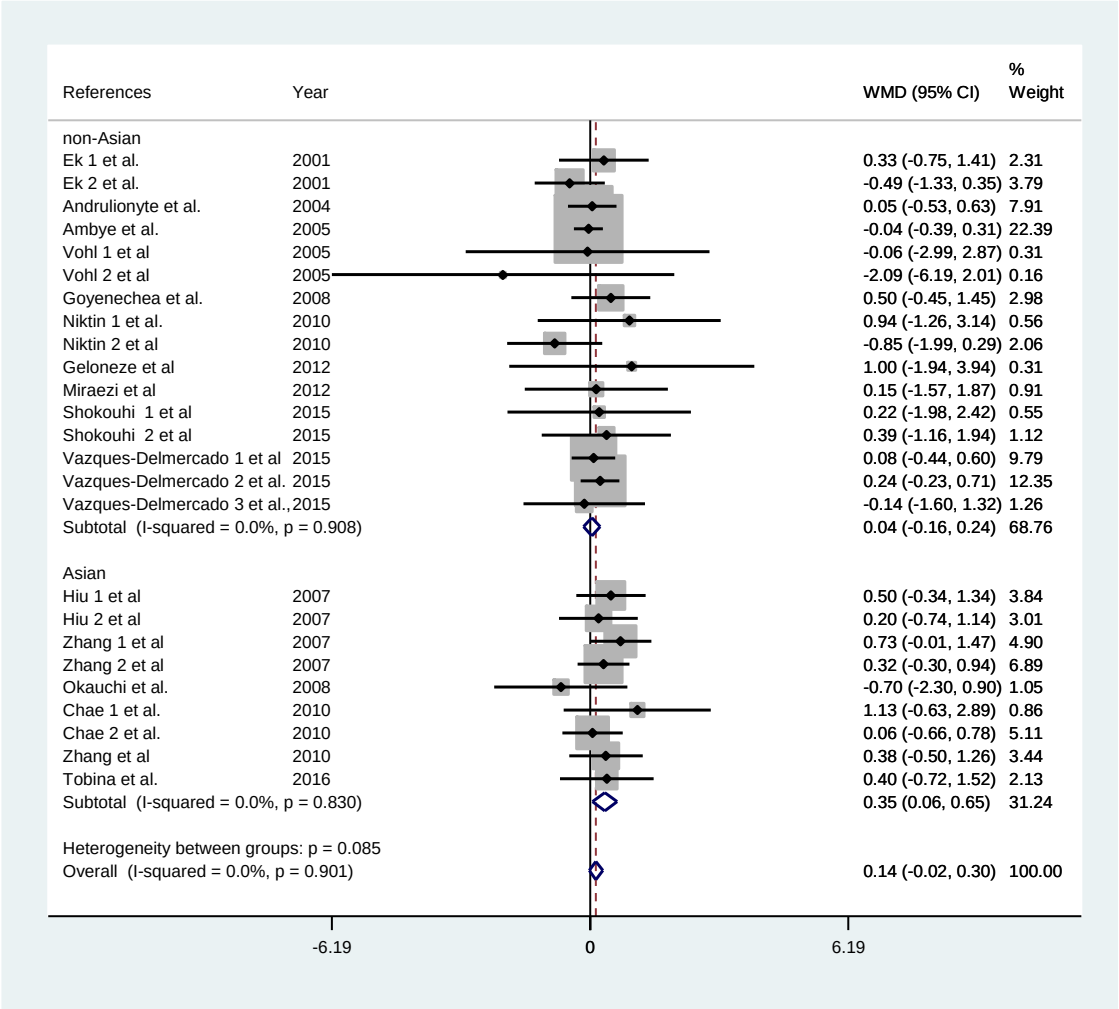

Figure 3.03: Forest plot showing difference in BMI between two genotype groups (dominant genetic model: GG versus GA+AA) of the Gly482Ser (rs8192678) polymorphism under the continuous fixed effect model after exclusion of Nishida et al, 2015 and Tai et al, 2016.

3.3.4 Association between PPARGC1A Gly482Ser polymorphism and BMI under recessive model.

In the recessive model, "GG+GA" genotypes had higher BMI in comparison to "AA" genotypes (WMD = -0.08, 95%CI = -0.34 to 0.18, p-Value = 0.54) (Figure 3.04). No statistical significance was achieved in difference in BMI between two genotype groups under recessive genetic model using the fixed effect model. The heterogeneity was not statistically significant in this outcome (Het.Chi-squared = 15.14 (df = 18), Het.p-Value = 0.65; I^2 = 0.0%).

In non-Asian population, higher BMI was observed in “AA” genotype group in comparison to “GG + GA” genotype group, but no statistically significant difference was observed (WMD = 0.08, 95% CI = -0.25 to 0.40, p-Value = 0.65) (Figure 3.04). The llevels of BMI were found to be significantly higher without being significant in “GG+GA” genotypes in comparison to the “AA” genotype group (under recessive genetic model) in Asian populations (WMD = 0.34 95% CI = -0.76 to 0.08, p-value = 0.12) (Figure 3.04). No significant heterogeneity was observed both in non-Asian (Het.Chi-squared = 10.89 (df = 11), Het.p-Value = 0.45; I^2 = 0.00%) and Asian (Het.Chi-squared = 7.69) (df = 10), Het.p-Value = 0.66; I^2 = 0.00%) populations.

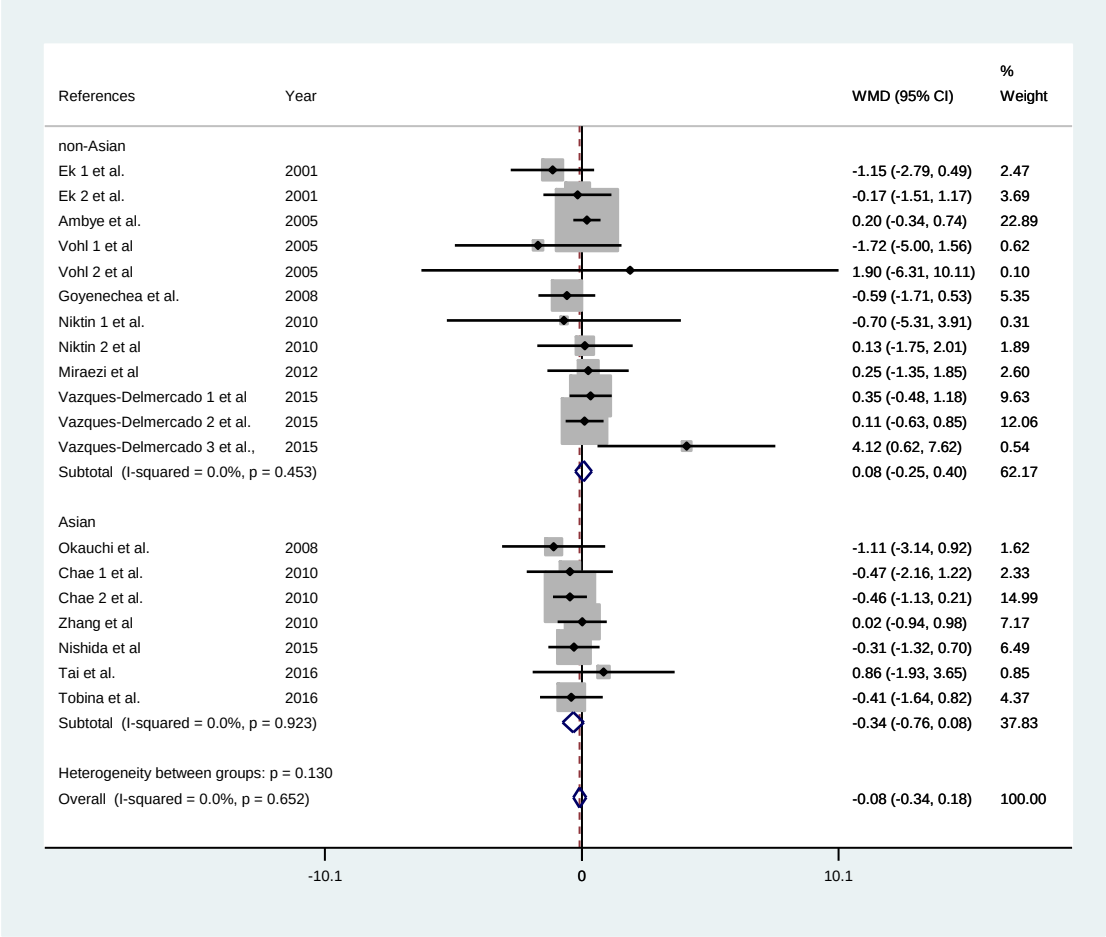

Figure 3.04: Forest plot showing difference in BMI between two genotype groups (recessive genetic model: AA versus GG+GA) of the Gly482Ser (rs8192678) polymorphism under continuous fixed effect model.

3.3.5 Association between *PPARGC1A* Gly482Ser polymorphism and plasma HDL-C under dominant model.

Under the dominant model "GG" genotypes had lower levels of HDL-C in comparison to combined (GA+AA) genotypes (WMD = -0.02, 95%CI = -0.04 to 0.0, p-Value = 0.13) (Figure 3.05). No statistically significant difference in levels of HDL-C was found between two genotype groups under dominant genetic model using the random effect model. The heterogeneity was not statistically significant for this outcome (Het.Chi-squared =36.47; (df = 26), Het.p-Value = 0.08; I^2 = 28.70%).

In non-Asian population, lower levels of HDL-C was observed in "GG" genotype group in comparison to "GA + AA" genotype group, but no statistically significant difference was observed (WMD = -0.01, 95% CI = -0.04 to 0.01, p-Value = 0.37) (Figure 3.05) between the "GG" and "GA+AA" genotype groups. Lower levels of HDL-C levels were found in "GG" genotype in comparison to "GA+AA" genotype group in Asian population, but no significant difference was observed (WMD = -0.03 95% CI = -0.08 to 0.01, p-value = 0.16) (Figure 3.05) between the "GG" and "GA+AA" groups. No significant heterogeneity was observed both in Asian (Het.Chi-squared = 14.23 (df = 10), Het.p-Value = 0.00; I^2 = 29.7%) and non-Asian (Het. Chi-squared = 21.36 (df = 15), Het.p-Value = 0.13; I^2 = 29.8%) (Figure 3.05) populations.

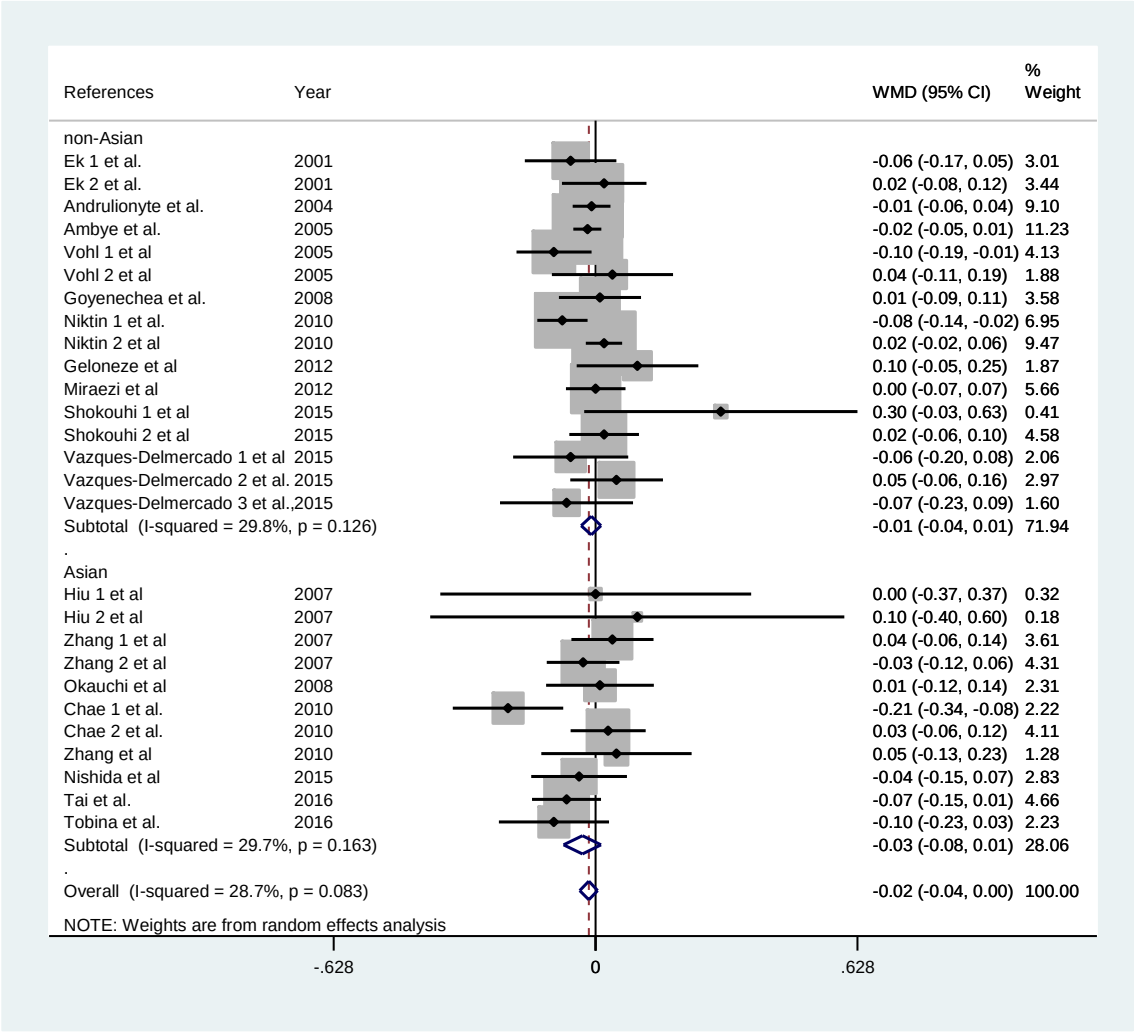

Figure 3.05. Forest plots showing difference in HDL-C levels between two genotype groups (genetic model: GG *versus* GA+AA) of the Gly482Ser (rs8192678) polymorphism under continuous random effect model.

3.3.6 Association between PPARGC1A Gly482Ser polymorphism and plasma HDL-C under recessive model.

The recessive model showed that "AA" genotypes had lower levels of HDL-C in comparison to combined (GG+GA) genotypes (WMD = -0.02, 95%CI = -0.06 to 0.02, p-Value = 0.38) (Figure 3.06). No statistically significant difference in levels of HDL-C was found between two genotype groups under recessive genetic model using the random effect model. The heterogeneity was statistically significant for this outcome (Het.Chi-squared = 44.39; (df = 18), Het.p-Value = 0.00; I^2 = 59.4 %).

In the sub-group analysis, no difference in levels of HDL-C levels were found in “AA” genotype in comparison to “GG+GA” genotype group in Asian population (WMD = -0.00 , 95% CI = -0.06 to 0.05 , p-value = 0.90 (Figure 3.06). In non-Asian population, lower levels of HDL-C was observed in “AA” genotype group in comparison to “GG+GA” genotype group, but no statistically significant difference was observed (WMD = -0.03, 95% CI = -0.08 to 0.03, p-Value = 0.33) (Figure 3.06). No significant heterogeneity was observed in Asian (Het.Chi-squared = 10.01 (df = 6), Het.p-Value = 0.12; I^2 = 40 %) population but significant heterogeneity was obtained in non-Asian populations (Het. Chi-squared = 34.37 (df = 11), Het.p-Value = 0.00; I^2 = 68.0%) (Figure 3.06).

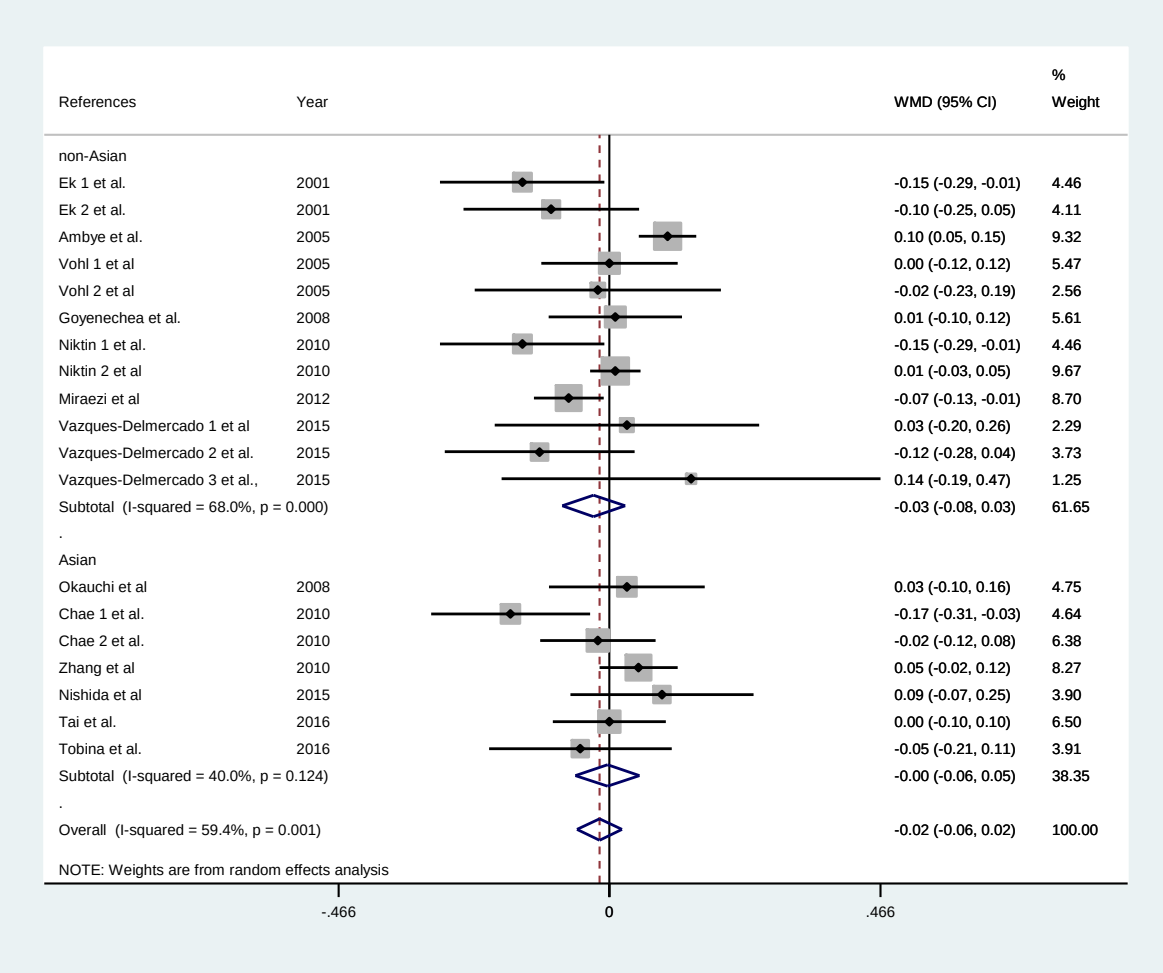

Figure 3.06. Forest plots showing difference in HDL-C levels between two genotype groups (recessive genetic model: AA *versus* GG+GA) of the Gly482Ser (rs8192678) polymorphism under the random effect model.

3.3.7 Association between PPARGC1A Gly482Ser polymorphism and plasma LDL-C under dominant model.

The outcome of the 15 studies with 22 data sets suggests that "GG" genotypes had higher levels of LDL-C in comparison to combined (GA+AA) genotypes (WMD = 0.01., 95%CI = 0.01 to 0.12, p-Value = 0.89) (Figure 3.07). No statistically significant difference in levels of LDL-C was found between two genotype groups under dominant genetic model using the random effect model. The heterogeneity was statistically significant for this outcome (Het.Chi-squared = 167.16; (df = 21), Het.p-Value = 0.00; I^2 = 87.4%).

In non-Asian population, higher levels of LDL-C was observed in "GG" genotype group in comparison to "GG + GA" genotype group, but no statistically significant difference was observed (WMD = 0.03, 95% CI = -0.11 to 0.18, p-Value = 0.67) (Figure 3.07). Lower levels of LDL-C levels were found in "GG" genotype in comparison to "GA+AA" genotype group in Asian population, but no statistically significant difference was observed (WMD = -0.03, 95% CI = -0.1 to 0.12, p-value = 0.63) (Figure 3.07). Statistically significant heterogeneity was observed in non-Asian (Het. Chi-squared = 154.50 (df = 13), Het.p-Value = 0.00; I^2 = 91.6%) (Figure 3.07) but not in Asian (Het.Chi-squared = 10.94 (df = 7), Het.p-Value = 0.14; I^2= 36.0%) populations.

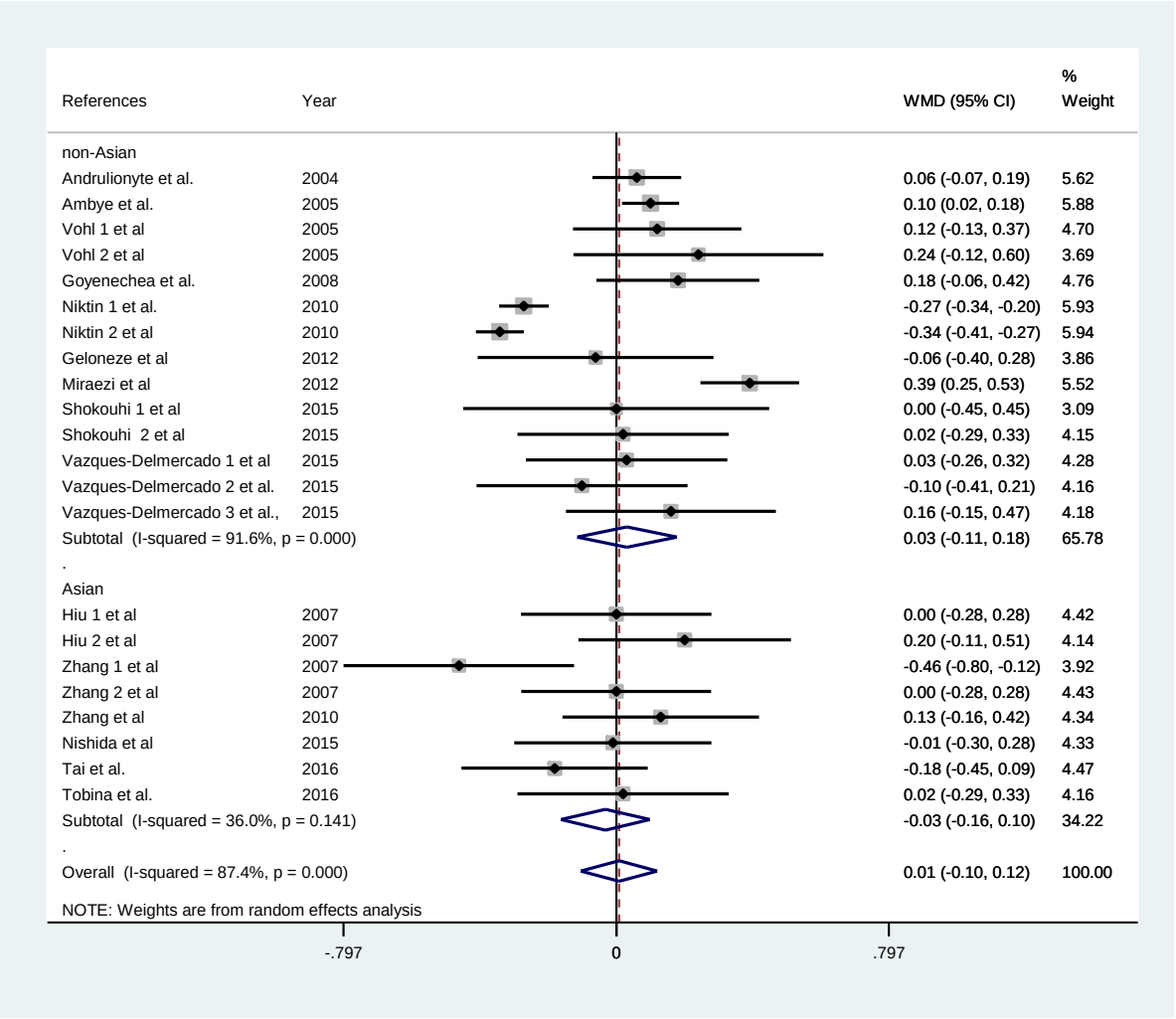

Figure 3.07. Forest plot showing difference in LDL-C levels between two genotype groups (dominant genetic model: GG versus GA+AA) of the Gly482Ser (rs8192678) polymorphism under the random effect model.

3.3.8 Association between PPARGC1A Gly482Ser polymorphism and plasma LDL-C under recessive model.

The "AA" genotypes had lower levels of LDL-C in comparison to combined (GG+GA) genotypes under a recessive model (WMD = -0.05, 95%CI = -0.11 to 0.11, p-Value = 0.10) (Figure 3.08). No statistically significant difference in levels of LDL-C was found between two genotype groups under dominant genetic model using a fixed effect model. The heterogeneity was not statistically significant for this outcome (Het.Chi-squared = 16.80; (df = 13), Het.p-Value = 0.21; I^2 = 22.6%).

In non-Asian population, lower levels of LDL-C was observed in “AA” genotype group in comparison to “GG+GA” genotype group, but no statistically significant difference was observed (WMD = -0.04, 95% CI = -0.11 to 0.02, p-Value = 0.20) (Figure 3.08). Lower levels of LDL-C levels were found in “AA” genotype in comparison to “GG+GA” genotype group in Asian population, but no significant difference was observed (WMD = -0.09, 95% CI = -0.24 to 0.06, p-value = 0.26 (Figure 3.08). Significant heterogeneity was observed in Asian (Het.Chi-squared = 6.88 (df =3), Het.p-Value = 0.08; I^2 = 56.4%) but not in non-Asian populations (Het. Chi-squared = 9.61 (df = 9), Het.p-Value = 0.38; I^2 = 6.3%) (Figure 3.08).

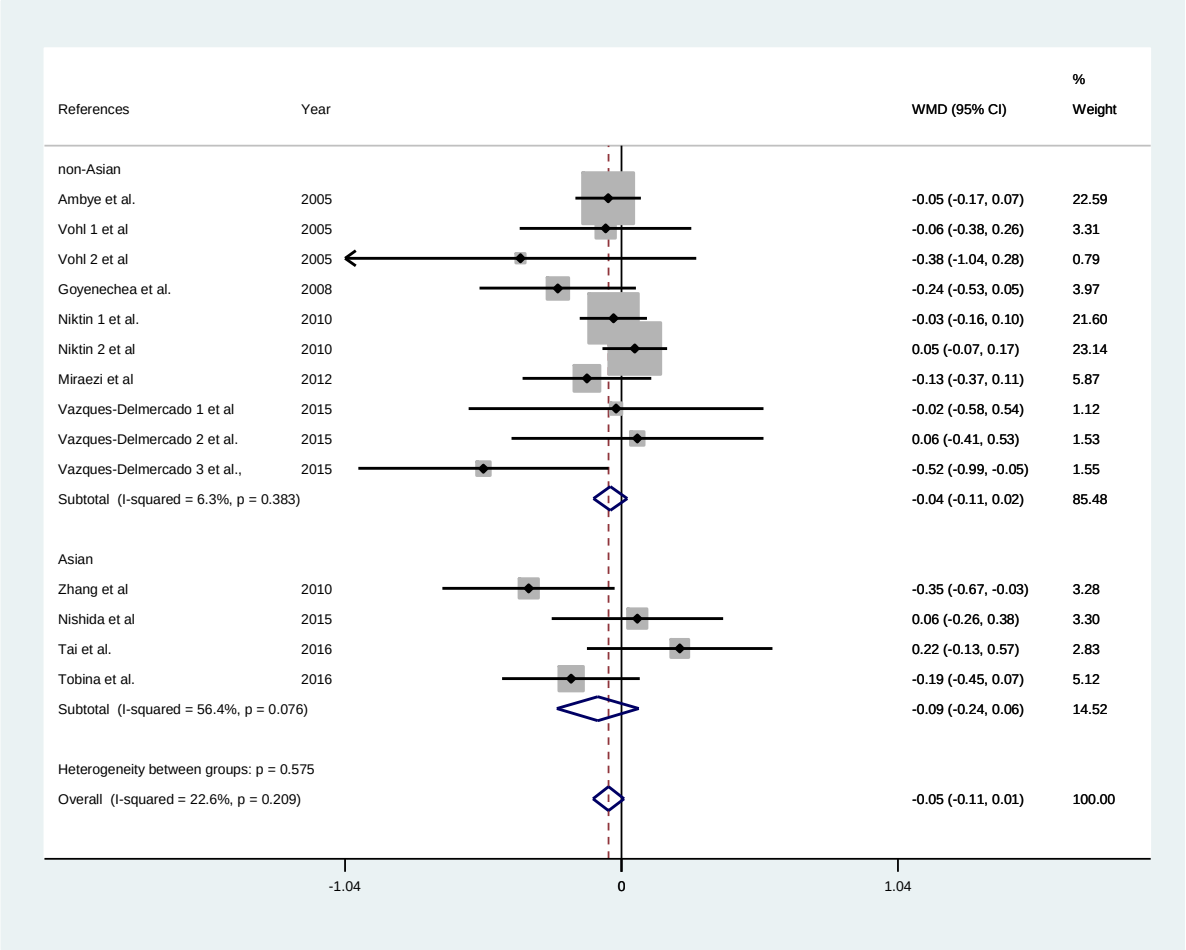

Figure 3.08. Forest plot showing difference in LDL-C levels between two genotype groups (recessive genetic model: AA versus GG+GA) of the Gly482Ser (rs8192678) polymorphism under fixed effect model.

3.3.9 Association between PPARGC1A Gly482Ser polymorphism and plasma TG under dominant model.

The dominant model shows that "GG" genotypes had higher levels of TG in comparison to combined (GA+AA) genotypes (WMD = 0.01., 95%CI = -0.04 to 0.06, p-Value = 0.74) (Figure 3.09). No statistically significant difference in levels of TG was found between two genotype groups under this model using the random effect model. The heterogeneity was statistically significant for this outcome (Het.Chi-squared = 59.02; (df = 25), Het.p-Value = 0.00; I^2 = 57.6%).

In non-Asian population, no difference in levels of TG were observed in "GG" and "GA + AA" genotype groups (WMD = 0.00, 95% CI = -0.06 to 0.06, p-Value = 0.92) (Figure 3.09) between the "GG" and "GA+AA" genotype groups. Higher levels of TG levels were found in "GG" genotype in comparison to "GA+AA" genotype group in Asian population, but no significant difference was observed (WMD = 0.04 ,95% CI = -0.03 to 0.11, p-value = 0.26) (Figure 3.09) between the "GG" and "GA+AA" groups. Statiscially significant heterogeneity was observed in non-Asian (Het. Chi-squared = 49.74 (df = 14), Het.p-Value = 0.00; I2 = 71.9 %) but not in Asian (Het.Chi-squared = 8.63 (df = 10), Het.p-Value = 0.56; I^2 = 0.0%)

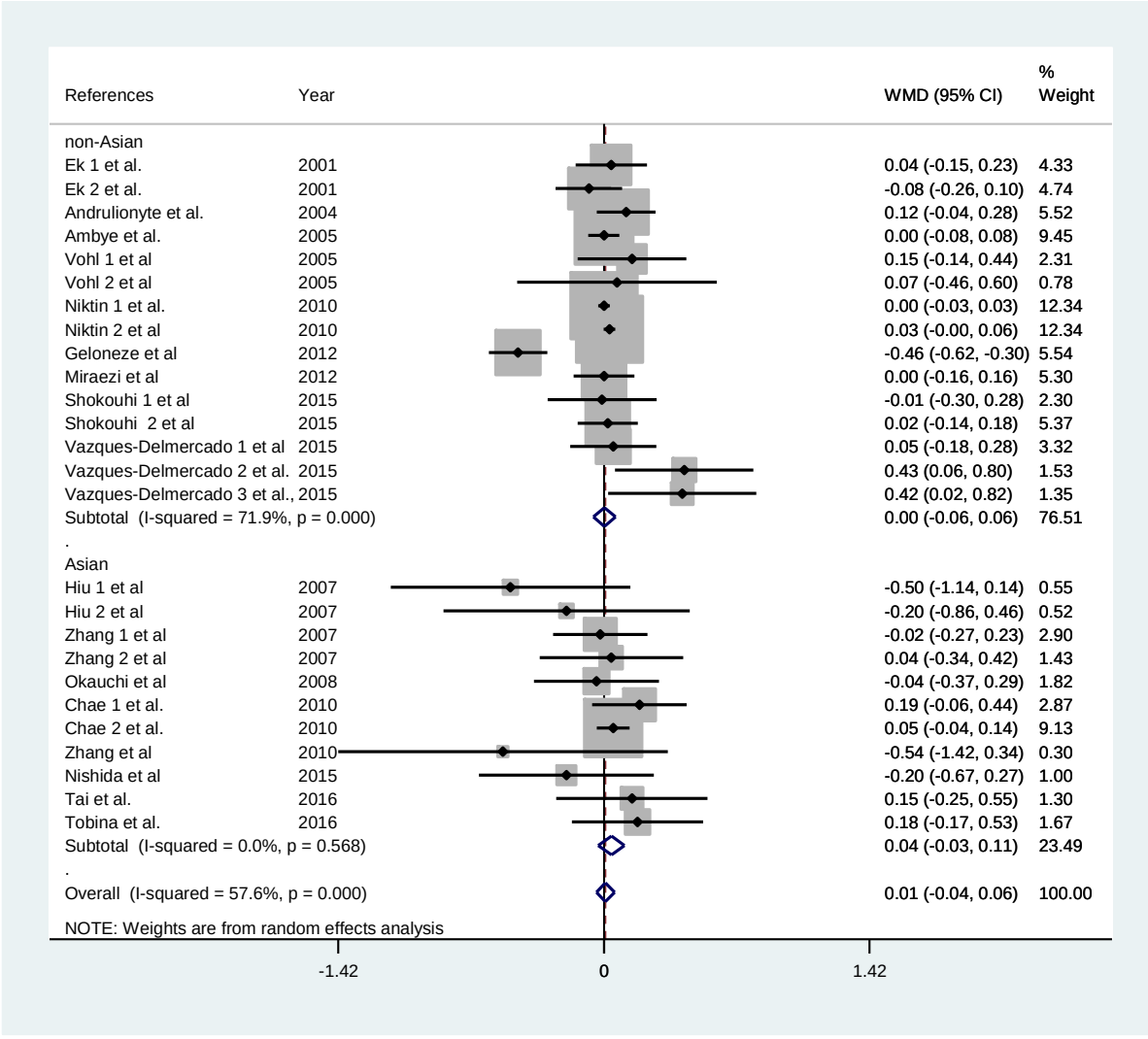

Figure 3.09. Forest plot showing difference in TG levels between two genotype groups (dominant genetic model: GG versus GA+AA) of the Gly482Ser (rs8192678) polymorphism under the random effect model.

3.3.10 Association between PPARGC1A Gly482Ser polymorphism and plasma TG under recessive model.

The outcome of the study under a recessive model suggests that "AA" genotypes had lower levels of TG in comparison to combined (GG+GA) genotypes (WMD = -0.02, 95%CI = -0.05 to 0.02, p-Value = 0.34) (Figure 3.10). No statistically significant difference in levels of TG was found between two genotype groups under this using a random effect model. The heterogeneity was not statistically significant from this outcome (Het.Chi-squared = 23.73; (df = 17), Het.p-Value = 0.13; I^2 = 28.3%).

In non-Asian population, lower levels of TG were observed in "AA" genotype group in comparison to "GG+GA" genotype group, but no statistically significant difference was observed (WMD = -0.02, 95% CI = -0.05 to 0.02, p-Value = 0.41) (Figure 3.10). Lower levels of TG levels were found in "AA" genotype in comparison to "GG+GA" genotype group in Asian population, but no significant difference was observed (WMD = -0.02, 95% CI = -0.08 to 005, p-value = 0.65 (Figure 3.10) between the "AA" and "GG+GA" groups. No significant heterogeneity was observed both in Asian (Het.Chi-squared = 2.19 (df = 6), Het.p-Value = 0.90; I^2 = 0.0%) but significant heterogeneity was observed in non-Asian populations (Het. Chi-squared 21.54 (df = 10), Het.p-Value = 0.02; I^2 = 53.6%) (Figure 3.10).

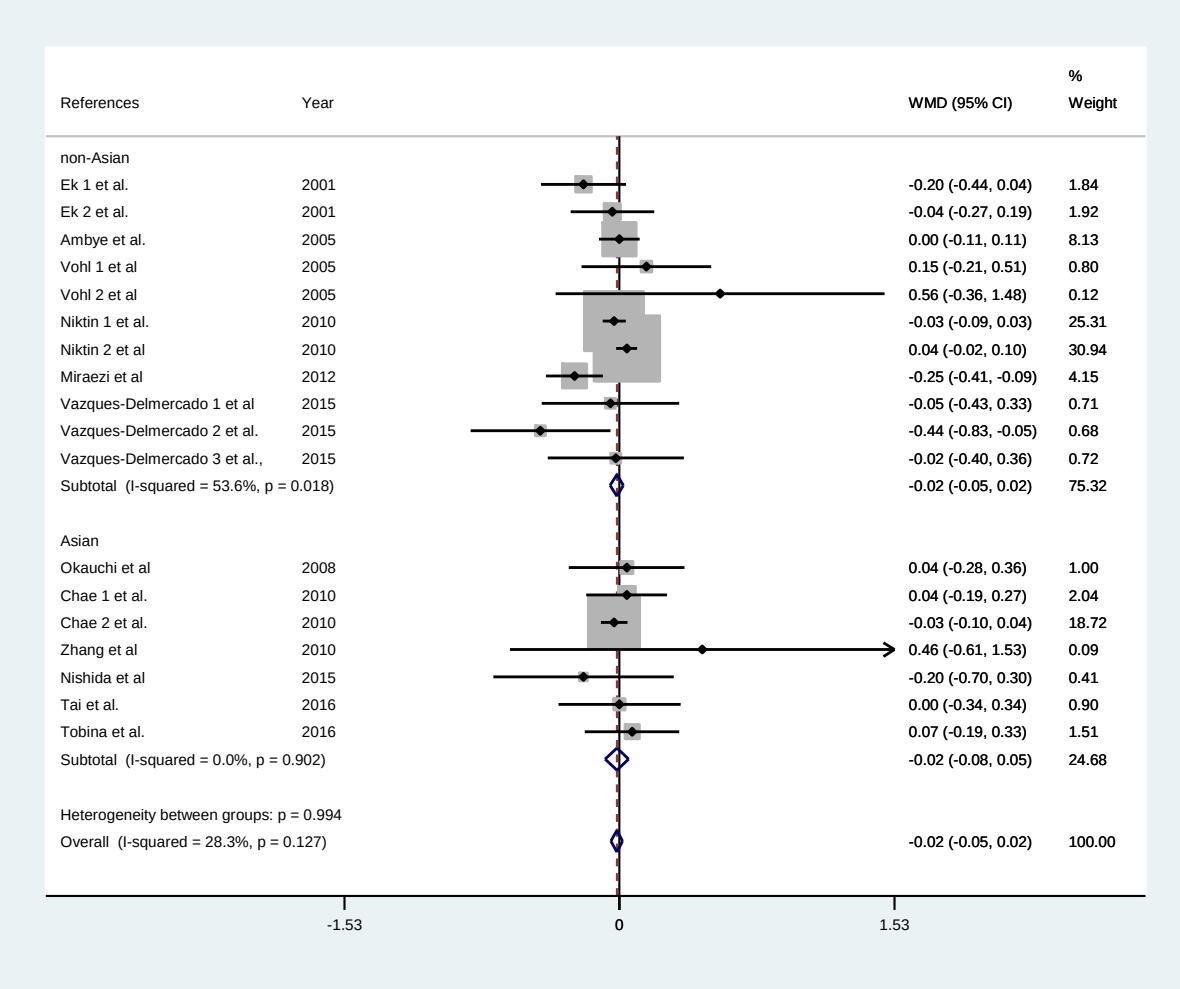

Figure 3.10. Forest plot showing difference in TG levels between two genotype groups (recessive genetic model: AA versus GG+GA) of the Gly482Ser (rs8192678) polymorphism under fixed effect model.

3.3.11 Association between PPARGC1A Gly482Ser polymorphism and plasma TC under dominant model.

The outcome of the dominant model showed that "GG" genotypes had higher levels of TC in comparison to combined (GA+AA) genotypes (WMD = 0.05., 95%CI = -0.05 to 0.18, p-Value = 0.34) (Figure 3.11). No statistically significant difference in levels of TC was found between two genotype groups under dominant genetic model using the random effect model. The heterogeneity was statistically significant for this outcome (Het.Chi-squared = 81.61; (df = 26), Het.p-Value = 0.0; I^2 = 68.1%).

In non-Asian population, higher levels of TC were observed in “GG” genotype group in comparison to “GA + AA” genotype group, but no statistically significant difference was observed (WMD = 0.08, 95% CI =-0.03 to 0.19, p-Value = 0.15) (Figure 3.11). Lower levels of TC levels were found in “GG” genotype in comparison to “GA+AA” genotype group in Asian population, but no significant difference was observed (WMD = -0.01 95% CI = -0.20 to 0.18, p-value = 0.92) (Figure 3.11) between the “GG” and “GA+AA” groups. Significant heterogeneity was observed both in Asian (Het.Chi-squared = 31.57 (df = 10), Het.p-Value = 0.0; I^2 = 68.3%) and non-Asian populations (Het. Chi-squared = 46.64 (df = 15), Het.p-Value = 0.0; I^2 = 67.8%) (Figure 3.11).

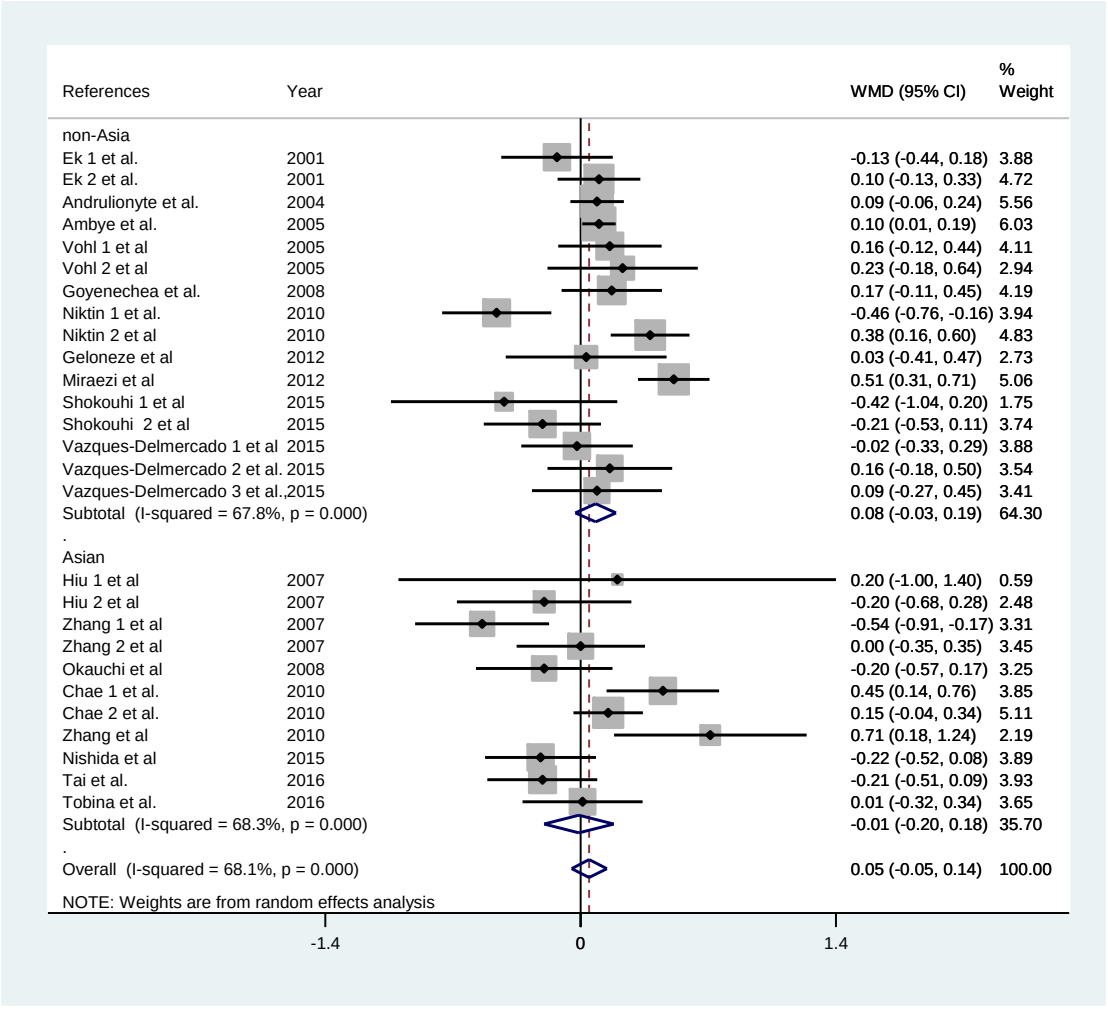

Figure 3.11. Forest plot showing difference in TC levels between two genotype groups (dominant genetic model: GG versus GA+AA) of the Gly482Ser (rs8192678) polymorphism under the random effect model

3.3.12 Association between PPARGC1A Gly482Ser polymorphism and plasma TC under recessive model.

The “AA” genotypes have lower levels of TC in comparison to combined “GG+GA” genotypes under a recessive model (WMD = -0.07, 95%CI = -0.14 to 0.01, p-Value = 0.07) (Figure 3.12). However, no statistically significant difference in levels of TC was found between two genotype groups under dominant genetic model using the random effect model. The heterogeneity was not statistically significant from this outcome (Het.Chi-squared =16.52; (df = 18), Het.p-Value = 0.56; I^2 = 0.0%).

In non-Asian population, significantly lower levels of TC were observed in “AA” genotype group in comparison to “GG+GA” genotype group (WMD = -0.11, 95% CI = -0.21 to -0.02, p-Value = 0.02) (Figure 3.12). No difference in levels of TC levels was found in “AA ” genotype in comparison to “GG+GA ” genotype group in Asian populations (WMD = 0.00, 95% CI = -0.11 to 0.12, p-value = 0.95 (Figure 3.12). No significant heterogeneity was observed both in non-Asian (Het. Chi-squared = 8.70 (df = 11), Het.p-Value =0.65; I^2 = 0.0 %) (Figure 3.12) and Asian populations (Het.Chi-squared = 5.53 (df = 18), Het.p-Value = 0.56; I^2 = 0.0%).

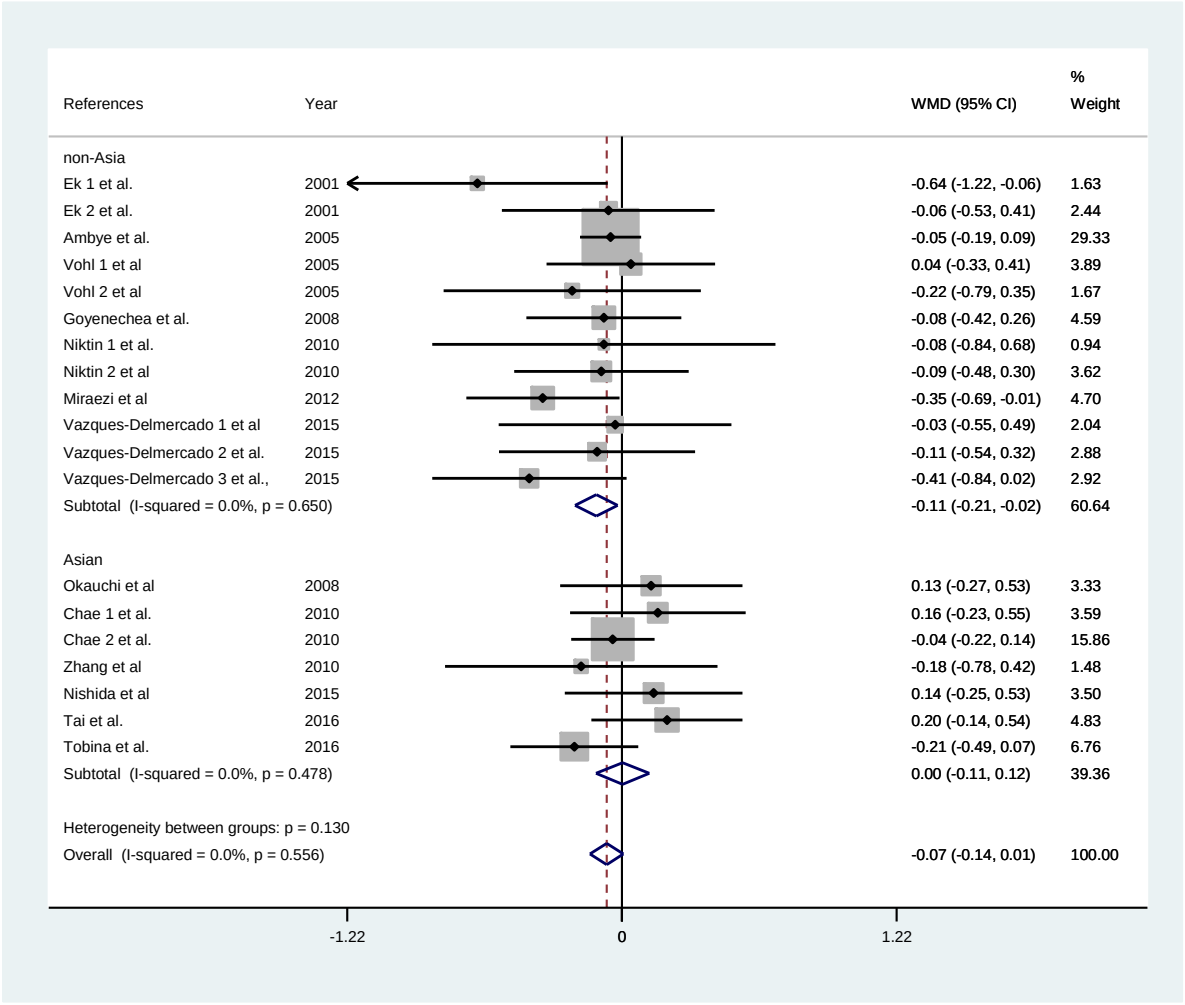

Figure 3.12. Forest plot showing difference in TC levels between two genotype groups (recessive genetic model: AA versus GG+GA) of the Gly482Ser (rs8192678) polymorphism under fixed effect model.

3.3.13 Association between PPARGC1A Gly482Ser polymorphism and Fasting plasma glucose (FPG) under dominant model.

Under the dominant genetic model the pooled weighted mean difference shows that the "GG" genotypes have a tendency towards lower levels of glucose in comparison to "GA+AA" genotypes but the value did not reach statistical significance (WMD = -0.01, 95%CI = - 0.07 to 0.05, p-Value = 0.69) (Figure 3.13). The overall between study heterogeneity was significantly different, therefore, the random effect model was selected for the analysis.

In non-Asian population the “GG” genotypes showed lower levels of fasting plasma glucose, however; the values were not statistically significant (WMD = -0.01, 95%CI = - 0.07 to 0.06, p-Value = 0.84) (Figure 3.13). In this group the between group heterogeneity was statistically significant (Het.Chi-squared = 28.25 (df = 13), Het.p-Value = 0.008; I^2 = 54.0 %).

In case of the Asian group the tendency of “GG” genotypes is similar to non-Asian group, but not statistically significant (WMD = -0.03, 95%CI = -0.17 to 0.10, p-Value = 0.64). The heterogeneity between the study in Asian group was not found statistically significant (Het.Chi-squared = 10.98 (df = 8), Het.p-Value = 0.20; I^2 = 27.1%).

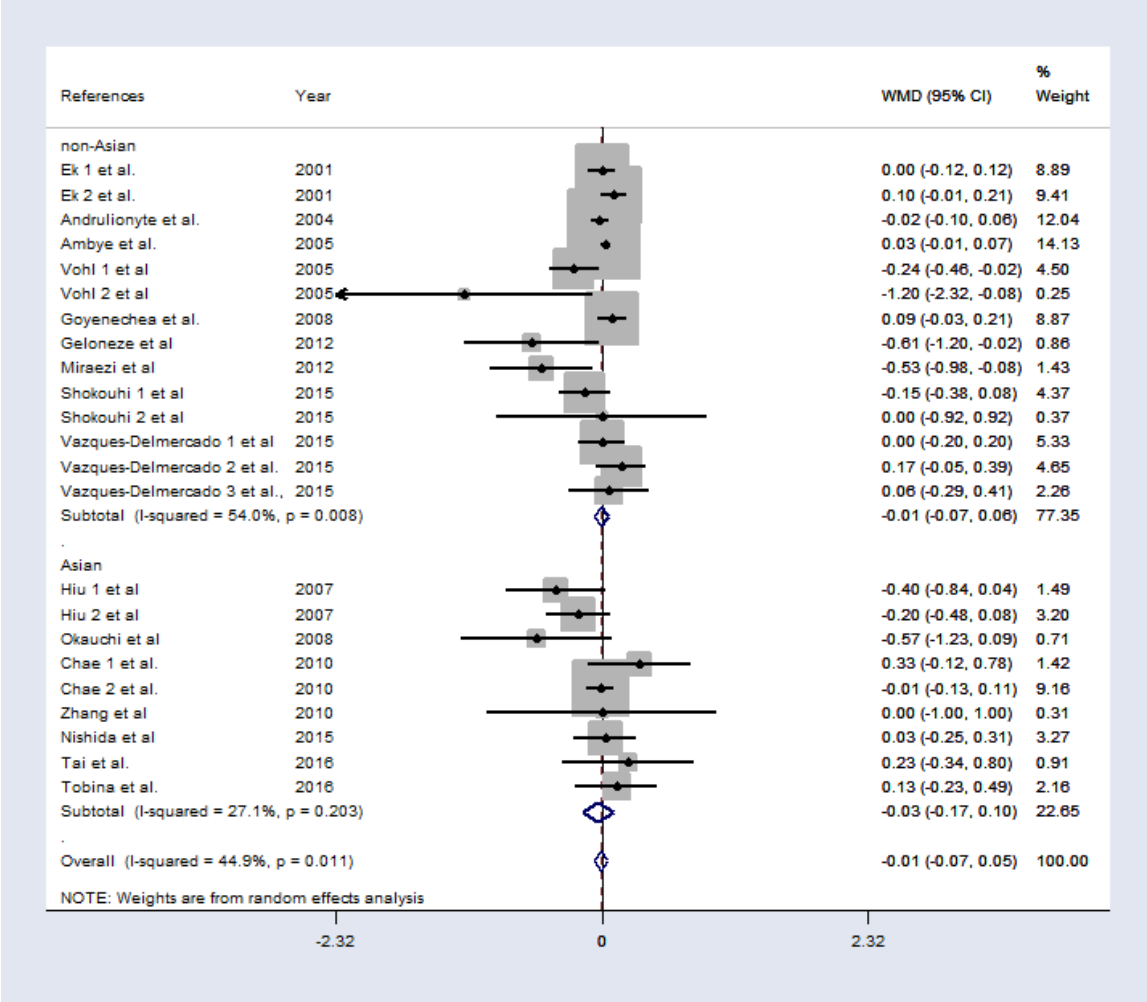

Figure 3.13. Forest plot showing difference in FPG levels between two genotype groups (dominant genetic model: GG versus GA+AA) of the Gly482Ser (rs8192678) polymorphism.

3.3.14 Association between PPARGC1A Gly482Ser polymorphism and Fasting plasma glucose (FPG) under a recessive model.

Under the recessive model, the pooled "AA" genotypes showed significantly lower levels of FPG in comparison to "GG+GA" genotypes (WMD = -0.08, 95%CI = - 0.13 to -0.03, p-Value = 0.00) (Figure 3.14). The levels of FPG were significantly lower as compared with "GG+GA" in non-Asian groups (WMD = -0.09, 95%CI = - 0.14 to – 0.04, p-Value = 0.001) (Figure 3.14).

In Asian group, the tendency of "AA" genotypes was similar as mentioned above, but the values were not statistically significant (WMD = -0.06, 95%CI = - 0.16 to 0.05, p-Value = 0.14) (Figure 3.14). The overall heterogeneity (Het.Chi-squared = 16.76 (df = 16), Het.p-Value = 0.40; I^2 = 4.6%) as well as those of Asian (Het.Chi-squared = 4.26% (df = 6), Het.p-Value = 0.64; I^2 = 0.0 %) and non-Asians (Het.Chi-squared = 12.20 (df = 9), Het.p-Value = 0.20; I^2 =26.20 %) were not statistically significant therefore, the fixed effect model was used for analysis.

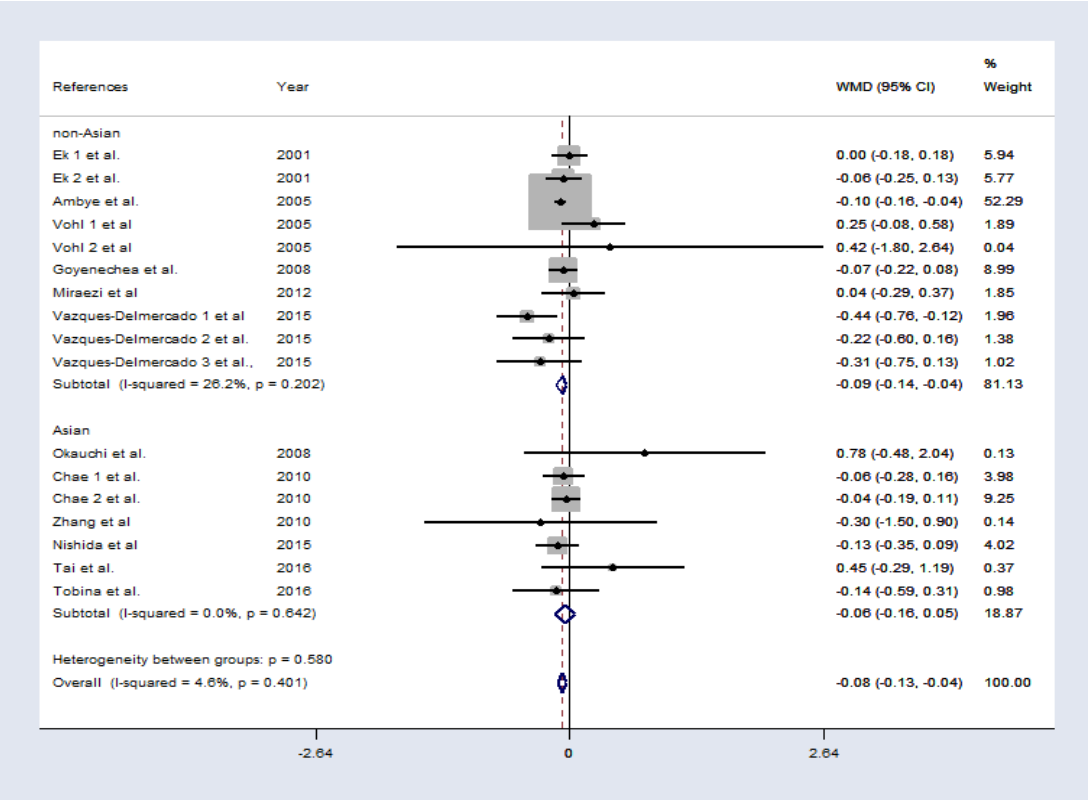

Figure 3.14. Forest plot showing difference in FPG levels between two genotype groups (recessive genetic model: AA versus GG+GA) of the Gly482Ser (rs8192678) polymorphism.

3.3.15 Association between PPARGC1A Gly482Ser polymorphism and Systolic blood pressure (SBP) under dominant model.

Under the dominant genetic model, the pooled "GG" genotypes showed lower levels of SBP in comparison to "GA+AA" genotypes without reaching statistical significance (WMD = -0.66, 95%CI = - 1.66 to 0.33, p-Value = 0.19) (Figure 3.15). No significant heterogeneity was found in the overall study (Het.Chi-squared = 8.75 (df = 12), Het.p-Value = 0.72; I^2 = 0.00 %). The non-Asians (WMD = -0.74, 95% CI = - 1.93 to 0.44, p-Value = 0.22) and Asians (WMD = -0.47, 95%CI = - 2.31 to 1.37, p-Value = 0.62) showed the similar tendency with no statistical significance. Heterogeneity did not reach statistical significance in both non-Asian (Het.Chi-squared = 3.25 (df = 7), Het.p-Value = 0.86; I^2 = 0.00 %) and Asian group (Het.Chi-squared = 5.45 (df = 4), Het.p-Value = 0.25; I^2 = 26.6 %).

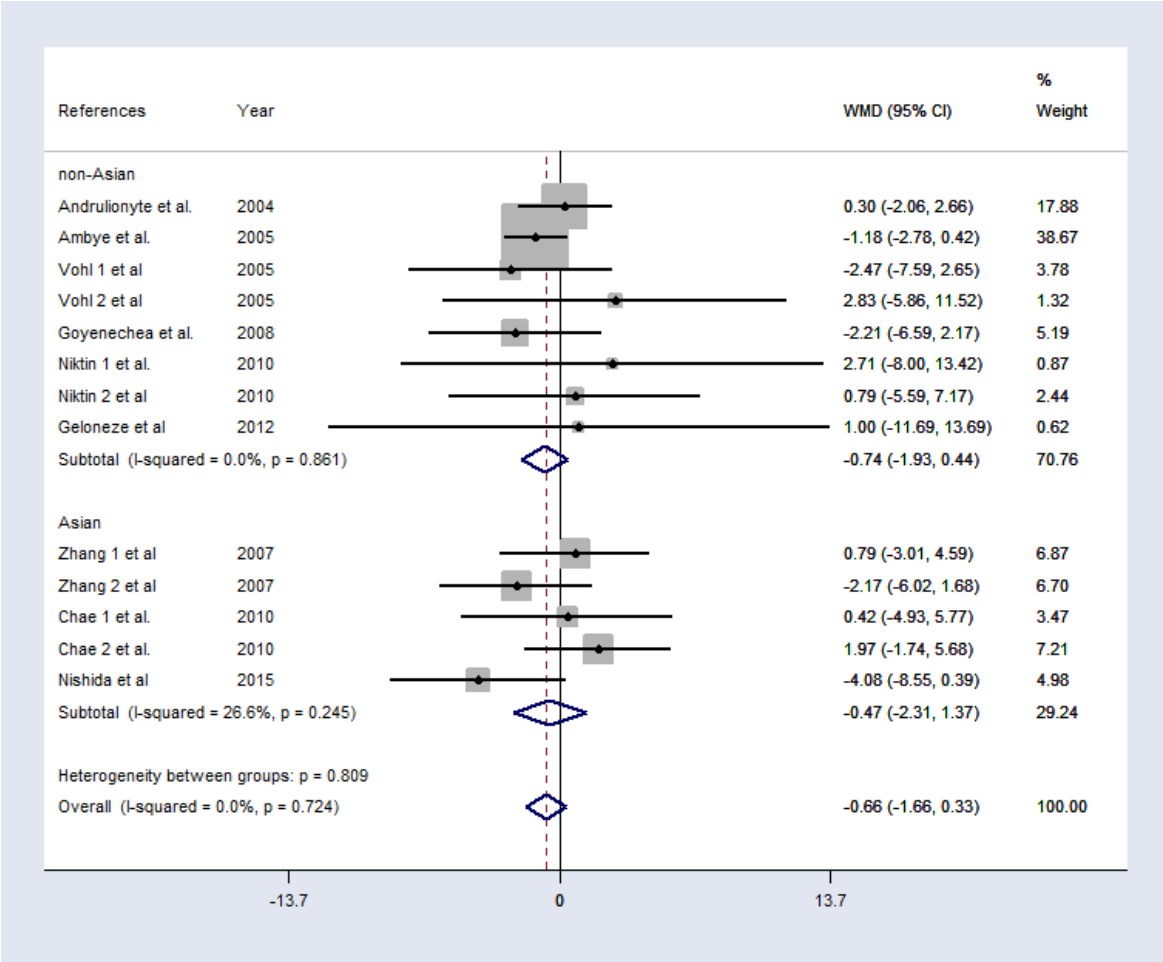

Figure 3.15. Forest plot showing difference in SBP levels between two genotype groups (dominant genetic model: GG versus GA+AA) of the Gly482Ser (rs8192678) polymorphism.

3.3.16 Association between PPARGC1A Gly482Ser polymorphism and Systolic blood pressure (SBP) under recessive model.

The overall pooled weighted mean difference showed that "AA" genotypes have a tendency towards lower levels of SBP in comparison to "GG+GA" under recessive genetic model (WMD = - 0.60, 95%CI = -2.17 to 0.97, p-Value = 0.46) (Figure 3.16). The overall heterogeneity was not statistically significant (Het.Chi-squared = 5.40 (df = 8), Het.p-Value = 0.71; I^2 = 0.00 %) therefore, the fixed effect model was used for the meta- analysis.

The similar tendency of "AA" genotype group was observed in non-Asian (WMD = - 0.79, 95%CI -2.79 = to 1.21, p-Value = 0.44) and Asian (WMD = -0.29, 95%CI = -2.84 to 2.25, p-Value = 0.82) subgroups. The heterogeneity in non-Asian (Het.Chi-squared = 4.82 (df = 5), Het.p-Value = 0.44; I^2 = 0.00 %) and Asian (Het.Chi-squared = 0.49 (df = 2), Het.p-Value = 0.78; I^2 = 0.00 %) was not statistically significant.

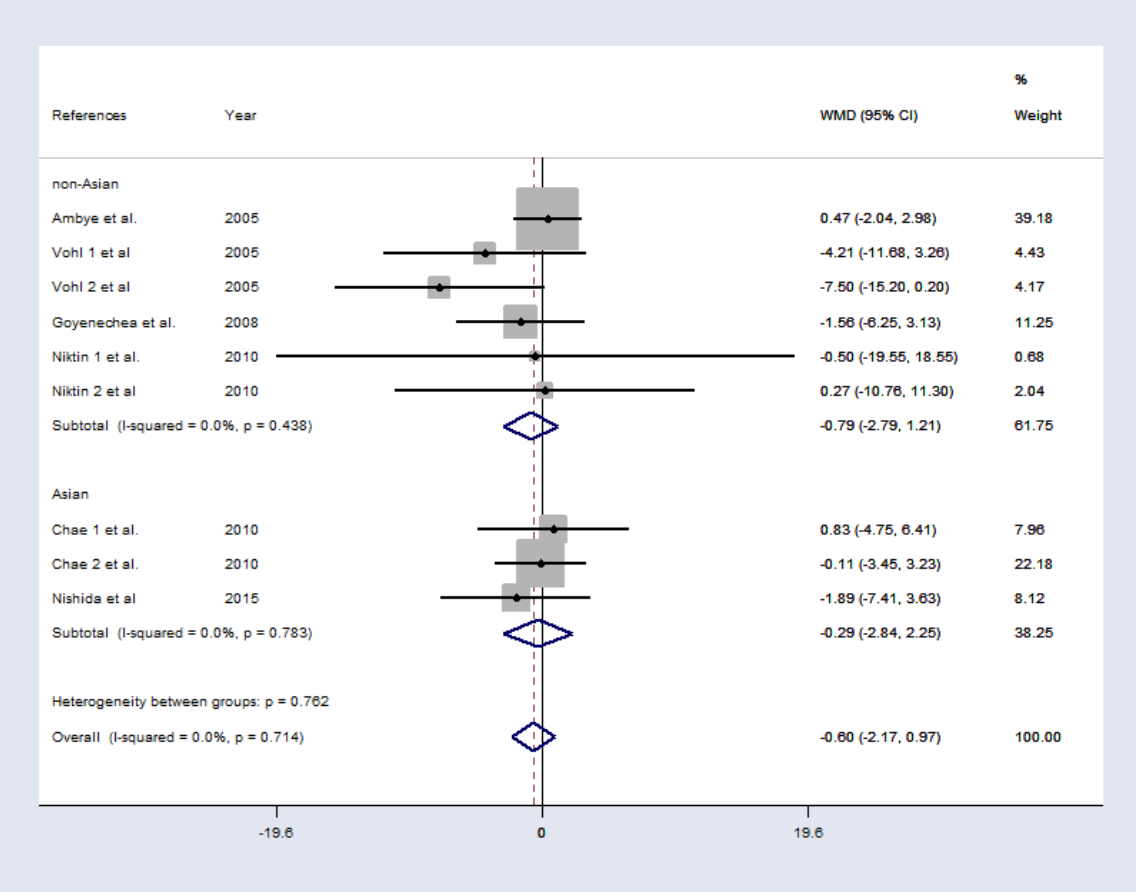

Figure 3.16. Forest plot showing difference in SBP levels between two genotype groups (recessive genetic model: AA versus GG+GA) of the Gly482Ser (rs8192678) polymorphism.

3.3.17 Association between PPARGC1A Gly482Ser polymorphism and diastolic blood pressure (DBP) under dominant model.

Under the dominant genetic model, the overall pooled weighted mean difference showed that "GG" genotypes have lower levels of DBP in comparison to "GA+AA" genotypes without reaching statistical significance (WMD = -0.20, 95%CI = - 0.76 to 0.36, p-Value = 0.48) (Figure 3.17). No significant heterogeneity was found in the pooled overall study (Het.Chi-squared = 8.04 (df =12), Het.p-Value = 0.78; I^2 = 0.00 %).

In subgroup analysis, the tendency of "GG" genotypes towards lower levels of DBP was evident in non-Asians (WMD = - 0.24, 95% CI = -0.87 to 0.38, p-Value = 0.45) and Asians (WMD = -0.04, 95%CI = - 1.31 to 1.24, p-Value = 0.96) without reaching statistical significance. Heterogeneity did not reach statistical significance in both non-Asian (Het.Chi-squared = 4.69 (df = 7), Het.p-Value = 0.70; I^2 = 0.00 %) and Asian group study (Het.Chi-squared = 3.27 (df = 4), Het.p-Value = 0.51; I^2 = 0.00 %).

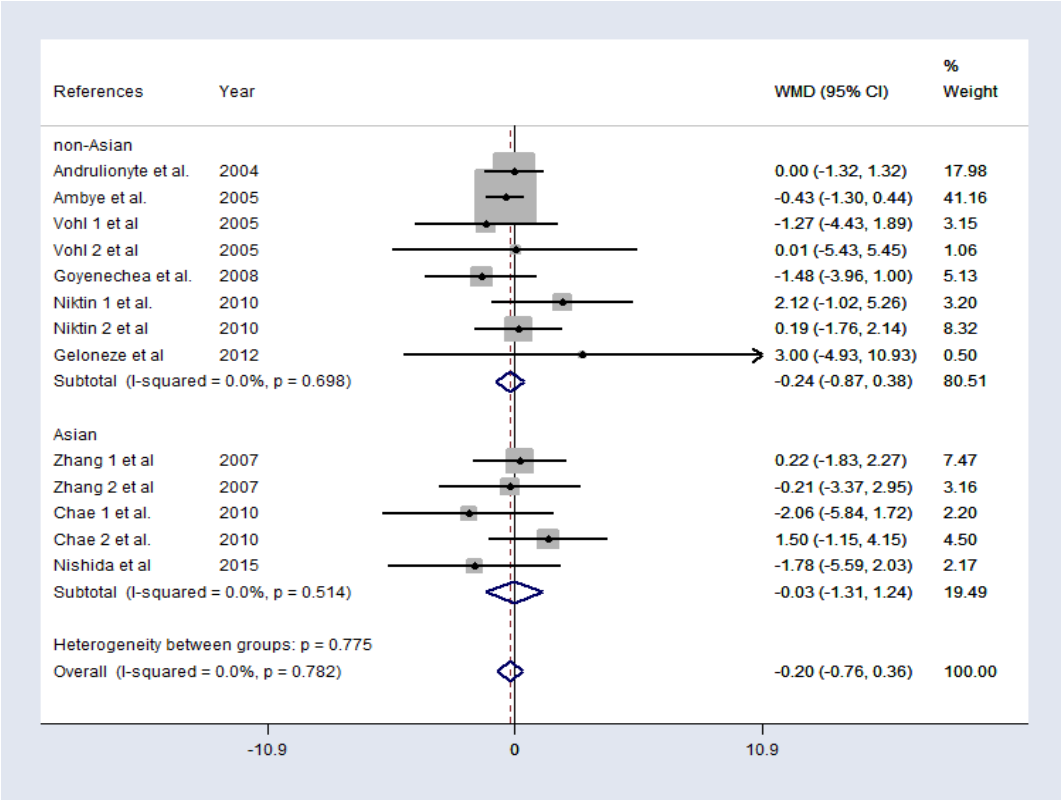

Figure 3.17. Forest plot showing difference in DBP levels between two genotype groups (dominant genetic model: GG versus GA+AA) of the Gly482Ser (rs8192678) polymorphism.

3.3.18 Association between PPARGC1A Gly482Ser polymorphism and diastolic blood pressure (DBP) under recessive model.

Under the recessive model the overall pooled WMD showed that "AA" genotypes had lower levels of DBP in comparison to combined (GG+GA) genotypes (WMD = - 0.22, 95%CI = - 1.17 to 0.72, p-Value = 0.64) (Figure 3.18). No statistical significance was observed in levels of DBP between two genotype groups under recessive genetic model using a fixed effect model. The heterogeneity was not statistically significant for this outcome (Het.Chi-squared =2.56; (df = 8), Het.p-Value = 0.96; I^2 = 0.00 %).

In the sub-group analysis, no difference in levels of DBP were found in "AA" genotype in comparison to " GG+GA " genotype group in Asian group (WMD = -0.22 , 95% CI = -1.64 to 20.7 , p-value = 0.82) (Figure 3.18) however; in non-Asian group the "GG" genotypes showed lower levels of DBP (WMD = -0.38, 95% CI = -1.48 to 0.72 , p-Value = 0.50) (Figure 3.18). But no statistically significant difference was observed in Asian (Het.Chi-squared = 0.16 (df = 2), Het.p-Value = 0.93; I^2 = 0.00 %) and non-Asian populations (Het. Chi-squared = 2.11 (df = 5), Het.p-Value =0.83; I^2 = 0.00 %) (Figure 3.18).

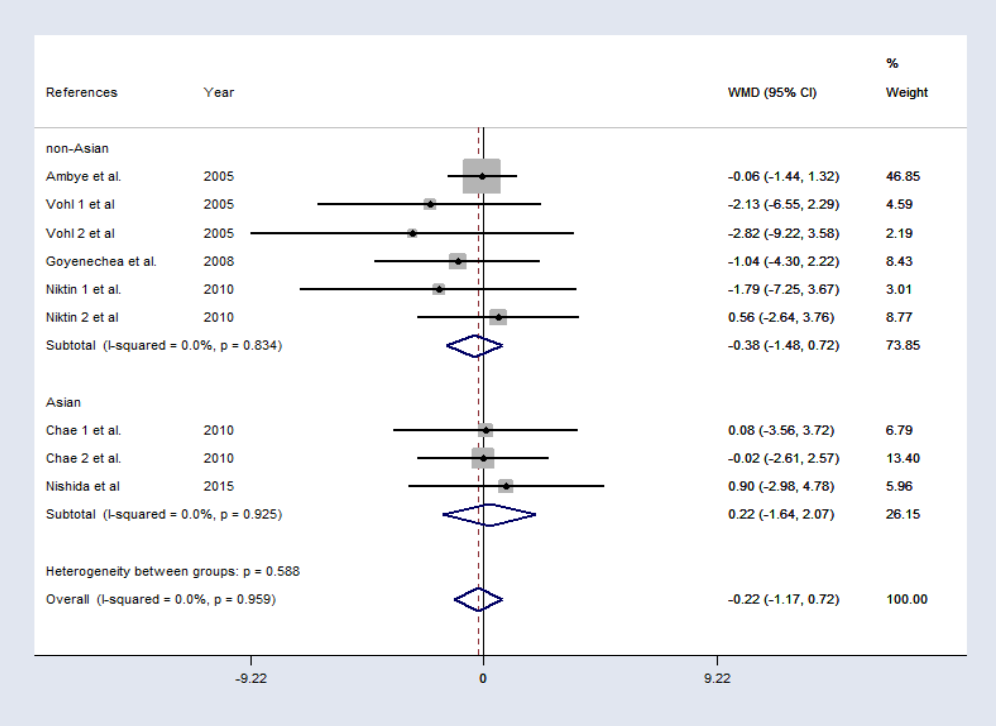

Figure 3.18. Forest plot showing difference in DBP levels between two genotype groups (recessive genetic model: AA versus GG+GA) of the Gly482Ser (rs8192678) polymorphism.

Table 3.02: Summary of the results of the meta-analysis.										
Variables	Genetic models	Heterogeneity	Statistical models	Population					
				Asian population		Non-Asian population		Overall population	
				WMD	p-value	WMD	p-value	WMD	p-value
BMI	Dominant (GG versus GA+AA)	No	Fixed	0.32	0.02*	0.04	0.68	0.13	0.10
	Recessive (AA versus GG+GA)	No	Fixed	-0.08	0.12	0.08	0.65	-0.08	0.54
HDL-C	Dominant (GG versus GA+AA)	Yes	Random	-0.01	0.16	-0.03	0.37	-0.02	0.13
	Recessive (AA versus GG+GA)	Yes	Random	-0.03	0.90	0.00	0.33	-0.02	0.38
LDL-C	Dominant (GG versus GA+AA)	Yes	Random	-0.03	0.63	0.03	0.67	0.01	0.89
	Recessive (AA versus GG+GA)	No	Fixed	-0.09	0.26	-0.04	0.20	-0.05	0.11
TG	Dominant (GG versus GA+AA)	Yes	Random	0.04	0.26	0.00	0.92	0.01	0.74
	Recessive (AA versus GG+GA)	No	Fixed	-0.02	0.65	-0.02	0.41	-0.02	0.35
TC	Dominant (GG versus GA+AA)	Yes	Random	-0.01	0.92	0.08	0.15	0.05	0.32
	Recessive (AA versus GG+GA)	No	Fixed	0.00	0.95	-0.11	0.02 *	-0.07	0.07
FPG	Dominant (GG versus GA+AA)	Yes	Random	-0.03	0.64	-0.01	0.84	-0.01	0.69
	Recessive (AA versus GG+GA)	No	Fixed	-0.06	0.14	-0.09	0.00*	-0.08	0.00*
SBP	Dominant (GG versus GA+AA)	No	Fixed	0.47	0.62	-0.74	0.22	-0.66	0.19
	Recessive (AA versus GG+GA)	No	Fixed	-0.29	0.82	-0.79	0.44	-0.60	0.46
DBP	Dominant (GG versus GA+AA)	No	Fixed	-0.04	0.96	-0.24	0.45	-0.20	0.48
	Recessive (AA versus GG+GA)	No	Fixed	-0.22	0.82	-0.38	0.50	-0.22	0.64

BMI = body mass index; HDL-C = high-density lipoprotein; LDL-C = low-density lipoprotein; TG = triglyceride; TC = total cholesterol; FPG = Fasting plasma glucose; SBP = systolic blood pressure; DBP = diastolic blood pressure; WMD = weighted mean difference. *Statistically significant at p-value ≤ 0.05.

3.4 Discussion:

The present study has shown that the polymorphism is significantly associated with body mass index (BMI) in an Asian subgroup under the dominant genetic model. The data suggest that the SNP is significantly associated with total cholesterol in non-Asians under recessive genetic model. In this study, the SNP was significantly associated with Fasting plasma glucose was in overall and non-Asian population.

Many emerging lines of evidence suggests an important role of PPARGC1A in lipid metabolism (Gulick et al., 1994; Zhang et al., 2004; Rufibach et al., 2006; Rhee et al., 2006; Wang et al., 2003) especially in skeletal muscle (Bentol et al, 2010), Beta- cells (Daniel et al, 2015) and liver (Zhang et al, 2004). PPARGC1A Gly482Ser is one of the most studied variants and some recent studies indicated its association with lipid levels (Franks et al, 2007; Esterbauer et al, 2002; Mirzaei et al, 2012; Shoukouhi et al, 2015 and Vazquez-de Mercado et al, 2015). To our knowledge, this meta-analysis has been conducted for the first time to evaluate the association between Gly482Ser genotypes and components of metabolic syndrome in humans. The present meta-analysis has been conducted to address conflicting results from the literature.

In this study, under the dominant genetic model, BMI of GG genotype has been found to be significantly higher in Asian subgroups. If we look at the individual studies included, the meta-analysis under Asian subgroup, none of them have been associated with BMI under either dominant or recessive model. The heterogeneity in this group was almost negligible, this could be due to ethnic similarity. The study remains significant even after the exclusion of Nishida et al., 2015 and Tai et al., 2016 and as a result of this exclusion the BMI comes under normal weight category (18.5- 24.9 Kg/m^2). This result seems to be consistent with the study carried out in Togans (Myles et al, 2011). With Togan population, the minor allele was significantly associated with BMI under both co-dominant and dominant model and the association even retained statistical significance even after adjustment with age and gender (Myles et al, 2011). The mean BMI (34 Kg/m^2) falls under the class I category in Togans. However, the minor allele of this SNP is associated with elevated body mass in Korean children (Ha et al, 2015) and non-diabetic overweight Chinese subjects (Weng et al, 2010). In this meta-analysis, the studies that contain Gly482Ser genotypes with lipid levels along with other metabolic variables were only included and the study without it has been excluded. To further analyse the association of the genotypes with BMI it would be necessary to conduct

further studies in the Asian population. This would allow to include more relevant studies conducted in Asian populations which might have been excluded from the present study. The “GG” genotype group of Gly482 polymorphism showed higher levels of LDL-C, TG and TC in comparison to “GA+AA” genotype group in overall studied populations, in contrast the “GG” genotype group showed lower levels of HDL-C under the dominant genetic model; however, none of the values were statistically significant.

Under the recessive genetic model, AA genotypes showed lower levels of HDL-C, LDL-C, TG and TC in comparison to GG+GA genotype groups with statistical significance in overall studied population.
To retain enough statistical power for the meta-analysis, the studied population has been divided into Asian and non-Asian subgroup populations even though this is very broad categorization. The Asian subgroup is more homogeneous as they are comprised of mostly East Asian populations. In Asian subgroup analysis, “GG” genotype group showed lower levels of HDL-C and LDL-C in comparison to “GA+AA” genotype group without reaching statistically significance. In case of non-Asian subgroup, “GG” genotype group showed higher levels of LDL-C and TC in comparison to “GA+AA” genotype group under dominant model. In Asian subgroup, under recessive model AA genotypes showed lower levels of HDL-C, LDL, TG. In non-Asian group, AA showed lower levels of LDL-C, TG but the statistical significance was achieved only in TC levels under a recessive model. Even though the non-Asian group is comprised of different ethnic groups, the heterogeneity was almost negligible. In contrary, Mirzaei et al, 2012 found a significant association of the genotypes with TC.

The association of the Gly482Ser genotypes with the components of metabolic syndrome in some of the above-mentioned studies might be due to the small sample size and other confounding factors. The primary aim of most of the studies selected for the meta-analysis was not to evaluate the association of the Gly482Ser genotypes with the components of metabolic syndrome and might lack adjustment of the essential confounding factors. In subgroup analysis, the broad categorization into Asian and non-Asian might have provided the heterogeneity.

In the meta-analysis, the studies with a larger number of samples are given more weight while calculating the pooled effect size. In this study, Ambye et al., 2005 has the highest number of samples and has been given more weight, which might have influenced the results especially in non-Asian subgroups. The other peculiar aspect of the study is that the studies vary not only according to the ethnicity, but also in terms of disease outcome. To analyse the study, according to healthy and disease group would lead to lack of power and very few studies could be categorized in this way.

The fasting plasma glucose in "AA" genotype is significantly reduced in comparison with "GG+GA" (i.e. recessive genetic model) in overall and non-Asian studies. However, no statistically significant association was observed under the dominant genetic model in any of the subgroups. The significant association achieved in this study might be due to the population stratification as multi-ethnic groups are included in the non-Asian population. Based on this result, it seems that the minor allele had a protective effect against elevated fasting glucose in non-Asian population, but again the data must have to be interpreted with caution because of the possibility of biases (publication bias, genotyping error, population stratification) in the study. This effect was not observed in previous meta-analysis (Barroso et al, 2006) even though the minor allele showed the tendency towards lower levels of fasting glucose.

The minor allele did not show any significant association with blood pressure levels under either of the genetic model. The current meta-analysis has not been stratified according to gender and it might be one of the reasons it failed to show any association. The previous meta-analysis showed the association of the minor allele in younger individuals (Vimaleswaran et al, 2006; however, the individuals below 18 years of age has been excluded from current studies and it might be the other reasons in failing to observe any association.

It is also important to consider the limitations of the present meta-analysis. Firstly, the study has been conducted in different population groups such as type 2 diabetes, CAD (coronary artery disease), morbidly obese, obese, pre-obese, NAFLD (Non-alcoholic fatty liver disease) and elderly age group which could be the main source of heterogeneity. Due to the difference in primary aim of the included study, the information regarding the dietary habit; medication intake; level of exercise and smoking status is not known which could potentially

modulate the level of lipids. Another source of heterogeneity might be due to the technique employed in genotyping the variant. The studies included in the meta-analysis were only published in English language which might probably skew the results by excluding the studies conducted in other languages. Gene-environment interaction has not been taken into consideration in this meta-analysis.

In conclusion, the present meta-analysis suggested that PPARGC1A Gly482Ser polymorphism is significantly associated with some components of metabolic syndrome under various genetic models. Further separate meta-analysis of the individual components of metabolic syndrome is warranted.

Chapter 4

Prevalence of Gly482Ser of PPARGC1A and its association with metabolic syndrome in the Nepalese population.

4.1 Introduction

The prevalence of MetS is increasing globally and the clustering of the metabolic risk factors is geographically unique (Eberly et al., 2006). (Metabolic syndrome is becoming a serious problem not only in developed countries, but also in developing countries. In Nepal, around 20.7% of the population have metabolic syndrome as assessed by the National Cholesterol Education Program (NCEP) criteria, in a study carried out in 14,425 subjects (Sharma et al., 2011). The people with metabolic syndrome are at greater risk of developing type 2 diabetes and cardiovascular disease posing a serious health economic burden to the nation.

Metabolic syndrome (MetS) is defined as a cluster of conditions which includes high blood pressure, impaired glucose tolerance, abdominal obesity and dyslipidaemia (Alberti et al., 1998, Grundy et al., 2005). Metabolic syndrome is a serious global public health problem and a major cause of morbidity and mortality.

The heritability estimate of MetS as defined by the National Cholesterol Education Program (NCEP) criteria is 30% (Kraja et al., 2005) and the heritability estimate of each of the components of MetS as defined by principal component analysis is: 43% to 54% for lipid and insulin, 47% to 66% for obesity and insulin and up to 37% for arterial blood pressure (Kraja et al., 2005). This clearly indicates the involvement of genetic factors in the development of MetS.

The role of Peroxisome proliferator-activated receptor-γ coactivator-1 (PPARGC1A) as a major regulator of energy expenditure and its role in metabolic disorder, such as type 2 diabetes and metabolic syndrome has already been explained in the introduction part of this thesis. Genetic susceptibility to metabolic syndrome is variable across populations and, to the best of our knowledge, no studies have been conducted on the association of PPARGC1A Gly482Ser variants with metabolic syndrome in Nepalese populations. Because of the distinct genetic background of Nepal, it is imperative to understand the role of the

Gly482Ser variant of this gene in Nepalese populations for early detection and management of metabolic syndrome.

4.2 Population and study design

A population from Attaria, Kalilali of the far western region of Nepal was selected for the study. Participants for the study were recruited following distribution of patient information sheet in Nepalese language and announcing by loudspeaker in a door-to-door visits with the help of trained facilitators. Participants with age ≥ 18 ≤ 40 years were recruited for the case-control study. The participants willing to take part in the research were handed over the participant information sheet written in Nepalese language. The eligible participants were instructed to fast overnight (at least 10-12 hours) and visit the clinic at J. K higher secondary school. A prior permission was granted from the school for using the school clinic for the research purpose. Participants were explained about the research in Nepalese or regional language after their arrival in the clinic and the informed voluntary consent in Nepalese language was obtained. The participants who could not read or write, were verbally explained about the research in local language and the thumb print of both the hands were taken in informed voluntary consent from in the presence of a witness. The demographic data were collected from the participants at the time of the visit to the clinic at J. K higher secondary school, Attaria, Kailali and the interview were conducted by the trained facilitators.

4.3 The anthropometric measurements

The height and weight were measured using height and weight stadiometer. The height in meter and weight in kilogram was used for the calculation of Body mass index (BMI) Kg/m^2. The waist circumference was measured with a non-stretchable measuring tape midpoint between the iliac crest and lowest rib at the end of a normal expiration while the participants were asked to relax. It was ensured that the measuring tape was perpendicular to the vertical long axis of the body and parallel to the floor. The hip circumference was measured around the widest part of the buttocks while the measuring tape was kept parallel to the floor. Arterial blood pressure and heart rate was measured after 10-12-minute rest with the help of digital blood pressure measuring instrument (micro life). Five ml of blood sample was collected from the participants by Dr Shiva Raj Paneru (a volunteer medical

doctor). Metabolic syndrome was diagnosed using Alberti et al., 2009, ethnic-specific criteria: elevated waist circumference ≥90 cm (men) or ≥80 cm (women) [for south Asians], triglycerides ≥1.70mmol/l or Hypertriglyceridemia or having medication for it, HDL-cholesterol < 1.0 mmol/l in men and < 1.30 mmol/l in women or on medication, arterial blood pressure ≥130/85 mmHg or hypertensive or having medication for it and fasting plasma glucose ≥ 5.6 mmol/l or diagnosed with type 2 diabetes (T2D) or having medication for T2D. The presence of any three of the five above mentioned criteria in participants was used for the diagnosis of metabolic syndrome and the absence of those characteristics was defined as control participants. Participants with acute or chronic illness, fever, pregnant women or lactating mother were excluded from the study.

4.4 Aims

The aim of the research presented in this chapter is as follows.

a. To analyse the prevalence of single nucleotide polymorphisms of the rs8192678 (Gly482Ser) of PPARGC1A gene in the Nepalese population.

b. To investigate the risk of the allele and genotypes of above mentioned SNPs to the MetS.

c. To investigate the possible association between the rs8192678 (Gly482Ser) of PPARGC1A gene and MetS

d. To analyse the strength of association between genotypes and components of MetS using various statistical models.

4.5 Ethics statement

The study was approved by the University Ethics committee (Robert Gordon University) and Nepal Health and research council (Nepal). All the documents related to ethical approval are included in the Appendix E. A signed informed consent was obtained from all the participants. The study was conducted according to the Helsinki declaration.

4.6 Estimation of plasma lipid levels

4. 6.1 Estimation of total Cholesterol from plasma using CHOD-PAP method (HUMAN, Germany).

It is an enzymatic colorimeter test for the determination of total cholesterol with lipid clearing factor. The cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator, quinoneimine, is formed in this enzymatic reaction from hydrogen peroxide and 4-aminophenazone in the presence of peroxidase and phenol.

Due to the accumulation of the lipoproteins in the blood samples causes a turbidity which is known as lipemia. The build-in-lipid clearing factor (LCF) clears up the turbidity of the reaction mixture in lipemic samples minimizing the false positive results. The reaction principle of the method is shown in figure 4.01.

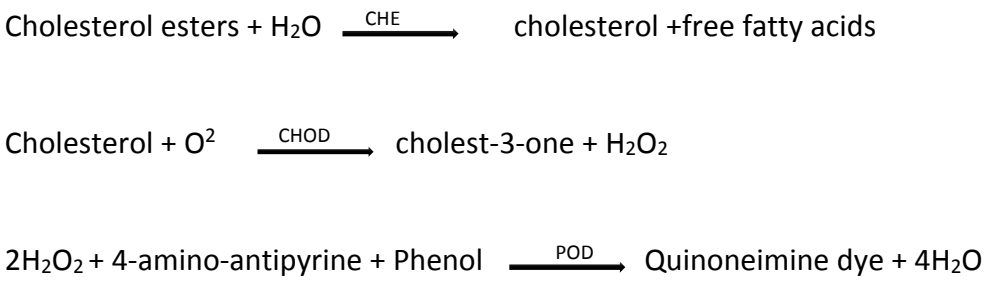

Figure 4.01: Reaction principle for the estimation of total cholesterol using CHOD-PAP method. CHE = Cholesterol esterase, CHOD = Cholesterol oxidase, POD = Peroxidase

Table 4.01: Pipetting scheme for the estimation of total Cholesterol using CHOD-PAP method.			
Pipetting scheme			
	Blank	Standard	Test
Sample (plasma)	-	-	10 µl
Standard (STD)(200mg/dl) (5.17mmol/l)	-	10 µl	-
Reagent (1000µl)	1000 µl	1000 µl	1000 µl

The reaction was incubated at 37°C for 5 minutes. The absorbance of the sample was measured against the blank reagent.

With the standard recommended by HUMAN (Germany) the following formula was given for the measurement.

$$\text{Concentration} = 5.17 \times \frac{A_{sample}}{A_{standard}} \text{ [mmol/l]}$$

4.6.2 Determination of Triglycerides by using a GPO-PAP Method [Enzymatic colorimetric test for triglycerides with lipid clearing factor (LCF)] (HUMAN, Germany).

This is an enzymatic colorimetric test for the estimation of triglycerides. In this method triglycerides are hydrolysed to glycerol and fatty acids by the enzyme lipases. Glycerol is converted to glycerol-3-phosphate and Adenosine diphosphate (ADP) in the presence of Adinosine triphosphate (ATP), which is oxidised by the glycerol phosphate oxidase to form dihydroxyacetone and hydrogen peroxidase (H_2O_2). In the presence of the enzyme peroxidase, an indicator quinoneimine is formed from hydrogen peroxidase, 4-amino-antipyrine and 4-chlorophenol. Triglycerides liquicolor (GPO-PAP method) reagent also comes with built-in-lipid clearing factor (LCF) which minimizes the turbidity in lipemic specimens. The reaction principle of the method is shown in figure 4.02.

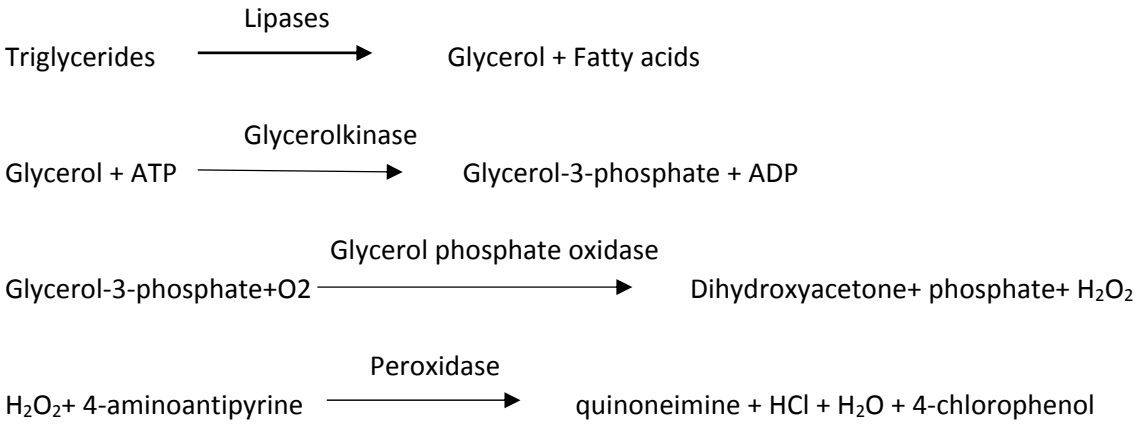

Figure 4.02: The reaction principle for the estimation of triglycerides using GPO-PAP method.

Table 4.02: Pipetting scheme for the estimation of triglycerides using GPO-PAP method.			
Pipetting scheme			
	Blank	Standard	Test
Sample (plasma)	-	-	10 µl
Standard Triglycerides (STD)(200mg/dl) (2.28mmol/l)	-	10 µl	-
Reagent (1000µl)	1000 µl	1000 µl	1000 µl

Calculation of the concentration of the triglycerides.

$$\text{Concentration} = 2.28 \times \frac{A_{sample}}{A_{standard}} \quad [\text{mmol/l}]$$

4.6.3 Determination of High density lipoprotein (HDL) by using CHOLESTEROL liquicolor test kit (HUMAN, Germany).

Phosphotungstic acid and magnesium chloride precipitates the chilomicrons, very low-density lipoproteins (VLDL) and Low-density lipoprotein (LDL). After centrifugation, the clear supernatant is formed from the precipitate. The supernatant contains high density lipoprotein (HDL) which estimated using CHOLESTEROL liquicolor test (HUMAN, Germany).

Reagent consists of Phosphotungstic acid (0.55mmol/l) and Magnesium chloride (25.00mmol/l) and was purchased from Human, Germany (Cat no: 10018). A standard cholesterol (1.29mmol/l) is also supplied in the test kit. The reagent was diluted to 4 parts of the bottle with 1 part of distilled water before using it.

The concentration of HDL in the plasma was determined in two stages: precipitation and HDL cholesterol determination stage. In the precipitation stage a 200 µl of the plasma along with 500 µl of the diluted reagent was pipetted into the centrifuge tube. The tube was mixed thoroughly and incubated for 10 minutes at room temperature. After that it was centrifuged for 10 minutes at 4000g. In the second stage, the clear supernatant the HDL (100 µl) was used for the determination of the concentration of HDL-C using CHOLESTEROL liquicolor reagent. After addition of 1000 µl of the reagent (CHOLESTEROL liquicolor) in 100 µl of HDL supernatant, it was mixed and incubated for 5 minutes at 37°C and the absorption was measured at 500nm.

Table 4.03: Assay for the determination of the concentration of HDL-C in the samples.			
1. precipitation stage (First stage)			
Reagent	Volume		
Sample	200 µl		
Diluted reagent (4+1)	500 µl		
2. HDL cholesterol determination using CHOLESTEROL liquicolor test (second stage)			
Reagent	Sample	Standard	Blank
Distilled water	-	-	100 µl
Sample (HDL supernatant)	100 µl	100 µl	-
Reagent	1000 µl	1000 µl	1000 µl

HDL = high-density lipoprotein

Calculation of HDL-C concentration with standard (known cholesterol concentration).

$$\text{Concentration} = 4.52 \times \frac{A_{sample}}{A_{standard}} \quad [\text{mmol/l}]$$

The concentration of LDL-C was calculated by using Friedewald et al, 1972 formula.

The following formulae was used for the calculation of concentration of LDL-C from the known concentration of total cholesterol, HDL-C and triglycerides.

$$\text{LDL-C} = \text{TC- HDL-C X } \frac{\text{TG [mmol/l]}}{2.2}$$

Where, TC represents total cholesterol, HDL-C and TG as high-density lipoprotein triglycerides respectively.

4.7 Estimation of blood glucose levels

The fasting blood glucose levels were measured by glucometer (Accu-Check) by using Accu-Check Inform II test trips by the finger pricking method. The performance and accuracy of the Accu-Check glucometer was checked by using the control provided with known glucose concentration. The index figure was wiped with disinfect wipes and was pricked and the first drop of blood was wiped and subsequent drop of blood was carefully drawn into the test stripes for measuring the blood glucose level.

4.8 DNA extraction

DNA was extracted by using QIAamp DNA Blood Mini kit (QIAGEN) catalogue no. 51304. In a 1.5ml micro centrifuge tube, 20 µl of QIAGEN protease and 200 µl of blood sample was pipetted. In the same micro centrifuge tube, 200µl of Buffer AL was added and mixed by pulse-vortexing for 15 seconds. After incubating the mixture at 56°C for 10 minutes, 200 µl if ethanol (96-100%), as provided by the manufacturer, was added and mixed again by pulse-vortexing for 15 seconds. The mixture was then carefully transferred to mini spin column and centrifuged at 6000Xg for 1 minutes by applying the 2ml collection tube and the filtrate was discarded. AW1 buffer supplied by QIAGEN was added into the column and again centrifuged at 6000xg for 1 minutes. The filtrate in the collection tube was discarded. In the same spin column, 500µl of AW2 buffer was added without wetting the rim and centrifuged at 20,000Xg for 3 minutes. The new mini spin column was placed in a clean micro centrifuge tube and 50 ml Buffer AW was added in the column and incubated at room temperature for 1 minute and centrifuged at 6000Xg for 1 minute. The eluted DNA sample was stored further quantified. This was conducted at Decode Genomic and Research Centre, Nepal.

4.9 DNA quantification

The quantification of the DNA was carried out by Nano drop Spectrophotometer (Thermo Fischer) by adding 1µl of the DNA directly into the measuring slot.

4.10 Polymerase chain reaction (PCR)

4.10.1 Primer design

Specific primers flanking the variants rs8192678 were designed using primer 3 and Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast). The information regarding the primers and the product size is given in table 4.06. The primers were synthesized by Xcelris labs (India).

The thermocycler conditions of the PCR were optimized for each pair of primers for each variant. The PCR was performed with a 25 μL reaction mixture by using 12.5 µl of the prepared 2X master mix with (20ng of DNA, 10µM of each primer, 0.4mM of each dNTP, 3mM of $MgCl_2$, 2U Taq polymerase, and 1XPCR buffer (Thermo Scientific).

PCR was carried out with initial denaturation at 94°C for 3 minutes, followed by denaturation at 94°C for 30 seconds, annealing of the primers with annealing temperature of 46°C for rs8192678 followed by Extension at 72°C for 45 seconds and final extension at 72°C for 10 minutes as shown in table 4.05.

4.10.2 Gel electrophoresis

Gel electrophoresis was carried out on 1.8% of agarose and the staining purpose gel was soaked in 0.5µg/ml solution of ethidium bromide and 5 µl of the PCR product was added in each lane and visualized under the UV light.

Table 4.04: The reagents and volume used for the PCR reaction

Components	Quantity/vol.
Nuclease free water	10.5-X* µl
Template DNA	20ng
Forward Primer (10pmole /µl)	1.0 µl
Reverse Primer (10pmole /µl)	1.0 µl
2 X PCR master mix (Taq polymerase = 2 units, dNTPs = 0.4mM, $MgCl_2$ = 3mM)	12.5 µl
Total Volume	25 µl

Note: X* represents the variable volume of genomic DNA.

Table 4.05: Thermocycler condition for the PCR.		
Condition	Temperature (°C)	Time
Initial Denaturation	94°C	3 minutes
Number of cycles	35	
Denaturation	94°C	30 seconds
		45 seconds
Annealing temperature (°C) used for the markers.		
rs8192678	46°C	
Extension	72°C	45 seconds
Final extension	72°C	10 minutes

Table 4.06: Primers used for the amplification and the size of the PCR product.					
Gene	SNP	Primer	Primer sequence (5'-3')	Length (bps)	Product size (bps)
PPARGC1A	rs8192678	Forward	TGACCATGACTATTGCCAGT	20	472
		Reverse	AGAAGGAGACACATTGAACA	20	

Figure 4.03 represents the image of the amplified PCR product of the SNP rs8192678 amplified using the specific primers as shown in table 4.06. The size of the product was estimated using the marker (M). The samples amplified as shown in the figure 4.03 are 1, 2, 3, 4, 5, 6, 7, 8, and 9.

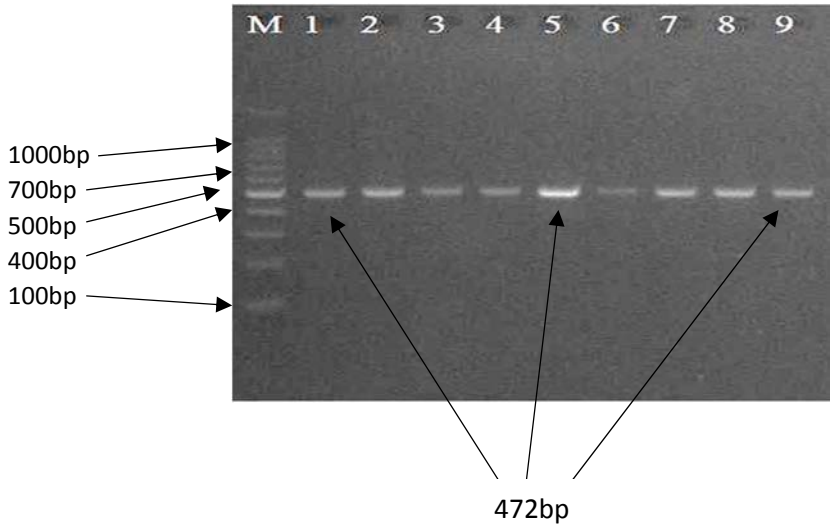

Figure 4.03: Gel image of the PCR amplified product of 472 bp using the primer for the marker rs8192678. Lane M represents 100bp marker (Xcelris labs); lane 1, 2, 3, 4, 5, 6, 7, 8 and 9 represents samples amplified.

4.11 Genotyping using Sanger sequencing using an ABI sequencer (3730xl).

The sequencing of the samples was carried out in Xcelris labs, India. Necessary training was provided by the lab manager to use the instrument for Sanger sequencing.

The following steps were observed for the genotyping using Sanger sequencing method.

a. PCR product clean up

First, the PCR products were cleaned by USB ExoSAP-IT (affymatrix) (product code: 78202) following the manufactures' protocol. A 5µl of USB ExoSAP-IT was mixed with 2µl of the PCR product. The reaction was incubated at 37°C for 4 minutes to degrade primers and excess dNTPs from the PCR products which is due to the presence of the enzymes Exo I and Shrimp Alkaline Phosphatase in the reagent. The second incubation was carried out at 80°C for 1 minute to inactive the USB Exo SAP-IT reagent.

b. Cycle sequencing

Table 4.07 refers the cycle sequencing reaction of the Sanger sequencing. Cycle sequencing was carried out in total volume of 20 µl. In a reaction plate, 8 µl of Big Dye Terminator 3.1 ready reaction mix (Applied Biosystem) was added along with 3.2 pmol of forward and reverse primer. A template PCR of 10 ng was added and the rest of deionize water was added to make it final volume of 20 µl.

Table 4.07: Cycle sequencing step of the sanger sequencing method using Big Dye terminator 3.1			
Standard reaction (20 µl)			
	Quantity per reaction	Forward reaction	Reverse reaction
Big Dye Terminator 3.1 ready Reaction mix (Applied Biosystem)	8 µl	8 µl	8 µl
Forward primer	3.2 pmol	1 µl	-
Reverse primer	3.2 pmol	-	1 µl
Deionized H_2O	Variable	9 µl	9 µl
Template (PCR product)	10 ng	2 µl	2 µl
Total volume		20 µl	20 µl

c. Data analysis

The genotype was assigned by using CLC Genomics Workbench 11.0 (QIAGEN). The forward and reverse sequence of the markers rs8192678, rs7903146, rs9939609 and rs1801282 were aligned with respect to the reference sequence of the respective gene to assign the genotype to each sample.

4.12 Statistical analysis

Hardy Weinberg equilibrium states that allele and genotype frequencies in a population in a successive generation if the following conditions are met: the population is large, no immigration and emigration occur, no mutation, random mating and natural selection occur in a population. The deviation of the expected allele frequencies might indicate genotyping error, inbreeding or population stratification.

If A represents the minor allele with frequency p and a represents a minor allele with frequency q, then under HWE the sum of the frequency of both the allele must equal to one i.e, p+q = 1. Similarly, if AA, Aa and aa represent homozygous dominant, heterozygote and homozygous recessive genotypes, then their frequencies will be p^2, 2pq, and q^2 respectively.

Under the HWE, the sum of the frequencies of three genotypes must add up to one, i.e., p2 + 2pq + q2 = 1. The HWE was analysed using chi-squared test with one degree of freedom

and the p-value ≤ 0.05 was considered significant. The statistical analysis was carried out by SPSS v 21. The sample size was calculated by using Quanto 1.2.4 (Gauderman et al, 2002).

Odds ratio under allelic and genotype models was calculated by using 2X2 contingency table using statistical software SPSS version 21.

An association under additive genetic model was analyzed by Chi- squared linear trend (Extended Mantel Haenszel) with one degree of freedom (http://www.openepi.com/DoseResponse/DoseResponse.htm).

The continuous variables were tested for the normality using SPSS using Kolmogorov-Smirnov and Shapiro-Wilk test with statistical significance at p-value ≤ 0.05.

The anthropometric and biochemical variables between case and control group was analysed by using student t test if the variables were normally distributed and Mann-Whitney U test for the variables with significant deviation from normal distribution even after log transformation of the variables. Similarly, the non-parametric Kruskal –Wallis test was used instead of one-way anova if the variables were not normally distributed even after log transformation of the variables. The p-value were adjusted for the multiple comparison in the post-hoc Kruskal-Wallis test. All the parametric and non-parametric tests were analysed using SPSS (Version 21.0. Armonk, NY: IBM Corp).

The odds ratio from binomial and multinomial logistic regression were calculated using SPSS. The estimation of the model was based upon the criteria: Good-ness-of fit, 2 log likelihood of reduced model, pseudo R-squared (Nagelkerke and McFadden).

4.13 Results

The figures 4.04, 4.05, 4.06 represents chromatogram of the sequenced samples 157,127 and 134 identified as GG, GA and AA genotypes of rs 8192678 of PPARGC1A gene

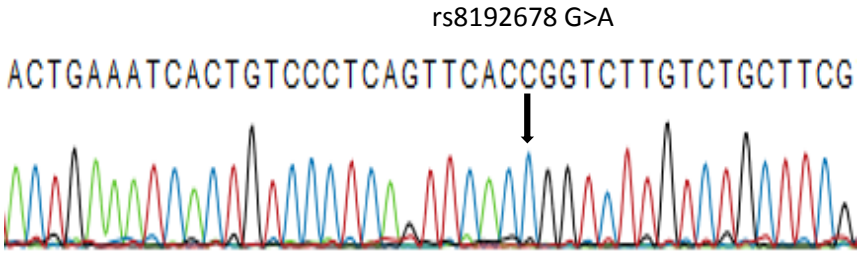

Figure 4.04: Homozygous dominant (GG) (as indicated by arrow) of Gly482Ser mutation in PPARGC1A gene of reverse sequencing. Chromatogram sequence derived from the sample 157 of control group.

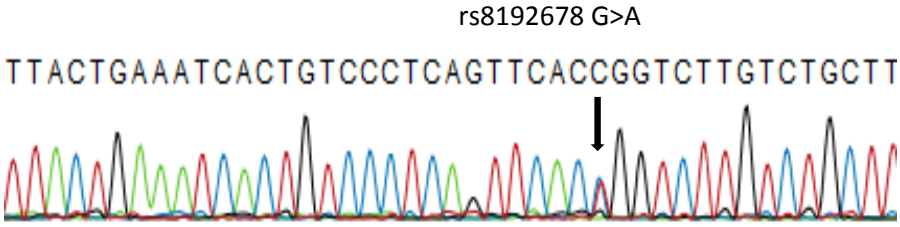

Figure 4.05: Heterozygous (AG) (as indicated by arrow) of Gly482Ser mutation in PPARGC1A gene of reverse sequencing. Chromatogram sequence derived from the sample 127 of control group.

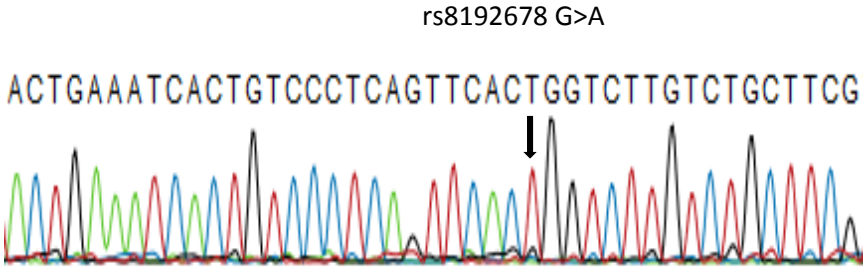

Figure 4.06: Homozygous recessive (AA) (as indicated by arrow) of Gly482Ser mutation in PPARGC1A gene of reverse sequencing. Chromatogram sequence derived from the sample 134 of control group.

4.13.1 Case-control population characteristics.

Table 4.08 shows the characteristics of the case (with metabolic syndrome) and control (without metabolic syndrome). The case and control group were matched by age with a mean age of 34.07 years in case group and 31.01 years in the control group. The number of males and females in the case group is almost similar but in the control group the number of males was higher than females. Table 4.08 shows the mean anthropometric and biochemical values of both case and control groups. The variables measured were not normally distributed and the log transformed data were also not normally distributed, therefore a non-parametric Mann-Whitney test was used instead of student t-test to determine any association the variables by comparing the case with the control group. A significant difference in age, weight, BMI, waist circumference, hip circumference, systolic blood pressure, diastolic blood pressure, fasting glucose, total cholesterol, TG and LDL-C was found between the two groups. The predominant subgroup populations found in the populations were: Bramhan, Chetri and Tharu.

Table 4.08: Anthropometrical and biochemical variables for case (Metabolic syndrome) and control groups.					
Variables	Metabolic syndrome (n = 88)		Control (n = 103)		p-value
Male/Female	43/45= 0.95		60/43 = 1.39		
	Mean	Std.	Mean	Std.	
Height (cm)	162.83	10.09	161.25	9.46	0.25
Weight (Kg)	66.73	13.30	59.23	11.06	p≤0.05*
BMI (Kg/m^2)	25.02	3.60	22.72	3.41	p≤0.05*
Waist circumference (cm)	91.65	10.55	83.39	10.47	p≤0.05*
Hip circumference (cm)	95.20	8.55	87.31	10.20	p≤0.05*
WHR (Waist-to-hip ratio)	0.96	0.06	0.96	0.06	0.69
Heart rate (beat per minute)	84.41	9.36	84.22	12.13	0.54
Systolic blood pressure (mm Hg)	124.99	11.25	118.75	7.84	p≤0.05*
Diastolic blood pressure (mm Hg)	83.81	8.62	77.62	6.94	p≤0.05*
fasting glucose (mmol/L)	5.86	0.86	5.40	0.38	p≤0.05*
TC (mmol/L)	4.84	1.25	4.38	1.01	p≤0.05*
HDL-C (mmol/L)	1.04	0.33	1.14	0.33	0.02*
TG (mmol/L)	1.80	0.53	1.45	0.49	p≤0.05*
LDL-C (mmol/L)	2.99	1.12	2.58	0.84	p≤0.05*

BMI = Body mass index; TC = Total cholesterol; HDL-C = High-density lipoprotein; TG = Triglycerides; LDL-C = Low-density lipoprotein; *statistically significant (p-value ≤ 0.05, Mann-Whitney U test); Std = Standard deviation.

4.13.2 Genotype and Allele frequency of rs8192678 marker in case (MetS), control and overall population.

Table 4.09, shows the number of genotypes of rs8192678 in case, control and overall population. The number of normal homozygous (GG), heterozygous (GA) and mutant homozygous (AA) genotypes are: 39, 42 and 7 respectively in case (subjects with Met) group. Similarly, the number of normal homozygous (GG), heterozygous (GA) and mutant homozygous (AA) genotypes are: 38, 52 and 13 in control (without MetS) group.

Table 4.09: Number of genotypes of rs8192678 of PPARGC1A gene in case and control groups.

Marker	Gene	Genotype	Sample size (n = 191)	
			Case	Control
rs8192678	PPARGC1A	GG	39	38
		AG	42	52
		AA	7	13

Table 4.10, shows the allele frequency of minor allele of rs8192678 in case, control and overall population. The frequency of minor allele in case, control and overall population is 0.31, 0.27 and 0.35 respectively. The allele frequency in control group is higher than the case group.

Table 4.10: Allele frequency of rs8192678 of PPARGC1A gene in case, control and overall populations.

Populations	Marker	Gene	Sample size	Allele	Allele type	Allele frequency
Overall population	rs8192678	PPARGC1A	191	A	Minor	0.31
				G	Major	0.69
Case (with MetS) group			88	A	Minor	0.27
				G	Major	0.73
Control (without MetS) group			103	A	Minor	0.35
				G	Major	0.65

MetS = Metabolic syndrome, PPARGC1A = Peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

The minor allele (A) frequency of rs8192678 (Gly482Ser) of the PPARGC1A gene in the overall population was found to be 0.31 whereas it was 0.27 and 0.35 in metabolic syndrome and control group respectively as shown in table 4.10. The highest allele frequency was found in the control group and the lowest was found in the case (MetS) group.

Table 4.11: Allele frequencies of rs8192678 in Tharu, Bramham and Chetri populations.

				Allele frequency		
Gene	G		Allele	Tharu	Chetri	Bramhan
PPARGC1A	rs8192678	Number		n = 25	n = 82	n = 81
		minor	A	0.48	0.34	0.31
		major	G	0.52	0.66	0.69
		minor	G	0.02	0.06	0.13
		major	C	0.98	0.94	0.87

PPARGC1A = Peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

The minor allele frequency (A) of rs8192678 in Tharu, Brahman and Chetri populations were 0.48, 0.34 and 0.31 respectively as shown in table 4.11.

4.13.3 Evaluation of genotype frequency in Hardy-Weinberg equilibrium (HWE) in a case and control population.

The observed genotype frequency of the markers was subjected to Hardy-Weinberg equilibrium test by using simple chi-squared test in case, control and overall populations and the results as shown in the table 4.12 were not significantly different. It showed that the genotype frequency of the markers does not violate the Hardy-Weinberg equilibrium assumptions. The minor allele frequencies in Tharu, Chetri and Brahman populations were all in HWE.

Table 4.12: Hardy-Weinberg equilibrium tests for rs8192678 polymorphism in overall, case, control, Tharu, Chetri and Bramhan populations.					
Population	SNP	Gene	ChiSq	p-value	Significance
Overall (N= 191)	rs8192678	PPARGC1A	0.03	0.88	Ns
Case (n = 88)	rs8192678	PPARGC1A	0.16	0.69	Ns
Control (n = 103)	rs8192678	PPARGC1A	0.06	0.80	Ns
Tharu (n = 25)	rs8192678	PPARGC1A	0.04	0.85	Ns
Chetri (n = 82)	rs8192678	PPARGC1A	3.05	0.08	Ns
Bramhan (n = 81)	rs8192678	PPARGC1A	0.00	0.99	Ns

PPARGC1A = Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, ChiSq = Chi squared test, p-value <0.05 is considered significant. Ns = nonsignificant

4.13.4 An association of minor allele and genotypes of rs8192678 using allelic, dominant, recessive and additive genetic model.

The allelic model of association of the minor allele of rs8192678 was analysed using the 2X2 contingency table and the obtained odds ratio (OR= 0.71, 95% CI = 0.46 -1.08, p-value=0.11) was not significantly associated with the metabolic syndrome as shown in table 4.13.

Table 4.13: Association of the minor allele of rs8192678 under allelic model.								
Gene	Case		Control			Odds Ratio ($OR_{A)}$	95% CI	Significance
PPARGC1A	Allele	N	Allele	N	test			
	A (minor)	56	A (minor)	78	A vs G	0.71	0.46-1.08	0.11
	G (major)	120	G (major)	128				

PPARGC1A = Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, OR = Odds ratio.

The association of the minor allele (A) of rs8192678 with the Case (MetS) under dominant (GG vs AG+AA), recessive (AA vs GG+AG) and co-dominant (AG vs GG vs AA) genetic model is shown in the table 4.14. Under the dominant genetic model, no significant association between the dominant genotype and the combined AG+AA genotype was achieved (OR = 1.36, 95% CI = 0.76-2.43, p-value = 0.30). An association analysed under a recessive model showed no significant difference (OR = 0.60, 95% CI = 0.23-1.57, p-value = 0.29). Similarly, no significant association was achieved under co-dominant model: heterogeneous co-dominant (AG vs GG) (OR = 1.05, 95% CI = 0.56-1.97, p-value = 0.89), heterogeneous co-dominant (AG

vs AA) (OR = 2.05, 95% CI = 0.74-5.68, p-value = 0.16), homogenous co-dominant (AA vs GG) (OR = 0.51, 95% CI = 0.18- 1.42, p-value = 0.19) as shown in table 4.14

Table 4.14: Association of rs8912678 under dominant, recessive and codominant model

Gene	Model	Case		Control			OR	95% CI	Chi sq (pearson)	Sig
PPARGC1A	Dominant model	Genotype	N	Genotype	N	test				
		GG	39	GG	38	GG vs AG+AA	1.36	0.76-2.43	1.09	0.30
		AG+AA	49	AG+AA	65					
	Recessive model	AA	7	AA	13	AA vs GG+AG	0.60	0.23-1.57	1.1	0.29
		GG+AG	81	GG+AG	90					
	Co-dominant model									
	heterogeneous co-dominant	GG	39	GG	38	AG vs GG	0.79	0.43-1.44	0.6	0.44
	heterogeneous co-dominant	AG	42	AG	52	AG vs AA	1.5	0.55-4.10	0.63	0.43
	homogenous co-dominant	AA	7	AA	13	AA vs GG	0.52	0.19-1.46	1.56	0.21

PPARGC1A = Peroxisome proliferator-activated receptor gamma coactivator 1-alpha,
OR = odds ratio, Chi sq = Chi squared test, sig = significance, p-value ≤ 0.05 was considered significant.

The analysis under the additive model of rs8192678 showed that the genotype with risk allele was not significantly associated case (MetS) (Chi sq (MH) = 1.65, p-value = 0.20). The baseline genotype is GG and the risk score has been assigned to the AG and AA according to the extra addition of the risk allele (A) as shown in table 4.15.

Table 4.15: Association of the genotypes of rs8192678 under additive genetic model using the chi squared test for linear trend.

Gene	Genotype risk score	Case		Control		Test	Mantel- Haenszel Odds ratio	Crude Odds ratio	Chi sq for linear trend (Extended Mantel-Haenszel) (df = 1)	P-value
PPARGC1A		Genotype	N	Genotype	N				1.65	0.20
	0	GG	39	GG	38	GG vs GG	1	1		
	1	AG	42	AG	52	AG vs GG	0.79	0.79		
	2	AA	7	AA	13	AA vs GG	0.53	0.53		

PPARGC1A = Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, Chi sq = Chi squared test, p-value ≤ 0.05 is considered significant. G is the major allele and A is the risk or minor allele. Normal homozygous genotype (GG) has been assigned as base line genotype with risk score as 0 and heterozygote and mutant homozygous genotype as 1 and 2 respectively.

The continuous metabolic variables were not normally distributed and neither the log transformed data. Therefore, the Kruskal-Wallis test was used for the difference in the variables in three genotype groups based on the rank and those found significant were only were further analysed by post-hoc test. The variables test for the Kruskal-Wallis test are: Height, weight, BMI, Waist circumference (WC), Hip circumference (HC), waist-hip-ratio, heart rate, systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting glucose, total cholesterol (TC), HDL-C, triglycerides and LDL-C is shown in the table 4.16.

Likewise, when the genotypes with SBP and DBP were compared among themselves (GG with AG, GG, with AA and AG with AA) according to the rank and no significant difference was observed as shown in table 4.16. Similarly, the difference in the fasting glucose in those genotype groups was not statistically significant.

When the lipid levels (TC, HDL-C, TG, LDL-C) of different genotype were compared as mentioned above, no statistical significance was observed.

Table 4.16: Independent samples Kruskal-Wallis test of the genotypes (GG, AG and AA) of rs8192678 of PPARGC1A gene.

Variables	GG (n = 77)			AG (n = 94)			AA (n = 20)			p-value
	Median	Minimum	Maximum	Median	Minimum	Maximum	Median	Minimum	Maximum	
Height (cm)	164	143	182	163.5	140	180	160	144	186	0.76
Weight (Kg)	61.9	41.75	95.05	61.1	42.45	92.65	60.3	37	94.7	0.75
Waist circumference (cm)	88	67.5	110	85	66	118	87	71	105	0.59
Hip circumference (cm)	91	68.5	110	90	67	122	90	73.75	107.5	0.47
Heart rate (beat per minute)	83	58	113	83	63	130	87.5	65	107	0.58
Systolic blood pressure (mm Hg)	123	105	148	118	102	149	120	93	143	0.83
Diastolic blood pressure (mm Hg)	80	64	98	79	62	100	80	66	105	0.57
fasting glucose (mmol/L)	5.5	4.4	10	5.43	3.7	8	5.5	4.8	7.5	0.66
TC(mmol/L)	4.53	2.59	8.41	4.42	2.66	12.93	4.75	2.77	7.11	0.78
HDL-C (mmol/L)	1.11	0.44	2.02	1.03	0.41	2.35	1.14	0.7	2.1	0.09
TG (mmol/L)	1.52	0.51	3.51	1.66	0.52	3.78	1.52	0.55	2.75	0.77
LDL-C (mmol/L)	2.6	1.2	6.2	2.68	1.2	9.9	2.70	0.96	5.15	0.99
BMI (kg/m2)	23.5	16.6	31.8	23	16.9	35.7	23.55	15.8	32	0.78
Waist-to-hip ratio	0.97	0.81	1.15	0.97	0.81	1.11	0.96	0.9	1.14	0.97

BMI = Body mass index; TC = Total cholesterol; HDL-C = High-density lipoprotein; TG = Triglycerides; LDL-C = Low-density lipoprotein. Post-hoc test could not be conducted as none of the genotype groups are significantly different.GG = normal homozygous genotype; AG = heterozygote and AA = mutant homozygous genotype. The p-value ≤ 0.05 was considered significant, Kruskal-Wallis test.

The genotypes of rs8192678 were regressed against the metabolic phenotypes using multinomial logistic regression as shown in table 4.17. In AG genotype group, no significant change in odds of the metabolic phenotypes was observed except systolic blood pressure (SBP). AG genotype was significantly associated with 1.04-fold decreased levels of SBP in comparison to GG genotype (β estimate = -0.04, OR = 0.96, 95%CI = 0.93-1.00, p-value = 0.03). This remained significant even after adjusting with age, BMI and gender as shown in

table 4.17. Similarly, AA genotype was associated with 1.02-fold decreased levels of SBP in comparison to GG genotype (β estimate = -0.08, OR = 0.93, 95%CI = 0.86-1.00, p-value = 0.05), however; no significant results were obtained after adjusting it with age, gender and BMI as shown in table 4.17.

Table 4.17: Multinomial logistic regression of PPARGC1A genotypes with systolic blood pressure after adjusting with age, gender and BMI.

PPARGC1A		β estimate	Std. Error	Wald	df	Sig.	OR	95% CI	
								Lower Bound	Upper Bound
AG	Intercept	3.72	2.08	3.20	1.00	0.07			
	SBP (mm Hg)[a]	-0.04	0.02	4.86	1.00	0.03*	0.96	0.93	1.00
	BMI (kg/m2)	0.00	0.05	0.00	1.00	0.97	1.00	0.91	1.10
	Age	0.03	0.03	1.68	1.00	0.20	1.03	0.98	1.09
	Male	0.29	0.34	0.74	1.00	0.39	1.34	0.69	2.60
	Female	0[b]	.	.	0.00	.	.	.	.
AA	Intercept	1.75	3.37	0.27	1.00	0.60			
	SBP (mm Hg)a	-0.02	0.03	0.68	1.00	0.41	0.98	0.93	1.03
	BMI (kg/m2)	-0.05	0.08	0.37	1.00	0.55	0.95	0.81	1.12
	Age	0.03	0.04	0.46	1.00	0.50	1.03	0.95	1.12
	Male	0.02	0.54	0.00	1.00	0.97	1.02	0.35	2.97
	Female	0[b]	.	.	0.00	.	.	.	.

BMI = Body mass index, SBP = systolic blood pressure, PPARGC1A = peroxisome proliferator-activated receptor gamma coactivator-1alpha, CI = confidence interval. The reference category is GG, [b]This parameter is set to zero because it is redundant. (SBP)[a] = adjusted with BMI, age and gender.

Table 4.18 shows the interaction of SBP with age, gender and BMI in AG and AA genotype group. In an AG genotype group, it is apparent that there is a significant interaction between SBP and BMI where the BMI has almost nullified the effect (OR = 1.00, 95%CI = 1.00-1.00, p-value = 1.00). The interaction between SBP and gender (male and female) in the AG genotype group in comparison to the GG genotype group, no significant interaction was evident. A significant interaction between SBP and age was evident in this genotype group in comparison to the reference genotype GG (p-value = 0.18) as shown in table 4.18. Similarly, a significant interaction of SBP with age, gender and BMI was evident in AA genotype group in comparison with the reference genotype group GG where all of these interactions has neutralized the effect observed without interaction.

Table 4.18: Interaction of systolic blood pressure of genotype group AG and AA group with age, gender and BMI.								
PPARGC1A[a]		β estimate	Std. Error	df	Sig.	OR	95% Confidence Interval	
							Lower Bound	Upper Bound
AG	Intercept	4.98	2.16	1.00	0.02			
	SBP X BMI	0.00	0.00	1.00	1.00	1.00	1.00	1.00
	SBP X Male	-0.05	0.02	1.00	0.03*	0.95	0.91	1.00
	SBP X Females	-0.05	0.02	1.00	0.03*	0.95	0.91	0.99
	SBP X Age	0.00	0.00	1.00	0.18	1.00	1.00	1.00
AA	Intercept	1.58	3.43	1.00	0.65			
	SBP X BMI	0.00	0.00	1.00	0.52	1.00	1.00	1.00
	SBP X Male	-0.02	0.03	1.00	0.52	0.98	0.92	1.05
	SBP X Females	-0.02	0.04	1.00	0.53	0.98	0.91	1.05
	SBP X Age	0.00	0.00	1.00	0.44	1.00	1.00	1.00

Reference category is GG genotype. SBP = systolic blood pressure, BMI = body mass index, GG = Homozygous dominant, AG = heterozygote, AA = homozygous recessive. *Significant difference between two genotype groups ($p \leq 0.05$, Mann-Whitney U test. BMI = body mass index; HDL-C = high density lipoprotein; LDL-C = low density lipoprotein; TG = triglyceride; TC = total cholesterol; FPG = Fasting plasma glucose; SBP = systolic blood pressure; DBP = diastolic blood pressure; statistically significant ($p \leq 0.05$, Mann-Whitney U test).

Table 4.19 and 4.20 refers to the difference in the variables in two different genotype groups under dominant and recessive genetic model. Under the dominant model, systolic blood pressure in GG genotype group was significantly higher in comparison to the GA and AA genotype group (p-value = 0.04). However, under the recessive genetic model no significant difference was observed in any of the variables.

Table 4.19: Anthropometric and biochemical characteristics by genotype groups of rs8192678 under dominant genetic model.					
Variables	Genotype groups				p-value
	GG (n = 77)		GA+AA (n = 114)		
	Mean	Standard. dev	Mean	Standard. dev	
Height (cm)	162.50	9.20	161.62	10.15	0.52
Weight (Kg)	63.32	12.38	62.26	12.91	0.53
Waist circumference (cm)	88.03	10.35	86.63	11.85	0.32
Hip circumference (cm)	92.01	8.83	90.23	11.07	0.25
Heart rate (beat per minute)	83.87	10.49	84.61	11.23	0.69
Systolic blood pressure (mm Hg)	123.40	10.18	120.42	9.80	0.04*
Diastolic blood pressure (mm Hg)	80.79	7.36	80.25	8.96	0.41
fasting glucose (mmol/L)	5.64	0.77	5.59	0.61	1.00
TC (mmol/L)	4.58	1.08	4.61	1.20	0.99
HDL-C (mmol/L)	1.09	0.30	1.10	0.35	0.93
TG (mmol/L)	1.61	0.50	1.61	0.56	1.00
LDL-C (mmol/L)	2.77	0.92	2.77	1.05	0.95
BMI (kg/m2)	23.82	3.16	23.75	4.00	0.52
Waist-to-hip ratio	0.96	0.06	0.96	0.06	0.83

GG = Homozygous dominant, AG = heterozygote, AA = homozygous recessive. BMI = body mass index; HDL-C = high density lipoprotein; LDL-C = low density lipoprotein; TG = triglyceride; TC = total cholesterol; FPG = Fasting plasma glucose; SBP = systolic blood pressure; DBP = diastolic pressure; statistically significant ($p \leq 0.05$, Mann-Whitney U test).

Table 4.20: Anthropometric and biochemical characteristics by genotype groups of rs8192678 under recessive genetic model.					
Variables	Genotype group				p-value
	AA (n = 20)		GG + AG (n = 171)		
	Mean	Standard. dev	Mean	Standard. dev	
Height (cm)	161.15	10.12	162.07	9.75	0.57
Weight (Kg)	60.59	12.28	62.93	12.73	0.57
Waist circumference (cm)	86.01	9.52	87.33	11.47	0.64
Hip circumference (cm)	89.09	9.95	91.16	10.28	0.49
Heart rate (beat per minute)	86.25	10.89	84.08	10.93	0.30
Systolic blood pressure (mm Hg)	120.85	10.78	121.71	9.98	0.82
Diastolic blood pressure (mm Hg)	81.85	10.90	80.31	8.01	0.67
fasting glucose (mmol/L)	5.65	0.62	5.61	0.69	0.38
TC(mmol/L)	4.65	1.09	4.59	1.16	0.49
HDL-C (mmol/L)	1.24	0.35	1.08	0.32	0.06
TG (mmol/L)	1.53	0.59	1.62	0.53	0.48
LDL-C (mmol/L)	2.71	0.92	2.78	1.01	0.95
BMI (kg/m2)	23.30	3.96	23.84	3.65	0.69
Waist-to-hip ratio	0.97	0.05	0.96	0.06	0.84

GG = Homozygous dominant, AG = heterozygote, AA = homozygous recessive. BMI = body mass index; HDL-C = high density lipoprotein; LDL-C = low density lipoprotein; TG = triglyceride; TC = total cholesterol; FPG = Fasting plasma glucose; SBP = systolic blood pressure; DBP = diastolic pressure; statistically significant ($p \leq 0.05$, Mann-Whitney U test).

4.14 Discussion

It is very important to understand the genetic factors associated with metabolic syndrome and its components in order to ascertain the extent of genetic risk posed in the Nepalese population. Metabolic syndrome is a cluster of factors which includes: obesity, hypertension, hyperglycemia and lipidemia. By using appropriate statistical models, in addition to MetS, the genetic risk associated with other metabolic factors can also be comprehended. Even though this is a small sample sized study conducted in the Far West region of Nepal, it is vital to understand the genetic underpinning of the disease for the better screening, intervention

and management of the disorder in the future. This thesis has attempted to analyze the prevalence of the minor allele frequency of rs8192678 (Gly482Ser) of the PPARGC1A gene in the Nepalese population. It has also sought to analyse an association of the variants with metabolic syndrome and its components in the same population. The strength of association between the genotypes of the SNPs and the components of metabolic syndrome has also been analysed. To the best of our knowledge, this study has not been conducted before in the Nepalese population.

All the variants studied, followed the Hardy-Weinberg equilibrium (HWE) in the overall and both cases (subjects with MetS) and control (subjects without MetS) population. The departure of the variants from HWE might suggest, the selective pressure, inbreeding, population stratification and also the genotyping error (Wigginton et al, 2005).

The minor allele frequencies (MAF) of rs8192678 (Gly482Ser) were 0.31 in the overall population to 0.27 in cases and 0.35 in control groups. The MAF of the variant was 0.29 in South Asian population analysed from 1000 genome project (https://www.ncbi.nlm.nih.gov). The MAF of the variant was found 0.28 in the neighboring Indian population (Vimaleswaran et al, 2005). Similarly, a study carried out by Gayatiri et al, 2009 found MAF 0.28, 0.26 and 0.54 in subjects with normal glucose tolerance, type 2 diabetes and diabetic nephropathy in Indian population. Likewise, Bhat et al, 2007 found MAF 39.9% and 28.6% in case (T2D) and control group in Kashmiri population and 26.7% and 17.5% of cases (T2D) and control group in Punjab and Jammu population. Even though the MAF of the variant (rs8192678) tends to be population specific, the average MAF is very close to the population studied in South Asian region. The frequency of the minor allele is higher (0.36) in both subjects with and without metabolic syndrome in the European population when compared with our study (Ambye et al, 2004).

Under the allelic model, no significant association of the variant was found with the metabolic syndrome and the odds ratio (0.79, 95% CI = 0.46-1.08) suggests a rather protective effect but it is not statistically significant. An association of the genotypes of the variant was tested using dominant, recessive and co-dominant models and none of them were significantly associated with metabolic syndrome.

Under the additive genetic model, in which the Odds ratio was calculated by using Mantel-Haenszel formula, an addition of one extra minor allele (A) decreases the odds of the metabolic syndrome substantially and showing a trend of protective effect without being statistically significant. Mantel-Haenszel formula is a powerful method of minimizing the confounding factors (Tripepi et al, 2010) and has been used in this population in conjunction with other models.

The protein encoded by the PPARGC1A gene has been considered as a master regulator of the gene involved in energy metabolism .The minor allele of rs8192678 has been associated with metabolic disease such as type 2 diabetes in various populations including the South Asian. Prediabetes, obesity and elevated blood pressure are the risk factor for type 2 diabetes and the main components of metabolic syndrome. This intercontivity of the gene with various metabolic factors and ultimately a greater contributor to the metabolic syndrome made it a potential candidate for association study in the Nepalese population.

There might be various contributing factors that could have influenced the result, for example, in a complex metabolic disease like metabolic syndrome, various variants might contribute to the manifestation of the disease, therefore individual SNP might have small contribution. It is not only the variant itself, but its interaction with other factors, including environment might play a greater role in association with the metabolic traits. The effect size, which in the strength of association between the two variables, obtained in our study might be due to small sample size or even the possibility of population stratification cannot be ignored.

To address these issues to some extent, an association between genotypes and phenotypes have also been studied. The binomial and multinomial logistic regression have been used whenever required to analyze the strength of association between the genotypes and phenotype continuous variables. In a multinomial logistic regression, genoyptes of rs8192678 (GG, GA and AA) were regressed against the phenotypic variables, except for the systolic blood pressure, the explanatory power was poor for other variables. The genotypes (AG and AA) of rs8192678 had a statistically significant protective effect of systolic blood pressure (SBP) (OR = 0.96, 95% CI = 0.93-1.00, p-value = 0.03). Similar results were obtained by Anderson et al, 2005 in Caucasians and the protective effect was gender biased with women being more protective against it in comparison to men. The essential difference

between Anderson et al, 2005 and our study is that, in our study this effect was observed only in SBP, but not in diastolic blood pressure and the subjects were with and without metabolic syndrome.

The findings of the present study are consistent with those found by Oberkofler et al, 2003 where the AA genotype showed a significant protective effect against hypertension in males but not in female subjects. Similar results were obtained by Ingelsson et al, 2008 where the minor allele was associated with the decreased risk of diastolic dysfunction and the dose dependent effect was also observed.

In contrary to our results, the minor allele has been significantly associated with the risk of hypertension in Argentine (Sookoian et al, 2005) and French population (Cherufa et al, 2004). However, there was no evidence of statistically significant association of the minor allele with hypertension in Chinese (Chen et al, 2004) and Indian (Vimaleswaran et al, 2005) population.

It was observed that under the dominant genetic model (GG vs GA+AA), the SBP was found significantly different (p-value = 0.04) when analyzed using the Mann-Whitney U test. Again the GG genotype showed significantly higher levels of SBP in comparison to combined GA + AA group which is consistent with the result obtained from multinomial logistic regression. In this study it clearly indicates that GG genotype is the main culprit for higher SBP while GA and AA genotypes offers a protective effect. When the difference in SBP was looked at in two different genotype groups under a recessive model (AA vs GG + GA), no significant difference was observed, which could be due to small sample size of AA genotypes or it can be interpreted, in conjunction with the data from logistic regression and dominant model, that GA genotype offers better protection than AA group.

When AG and AA genotype group were predicted for interaction of SBP with age and BMI using multinomial logistic regression with reference to GG genotype, it was found that both age and BMI nullifies the protective effect of both AG and AA genotypes.

No evidence of association of lipid levels (HDL-C. LCL-C, TG, TC) with the minor allele has been found under either dominant (GG vs GA+AA) or recessive model (AA+ GG +GA). These variables were not fitted for the logistic regression model. Similarly, no association of the

minor allele with obesity indices (waist circumference and BMI) and fasting glucose level were observed in our study.

Chapter 5.

Prevalence and association of the SNPs rs7903146 of Transcription factor 7 like 2TCF7L2, rs9939609 of Fat mass and obesity-associated protein (FTO) and rs1801282 (Pro12Ala) of Peroxisome proliferator-activated receptor gamma (PPARG) with metabolic syndrome in the Nepalese population.

5.1 Introduction

The SNPs (rs7903146, rs9939609 and rs1801282) selected for the study have been found to be associated either with metabolic syndrome or its components which has already been described in the chapter 1. To the best of our knowledge, no literature was available regarding the association of these SNPs with metabolic syndrome and its components in the Nepalese population.

5.1.1 Aim

The aim of the research presented in this chapter is as follows:

a. To analyse the prevalence of single nucleotide polymorphisms rs7903146 of TCF7L2, rs9939609 of FTO and rs1801282 of PPARG gene in the Nepalese population.

b. To investigate the risk of the allele and genotypes of above mentioned SNPs to the MetS.

c. To investigate the possible association between the SNPs and MetS

d. To analyse the strength of association between genotypes of the SNPs and components of MetS using various statistical models.

5.2 Methods

The methods are as described in the chapter 4 in the sub-section 4.6, 4.7, 4.8, 4.9 and 4.10. Table 5.0a shows the prime sequences and the expected PCR product size of the SNPs: rs7903146, rs9939609 and rs1801282. Table 5.0b shows the thermocycler condition used for the amplification of the SNPs.

Table 5.0a: Primers used for the amplification and the size of the PCR product.

Gene	SNP	Primer	Primer sequence (5'-3')	Length (bps)	Product size (bps)
TCF7L2	rs7903146	Forward	ATTGGAGGGTTGCACATGTG	20	326
		Reverse	GCCCCTCTAACCTTTTCCTA	20	
FTO	rs9939609	Forward	TGGTGGTACGCTGCTATGGTTCTA	24	455
		Reverse	CAGCCTCTCTACCATCTTATGTCC	24	
PPARG	rs1801282	Forward	AGTGCCAGCCAATTCAAGCC	20	360
		Reverse	GGAAGACAAACTACAAGAGC	20	

Table 5.0b: Thermocycler condition for the PCR.

Condition	Temperature (°C)	Time
Initial Denaturation	94°C	3 minutes
Number of cycles	35	
Denaturation	94°C	30 seconds
		45 seconds
Annealing temperature (°C) used for the markers.		
rs7903146	47°C	
rs9939609	53°C	
rs1801282	47°C	
Extension	72°C	45 seconds
Final extension	72°C	10 minutes

5.3 Results

The figure 5.01 shows the PCR amplified agarose gel image of the SNP rs7904146. The size of the PCR product (326bp) is indicated by arrow. Each lane represents the sample 151, 152, 153, 154, 155 and 156 as shown on top of the figure. It showed that the size of the PCR product was as expected as estimated from the marker represented as M.

Similarly, the figures 5.02 and 5.03 represent the PCR amplified agarose gel image of rs9939609 and rs1801282 with expected size of 455bp and 360bp respectively. The size of the amplified PCR products was estimated by the known size of the marker as shown in the figure 5.02 and 5.03.

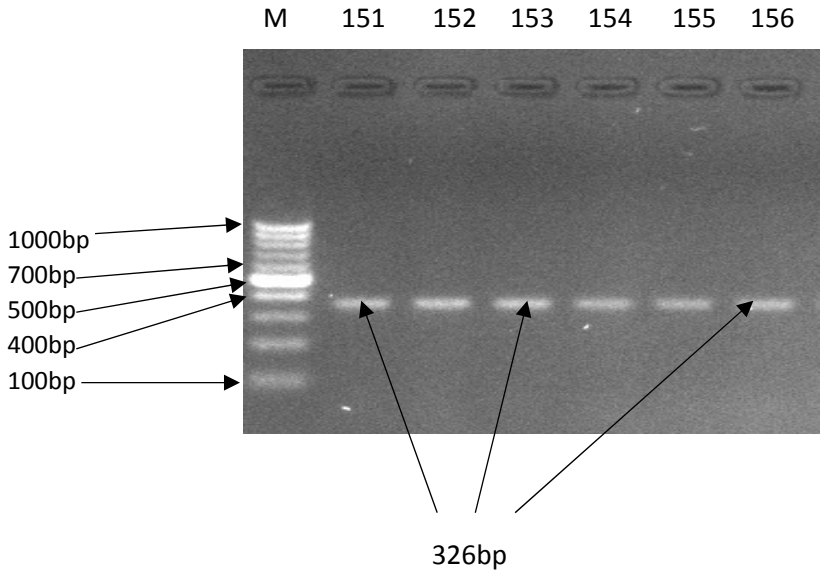

Figure 5.01: Gel image of the PCR amplified product of 326 bp using the primer for the marker rs7903146. Lane M represents 100bp marker (Xcelris labs); lane 151, 152, 153, 154, 155 and 156 represent the samples amplified.

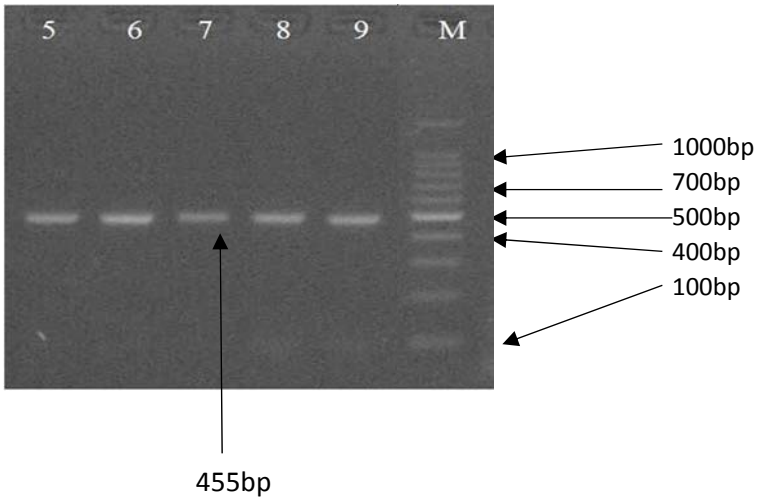

Figure 5.02: Gel image of the PCR amplified product of 455 bp using the primer for the marker rs9939609. Lane M represents 100bp marker (Xcelris labs); lane 5, 6, 7, 8 and 9 represent the samples amplified.

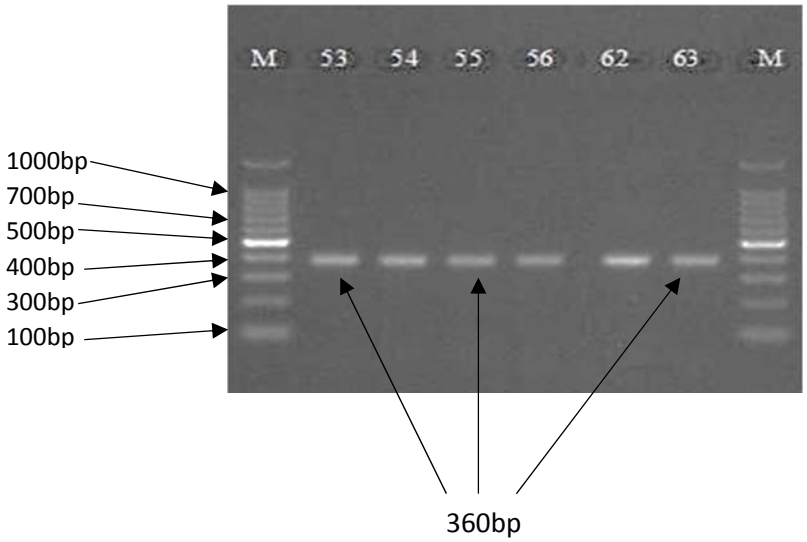

Figure 5.03: Gel image of the PCR amplified product of 455 bp using the primer for the marker rs1801282. Lane M represents 100bp marker (Xcelris labs); lane 53, 54, 55, 56, 62 and 63 represent the samples amplified.

The figure 5.04, 5.05 and 5.06 are the chromatogram image of the sequenced PCR product of SNP rs7903146 they represent CC (homozygote dominant), CT (heterozygote) and TT (homozygote recessive) genotype. The genotype of the sequenced SNP region is shown by arrow. They represent the sample 35, 11 and 20 respectively.

Similarly, the figure 5.07, 5.08 and 5.09 are the chromatogram image of the sequenced PCR product of SNP rs9939609 they represent TT (homozygote dominant), AT (heterozygote) and AA (homozygote recessive) genotype. The genotype of the sequenced SNP region is shown by arrow. They represent the sample 218, 179 and 187 respectively.

Likewise, the figure 5.10 and 5.11 are the chromatogram image of the sequenced PCR product of SNP rs1801282 they represent CC (homozygote dominant), CG (heterozygote) genotype. The genotype of the sequenced SNP region is shown by arrow. They represent the sample 109 and 96 respectively. The genotype GG (homozygote recessive) was not found in the population studied.

rs7903146 C>T

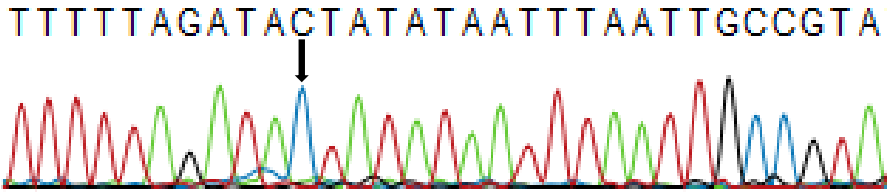

Figure 5.04: Homozygous dominant (CC) (as indicated by arrow) of rs7903146 C>T mutation in TCF7L2 gene of forward sequencing. Chromatogram sequence derived from the sample 35 of case group.

rs7903146 C>T

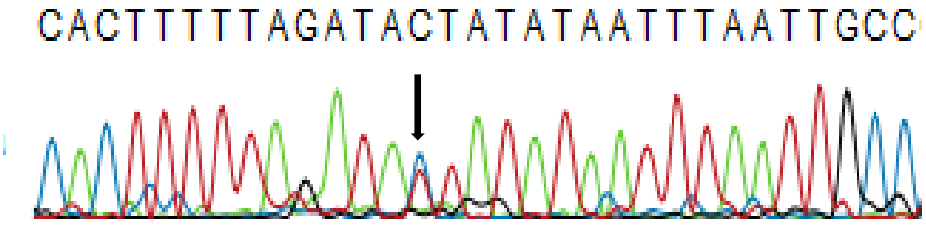

Figure 5.05: Heterozygous (CT) (as indicated by arrow) of rs7903146 C>T mutation in TCF7L2 gene of forward sequencing. Chromatogram sequence derived from the sample 11 of case group.

rs7903146 C>T

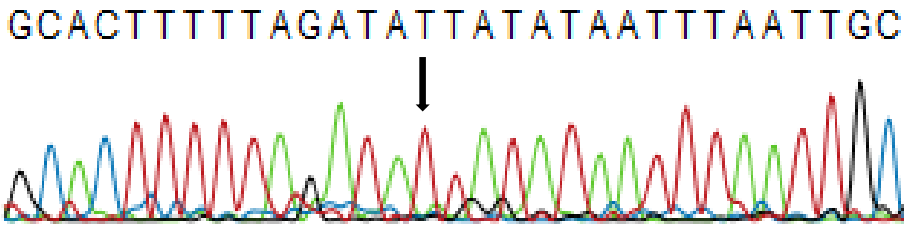

Figure 5.06: Heterozygous recessive (TT) (as indicated by arrow) of rs7903146 C>T mutation in TCF7L2 gene of forward sequencing. Chromatogram sequence derived from the sample 20 of case group.

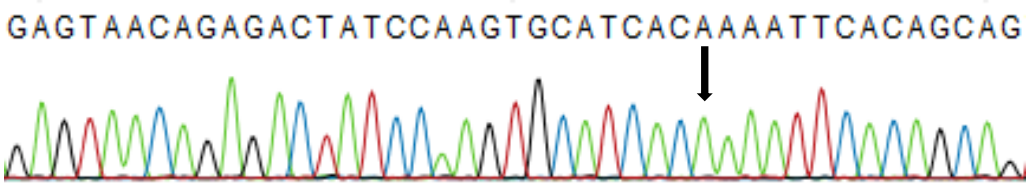

Figure 5.07: Homozygous dominant (TT) (as indicated by arrow) of rsrs9939609 T>A mutation in FTO gene of reverse sequencing. Chromatogram sequence derived from the sample 218 of control group.

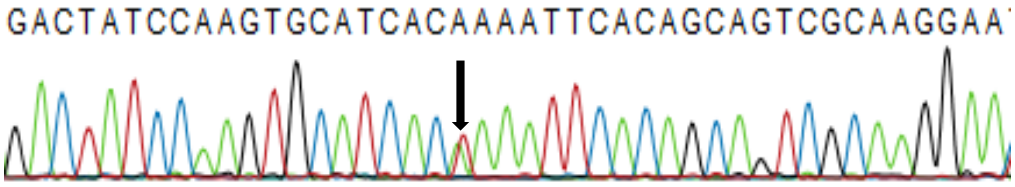

Figure 5.08: Heterozygote (AT) (as indicated by arrow) of rsrs9939609 T>A mutation in FTO gene of reverse sequencing. Chromatogram sequence derived from the sample 179 of control group.

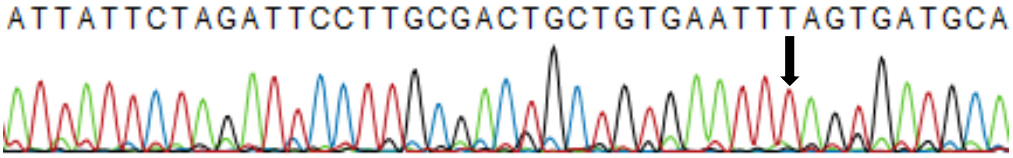

Figure 5.09: Homozygous recessive (AA) (as indicated by arrow) of rsrs9939609 T>A mutation in FTO gene of forward sequencing. Chromatogram sequence derived from the sample 187 of control group.

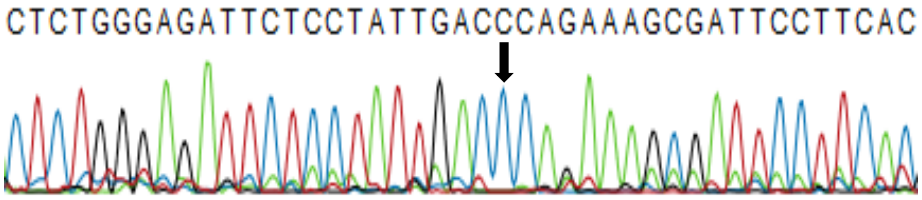

Figure 5.10: Homozygous dominant (CC) (as indicated by arrow) of rs1801282C >G (Pro12Ala) mutation in PPARG gene of forward sequencing. Chromatogram sequence derived from the sample 109 of control group.

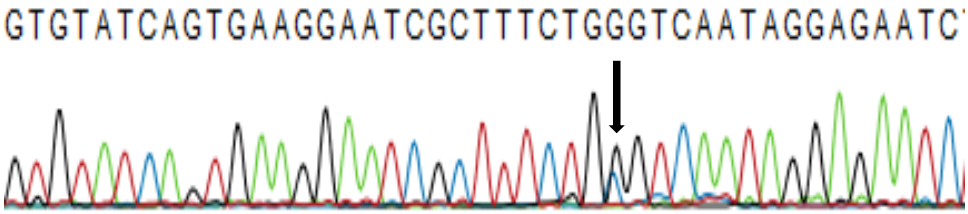

Figure 5.11: Heterozygote (CG) (as indicated by arrow) of rs1801282 C>G (Pro12Ala) mutation in PPARG gene of reverse sequencing. Chromatogram sequence derived from the sample 96 of control group.

5.3.1 Genetic association of rs7903146 of TCF7L2 gene with MetS

Table 5.01 shows the allelic model of association of the minor allele (T) of TCF7L2 gene with the metabolic syndrome (MetS). It showed that the minor allele is not significantly associated with MetS) (OR = 1.14, 95% CI = 0.69-1.88, p-value = 0.60).

Table 5.01: Association of the minor allele of sr7903146 under allelic model.

Gene	Case		Control			Odds Ratio ($OR_{T)}$	95% CI	Z-statistics	Significance
TCF7L2	Allele	N	Allele	N	test				
	T (minor)	38	T (minor)	40	T vs C	1.14	0.69-1.88	0.53	0.60
	C (major)	138	C (major)	166					

TCF7L2 = transcription factor 7-like 2, CI = confidence interval

Similarly, the table 5.02 shows the dominant, recessive and co-dominant model of association of rs7903146 with metabolic syndrome. Under the dominant model (CC vs CT+TT) of association no significant association was observed (OR = 0.82, 95% CI = 0.45-1.49, p-value = 0.52). The association under recessive model (TT vs CC+CT) also did not reach statistical significance (OR =0.97, 95% CI = 0.29-3.30, p-value = 0.97). Likewise, no significant association was achieved under co-dominant model: heterogeneous co-dominant (CT vs CC) (OR = 1.19, 95% CI = 0.64-2.24, p-value = 0.58), heterogeneous co-dominant (CT vs TT) (OR = 1.16, 95% CI = 0.32-4.23, p-value = 0.82), homogenous co-dominant (TT vs CC) (OR = 1.03, 95% CI = 0.3-3.56, p-value = 0.96) as shown in table 5.02

Table 5.02: Association of rs7903146 under dominant, recessive and co-dominant model										
Gene	Model	Case		Control			Odds Ratio	95% CI	Z statistics	Significance
TCF7L2	Dominant model	Gt.	N	Gt.	N	test				
		CC	55	CC	69	CC vs CT+TT	0.82	0.45-1.49	0.65	0.52
		CT+TT	33	CT+TT	34					
	Recessive model	TT	5	TT	6	TT vs CC+CT	0.97	0.29-3.30	0.04	0.97
		CC+CT	83	CC+CT	97					
	co-dominant model									
	heterogeneous co-dominant	CC	55	CC	68	CT vs CC	1.19	0.64-2.24	0.3	0.58
	heterogeneous co-dominant	CT	28	CT	29	CT vs TT	1.16	0.32-4.23	0.05	0.82
	homogenous co-dominant	TT	5	TT	6	TT vs CC	1.03	0.3-3.56	0.05	0.96

Gt = Genotype

Table 5.03 shows findings of additive model of association of rs7903146 using chi squared test for linear trend (Mantel-Haenszel). Under the additive model, no significant association with MetS was observed [Chi Sq (MH)] = 1.32, p-value = 0.25).

Table 5.03: Association of the genotypes of rs7903146 under additive genetic model using chi square for linear trend.

Gene	Genotype risk score	Case		Control		Exposure	Mantel- Haenszel Odds ratio	Crude Odds ratio	Chi sq for linear trend (Extended Mantel-Haenszel) (df = 1)	P-value
TCF7L2		Genotype	N	Genotype	N				1.32	0.25
	0	CC	55	CC	69	CC vs CC	1.00	1.00		
	1	CT	28	CT	28	CT vs CC	1.26	1.26		
	2	TT	5	TT	6	TT vs CC	1.05	1.05		

TCF7L2= Transcription factor 7 like 2

Table 5.04 shows the comparison of the genotypes with the mean rank of the metabolic phenotypes (Height, weight, HC, HC, BMI, waist hip ratio, heart rate, systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting glucose, total cholesterol (TC), HDL-C, triglycerides and LDL-C) using non-parametric Kruskal-Wallis test. The genotypic difference in the heart rate was not statistically significant after adjusting for multiple comparison.

Table 5.04: Independent samples Kruskal-Wallis test for the genotypes (CC, CT and TT) of rs7903146 of TCF7L2 gene.

Variables	CC (n = 124)			CT (n = 56)			TT (n = 11)			p-value	Genotype	Average rank of each genotype group	Post-hoc test based on average rank	P-value	Adjusted p-value of multiple comparison
	Med	Min	Max	Med	Min	Max	Med	Min	Max						
Height (cm)	164.0	140.0	186.0	162.0	147.0	182.0	159.0	146.0	178.0	0.70					
Weight (Kg)	61.48	37.00	95.05	61.20	41.75	94.70	56.80	41.80	90.00	0.42					
WC (cm)	87.50	67.50	112.50	87.50	66.00	118.00	90.00	70.00	107.00	0.91					
HC (cm)	90.00	68.50	115.00	90.00	67.00	122.00	92.00	76.00	108.00	0.91					
Heart rate (beat per minute)	81.00	58.00	104.00	86.50	65.00	111.00	88.00	76.00	130.00	0.01	CC	87.87	CT-CC	0.02	0.07
											CT	107.87	TT-CC	0.03	0.08
											TT	126.68	TT-CT	0.91	0.91
SBP (mm Hg)	119.0	93.0	149.0	117.0	102.0	148.0	118.0	106.0	133.0	0.75					
DBP (mm Hg)	79.00	62.00	103.00	80.00	65.00	105.00	81.00	68.00	89.00	0.96					
FPG (mmol/L)	5.50	3.70	8.20	5.48	4.60	10.00	5.70	4.80	7.50	0.83					
TC (mmol/L)	4.44	2.59	12.93	4.52	2.79	6.88	4.84	3.16	7.11	0.67					
HDL-C (mmol/L)	1.09	0.41	2.35	1.11	0.44	1.99	1.02	0.70	1.81	0.86					
TG (mmol/L)	1.62	0.51	3.78	1.67	0.52	3.51	1.60	0.65	2.33	0.83					
LDL-C (mmol/L)	2.61	0.96	9.90	2.66	1.20	4.70	2.90	1.90	5.15	0.58					
BMI (kg/m2)	23.50	15.80	34.10	22.95	16.90	35.70	23.30	18.20	28.40	0.41					
Waist-to-hip ratio	0.97	0.84	1.15	0.97	0.81	1.14	0.95	0.87	1.03	0.94					

WC = waist circumference, HC = hip circumference, BMI = body mass index; HDL-C = high density lipoprotein; LDL-C = low density lipoprotein; TG = triglyceride; TC = total cholesterol; FPG = Fasting plasma glucose; SBP = systolic blood pressure; DBP = diastolic pressure. Med = Median, Min = minimum value, Max = maximum value. The p-value are calculated from non-parametric Kruskal-Wallis test. CC = homozygous dominant, CT = heterozygote, TT = homozygous recessive. CT-CC, TT-CC, TT-CT represents post hoc test (multiple comparison) of genotype groups based upon average rank

Table: 5.05: Anthropometric and biochemical characteristics by genotype groups of rs7903146 under dominant genetic model.					
Variables	Genotype groups				p-value
	CC (n = 67)		CT + TT (n = 124)		
	Mean	St.dev	Mean	St.dev	
Height (cm)	161.95	10.25	162.01	8.86	0.97
Weight (Kg)	63.09	12.41	61.94	13.22	0.48
WC (cm)	87.40	10.97	86.82	11.87	0.74
HC (cm)	90.96	10.00	90.91	10.75	0.75
Heart rate (beat per minute)	82.65	10.29	87.39	11.43	0.01*
SBP (mm Hg)	121.65	9.37	121.57	11.23	0.47
DBP (mm Hg)	80.50	8.11	80.42	8.79	0.83
FPG (mmol/L)	5.60	0.63	5.63	0.78	0.70
TC (mmol/L)	4.59	1.25	4.60	0.96	0.72
HDL-C (mmol/L)	1.10	0.35	1.08	0.30	0.83
TG (mmol/L)	2.77	1.06	1.61	0.56	0.74
LDL-C (mmol/L)	2.77	1.06	2.77	0.89	0.76
BMI (kg/m2)	23.96	3.59	23.45	3.83	0.23
Waist-to-hip ratio	0.96	0.06	0.96	0.06	0.74

BMI = body mass index; HDL-C = high density lipoprotein; LDL-C = low density lipoprotein; TG = triglyceride; TC = total cholesterol; FPG = Fasting plasma glucose; SBP = systolic blood pressure; DBP = diastolic pressure. St.dev = standard deviation, *Statistical significance (p-value ≤ 0.05, Mann-Whitney U test).

Table 5.06: Anthropometric and biochemical characteristics by genotype groups of rs7903146 under recessive genetic model.					
Variables	Genotype group				p-value
	TT (n = 11)		CT + CC (n = 180)		
	Mean	St. dev	Mean	St. dev	
Height (cm)	159.91	8.90	162.10	9.82	0.42
Weight (Kg)	58.37	13.84	62.95	12.59	0.20
WC (cm)	87.36	12.53	87.18	11.22	0.90
HC (cm)	91.18	10.28	90.93	10.27	0.86
Heart rate (beat per minute)	93.55	16.83	83.74	10.25	0.06
SBP (mm Hg)	119.55	7.63	121.75	10.17	0.68
DBP (mm Hg)	79.82	6.56	80.51	8.44	1.0
FPG (mmol/L)	5.70	0.82	5.61	0.67	0.75
TC(mmol/L)	4.89	1.20	4.58	1.15	0.38
HDL-C (mmol/L)	1.06	0.29	1.10	0.33	0.58
TG (mmol/L)	1.49	0.55	1.62	0.54	0.71
LDL-C (mmol/L)	3.10	1.10	2.75	0.99	0.29
BMI (kg/m2)	22.59	3.42	23.85	3.68	0.33
Waist-to-hip ratio	0.96	0.06	0.96	0.06	0.98

BMI = body mass index; HDL-C = high density lipoprotein; LDL-C = low density lipoprotein; TG = triglyceride; TC = total cholesterol; FPG = Fasting plasma glucose; SBP = systolic blood pressure; DBP = diastolic pressure. St.dev = standard deviation, *Statistical significance (p-value ≤ 0.05, Mann-Whitney U test).

5.3.2 Genetic association of rs9939609 of FTO gene with MetS

An analysis of an association of the minor allele (A) with the metabolic syndrome under an allelic model is shown in table 5.07, however; it did not reach statistical significance (OR = 1.31, 95% CI = 0.83-2.06, p-value = 0.25).

Table 5.07: Association of minor allele (A) of rs9939609 with MetS under Allelic model

Gene	Case		Control			Odds Ratio ($OR_{A)}$	95% CI	Z-statistics	p-value
FTO	Allele	N	Allele	N	Test				
	A (minor)	51	A (minor)	49	A vs T	1.31	0.83-2.06	1.15	0.25
	T (major)	125	T (major)	157					

Similarly, table 5.08 shows an analysis of an association of rs9939609 with MetS under dominant (TT vs AT+AA), recessive (AA vs TT+AT) and co-dominant model (AT vs TT, AA vs AT and AA vs TT). Under the dominant model, no significant association with MetS was observed (OR = 0.66, 95% CI = 0.37-1.17, p-=value = 0.15) but the result rather showed protective tendency under this model. Likewise, no statistical significance reached under the recessive model (OR = 1.00, 95% CI = 0.32-3.10, P-value = 1.00). In the co-dominant model, highest odds ratio (OR = 1.63, 95% CI = 0.89-2.97) was observed under heterogeneous co-dominant model (AT vs TT) followed by homogenous Co-dominant model (AA vs TT) (OR = 1.06, 95% CI = 0.34-3.29) and heterogeneous co-dominant (AA vs AT) (OR = 0.65, 95% CI = 0.21-2.07) where none of the model reached statistical significance.

Table 5.08: Association of rs9939609 with MetS under dominant, recessive and co-dominant model.										
Gene	Model	Case		Control			Odds Ratio	95% CI	Chi sq	Significance
FTO	Dominant model	Genotype	N	Genotype	N	test				
		TT	43	TT	61	TT vs AT+AA	0.66	0.37-1.17	2.05	0.15
		AT+AA	45	AT+AA	42					
	Recessive model	AA	6	AA	7	AA vs TT+AT	1.00	0.32-3.10	0.00	1.00
		TT+AT	82	TT+AT	96					
	Co-dominant model									
	Heterogenous co-dominant model	TT	43	TT	61	AT vs TT	1.63	0.89-2.97	2.52	Ns
	Heterogenous co-dominant model	AT	39	AT	34	AA vs AT	0.65	0.21-2.07	0.53	Ns
	Homogenous Co-dominant model	AA	6	AA	8	AA vs TT	1.06	0.34-3.29	0.0	Ns

The analysis of an association of the genotypes with MetS under additive genetic model in shown in the table 5.09. Chi squared test for linear trend (extended Mantel Haenszel) was used and the genotypes were assigned the risk score according to the presence of minor allele (A). The baseline or reference genotype was TT and it was compared with other genotypes and the test did not confer any significant association.

Table 5.09: Association of rs9939609 with MetS under additive model.

Gene	Genotype risk score	Case		Control		Exposure	Mantel-Haenszel Odds ratio	Crude Odds ratio	Chi sq for linear trend (Extended Mantel-Haenszel) (df = 1)	P-value
FTO		Genotype	N	Genotype	N				1.32	0.25
	0	TT	43	TT	61	TT vs TT	1.00	1.00		
	1	AT	39	AT	35	AT vs TT	1.58	1.58		
	2	AA	6	AA	7	AA vs TT	1.22	1.22		

Table 5.10 shows the results from Kruskal-Wallis test. The Genotype groups with metabolic parameters are compared among themselves according to the rank. The weight of AA genotype was significantly higher in comparison with TT genotype (p-value =0.03). Similarly, AA genotype showed higher weight when compared with AT genotype but did not reach statistical significance (P-value = 0.32). Likewise, the AT genotype shows higher weight when compared with TT genotypes without reaching statistical significance (p-value = 0.20). The result clearly shows the dose-response effect for the A allele. The difference in BMI between AA and TT genotype shows the higher BMI tendency of AA genotype and the effect was statistically significant (p-value = 0.03). The dose-dependent effect of the minor allele A was clearly evident from the mean difference between AA and AT genotypes and AT and TT genotype but this difference did not reach statistical significance. However, the waist circumference, Hip circumference, heart rate, systolic blood pressure, diastolic blood pressure, fasting glucose, TC, HDL-C ,TG, LDL-C, Waist-to-hip ratio in genotype groups did not produce any significant difference after adjusting with multiple comparison as shown in table 5.10.

Table 5.10: Independent samples Kruskal-Wallis test of the genotypes (TT, AT and AA) of rs9939609 of FTO gene.															
Variables	Genotype groups										Kruskal-Wallis post hoc test				
	TT (n = 104)			AT (n = 74)			AA (n = 13)			p-value	Genotype	Average rank	Post-hoc test	P-value	Adjusted p-values for multiple comparisons
	Med	Min	Max	Med	Min	Max	Med	Min	Max						
Height (cm)	160	140	186	165	144	180	170	144	178	0.38					
Weight (Kg)	57.975	37	94.7	63	45	90	70.2	42.45	95.05	0.03*	TT	87.38	AT-TT	0.05*	0.16
											AT	103.56	AA-TT	0.03*	0.10
											AA	121.92	AA-AT	0.27	0.81
Waist circumference (cm)	85	67.5	118	89.5	66	110	91	76	110	0.02*	TT	86.57	AT-TT	0.03*	0.10
											AT	104.62	AA-TT	0.03*	0.08
											AA	122.38	AA-AT	0.29	0.86
Hip circumference (cm)	90	68.5	122	92	67	108	93	79	109	0.07					
Heart rate (beat per minute)	82	63	130	84	66	111	84	58	103	0.51					
Systolic blood pressure (mm Hg)	118	93	144	120.5	102	149	121	112	138	0.16					
Diastolic blood pressure (mm Hg)	78.5	62	105	80	64	103	80	71	100	0.07					
fasting glucose (mmol/L)	5.4	3.7	8.2	5.5	4.6	10	5.3	5	6.7	0.23					
TC(mmol/L)	4.42	2.59	8.41	4.5	2.77	12.93	4.76	3.04	7.73	0.44					
HDL-C (mmol/L)	1.11	0.44	2.1	1.06	0.41	2.25	1.09	0.62	2.35	0.67					
TG (mmol/L)	1.65	0.51	3.51	1.59	0.53	3.78	1.48	1.21	2.88	0.70					
LDL-C (mmol/L)	2.545	1.3	6.2	2.81	0.96	9.9	3.06	1.2	5.6	0.20					
BMI (kg/m2)	23	15.8	35.7	23.6	16.9	31.5	25.3	19.6	33.5	0.03*	TT	87.83	AT-TT	0.09	0.28
											AT	101.99	AA-TT	0.02*	0.05*
											AA	127.23	AA-AT	0.13	0.39
Waist-to-hip ratio	0.96	0.81	1.14	0.97	0.81	1.1	0.97	0.88	1.15	0.50					

BMI = Body mass index; TC = Total cholesterol; HDL-C = High-density lipoprotein; TG = Triglycerides; LDL-C = Low-density lipoprotein; statistical significance ≤ 0.05, Kruskal-Wallis).

Table 5.11 shows the findings of the multinomial logistic regression model used to identify the strength of association of the metabolic variables with the AA and AT genotype of FTO gene while TT being used as a reference genotype. The AT genotype is significantly associated with 1.04 increased levels of SBP in comparion to TT genotype (OR = 1.04, 95% CI = 1.01-1.08, p-value= 0.02); however, the statistical significance did not survive after adjusting with age, gender and BMI (Table 5.11).

Likewise, The AA genotype was significantly associated with 1.86-fold increased weight in comparison to TT genotype (OR = 1.86, 95%CI = 1.04-3.31, p-value = 0.04). When the weight was further subjected for adjustment with age, gender and BMI, the statistical significance was still maintained (OR = 1.07, 95%CI =1 .01-1.14, p-value = 0.02). As shown in the table 5.12, the genotype AA was significantly associated with 1.20-fold (OR = 1.20, 95% CI = 1.02-1.42, p-value = 0.03) increased BMI and 1.07-fold (OR = 1.07,95% CI = 1.01-1.14, p-value = 0.02) increased weight in comparison to TT genotype and both remained significant even after adjusting with age and genderi.

Table 5.11: Multinomial logistic regression of rs9939609 of FTO gene on the metabolic parameters.									
Genotype	Variables	B estimate	Std. Error	Wald	df	Sig.	OR	95% CI	
								Lower Bound	Upper Bound
AT	Intercept	19.52	20.16	0.94	1.00	0.33			
	Height	-0.16	0.12	1.76	1.00	0.18	0.85	0.67	1.08
	Weight	0.20	0.15	1.78	1.00	0.18	1.23	0.91	1.65
	WC	0.03	0.04	0.61	1.00	0.43	1.03	0.95	1.12
	HC	0.00	0.04	0.00	1.00	0.95	1.00	0.92	1.08
	HR	0.00	0.02	0.00	1.00	0.99	1.00	0.97	1.03
	SBP[b]	0.01	0.02	0.32	1.00	0.57	1.01	0.97	1.06
	SBP	.04	.02	5.25	1.00	0.02*	1.04	1.01	1.08
	DBP	0.02	0.03	0.52	1.00	0.47	1.02	0.97	1.08
	FPG	0.28	0.24	1.32	1.00	0.25	1.32	0.82	2.14
	HDL-C	-1.89	2.31	0.67	1.00	0.41	0.15	0.00	13.85
	TG	-1.08	1.08	1.00	1.00	0.32	0.34	0.04	2.83
	LDL-C	-1.74	2.31	0.57	1.00	0.45	0.18	0.00	16.16
	BMI	-0.58	0.40	2.11	1.00	0.15	0.56	0.25	1.23
AA	Intercept	71.57	40.36	3.15	1.00	0.08			
	Height	-0.49	0.25	3.95	1.00	0.05	0.61	0.38	0.99
	Weight	0.62	0.29	4.42	1.00	0.04*	1.86	1.04	3.31
	WC	0.06	0.09	0.55	1.00	0.46	1.07	0.90	1.27
	HC	-0.07	0.08	0.83	1.00	0.36	0.93	0.80	1.09
	HR	0.03	0.03	1.02	1.00	0.31	1.03	0.97	1.10
	SBP	0.00	0.05	0.00	1.00	0.98	1.00	0.91	1.10
	DBP	-0.01	0.06	0.02	1.00	0.88	0.99	0.89	1.11
	FPG	-0.46	0.61	0.56	1.00	0.46	0.63	0.19	2.11
	BMI	-1.37	0.77	3.19	1.00	0.07	0.25	0.06	1.14

The reference category genotype is TT. WC = waist circumference; HC = hip circumference; HR = heart rate; BMI = Body mass index; TC = Total cholesterol; HDL-C = High-density lipoprotein; TG = Triglycerides; LDL-C = Low-density lipoprotein; adjusting with (age, gender and BMI) [b]; OR = odds ratio; df = degree of freedom; *statistical significance at p-value ≤ 0.05

Table 5.12 : Multinomial logistic regression of rs9939609 of FTO gene on BMI and weight after adjusting with age and gender

FTO genotypes	Parameter	B estimate	Std. Error	Wald	df	Sig.	OR	95% Confidence Interval for OR	
								Lower Bound	Upper Bound
AT	BMI (kg/m2)	0.03	0.05	0.46	1.00	0.50	1.03	0.94	1.14
	Age	0.02	0.03	0.66	1.00	0.42	1.02	0.97	1.07
	Male	0.40	0.31	1.62	1.00	0.20	1.48	0.81	2.73
	Female[b]	0[b]			0.00				
	WEIGHT	0.02	0.02	0.90	1.00	0.34	1.02	0.98	1.05
	AGE	0.02	0.02	0.53	1.00	0.46	1.02	0.97	1.07
	Male	0.21	0.37	0.32	1.00	0.57	1.24	0.59	2.58
	Female[b]	0[b]			0.00				
AA	BMI (kg/m2)	0.18	0.08	4.65	1.00	0.03*	1.20	1.02	1.42
	Age	0.01	0.05	0.07	1.00	0.79	1.01	0.91	1.13
	Male	0.17	0.60	0.08	1.00	0.78	1.18	0.36	3.85
	Female[b]	0[b]			0.00				
	WEIGHT	0.07	0.03	5.73	1.00	0.02*	1.07	1.01	1.14
	AGE	0.01	0.05	0.04	1.00	0.85	1.01	0.91	1.12
	Male	-0.77	0.73	1.11	1.00	0.29	0.46	0.11	1.94
	Female[b]	0[b]			0.00				

a. The reference category is: TT.
b. This parameter is set to zero because it is redundant.
c. BMI and Weight were separately analysed and adjusted with age and gender
d. OR = Odds ratio

Table 5.13 shows the anthropometric and biochemical difference of the two genotype groups of rs9939609 under dominant genetic model (TT versus AT +AA) by using Mann-Whitney U test. It was found that waist circumference (WC), BMI and diastolic blood pressure (DBP) were found to be significantly different. It showed that AT and AA genotype group combined had significantly higher WC, BMI and DBP. Similarly, under the recessive model as shown in table 5.14, BMI was found to be significantly different in two genotype groups.

Table 5.13: Anthropometric and biochemical characteristics by genotype groups of rs9939609 under dominant genetic model.

Variables	Genotype group				p-value
	TT (n = 104)		AT + AA (n = 87)		
	Mean	Standard. dev	Mean	Standard. dev	
Height (cm)	161.17	9.67	162.94	9.84	0.17
Weight (Kg)	60.69	11.93	65.08	13.19	0.02*
WC (cm)	85.31	11.27	89.45	10.90	0.01*
HC (cm)	89.54	10.84	92.62	9.25	0.33
HR(beat per minute)	84.12	11.56	84.54	10.14	0.50
Systolic blood pressure (mm Hg)	120.41	9.93	123.07	10.03	0.06
Diastolic blood pressure (mm Hg)	79.11	7.96	82.10	8.52	0.02*
Fasting plasma glucose (mmol/L)	5.55	0.62	5.69	0.74	0.16
TC(mmol/L)	4.48	0.99	4.73	1.31	0.25
HDL-C (mmol/L)	1.10	0.31	1.09	0.35	0.40
TG (mmol/L)	1.62	0.51	1.59	0.57	0.45
LDL-C (mmol/L)	2.66	0.83	2.90	1.16	0.07
BMI (kg/m2)	23.33	3.88	24.32	3.35	0.03*
Waist-to-hip ratio	0.95	0.06	0.97	0.05	0.26

WC = waist circumference; HC = hip circumference; HR = heart rate; BMI = Body mass index; TC = Total cholesterol; HDL-C = High-density lipoprotein; TG = Triglycerides; LDL-C = Low-density lipoprotein; *statistically significant (p-value ≤ 0.05, Mann-Whitney U test)

WC = waist circumference; HC = hip circumference; HR = heart rate; BMI = Body mass index;

Table 5.14: Anthropometric and biochemical characteristics by genotype groups of rs9939609 under recessive genetic model.

Variables	Genotype group				p-value
	AA (n = 13)		TT + AT (n = 178)		
	Mean	Standard. dev	Mean	Standard. dev	
Height (cm)	163.00	12.55	161.90	9.57	0.55
Weight (Kg)	70.25	16.99	62.14	12.18	0.08
Waist circumference (cm)	92.92	10.88	86.78	11.21	0.07
Hip circumference (cm)	95.08	8.87	90.64	10.29	0.13
Heart rate (beat per minute)	86.46	12.51	84.15	10.81	0.25
Systolic blood pressure (mm Hg)	122.69	8.97	121.54	10.13	0.64
Diastolic blood pressure (mm Hg)	82.54	9.31	80.32	8.27	0.49
fasting glucose (mmol/L)	5.52	0.45	5.62	0.70	0.61
TC(mmol/L)	4.85	1.26	4.58	1.14	0.38
HDL-C (mmol/L)	1.13	0.47	1.09	0.32	0.98
TG (mmol/L)	1.61	0.45	1.61	0.54	0.55
LDL-C (mmol/L)	2.98	1.16	2.76	0.99	0.48
BMI (kg/m2)	26.15	4.25	23.61	3.58	0.04*
Waist-to-hip ratio	0.98	0.08	0.96	0.06	0.54

TC = Total cholesterol; HDL-C = High-density lipoprotein; TG = Triglycerides;
LDL-C = Low-density lipoprotein; *statistically significant (p-value ≤ 0.05, Mann-Whitney U test).

5.3.3 Genetic association of rs1801282 of PPARG gene with MetS

Table 5.15 shows the allelic model of association of the minor allele of rs1801282 with MetS. Under the allelic model, no significant association of the minor allele (G) with MetS was evident (OR = 0.79, 95% CI = 0.38-1.63, p-value = 0.52)

Table 5.15: Association of rs1801282 with MetS under Allelic genetic model

Gene	Case		Control			Odds Ratio ($OR_{G)}$	95% CI	Z statistics	Significance
PPARG	Allele	N	Allele	N	test				
	G (minor)	13	G (minor)	19	G vs C	0.79	0.38-1.63	0.65	0.52
	C (major)	163	C (major)	187					

The association of the analysis of rs1801282 with MetS under dominant and co-dominant model is shown in table 5.16. The recessive model was not possible as the homozygous recessive geotype (GG) was not found in any of the subjects studied. The heterogenous co-dominant model (CG vs CC) showed protective effects against MetS but the effect was not statistically significant (OR = 0.72, 95% CI = 0.33-1.55, p-value = 0.40).

Table 5.16: Association of rs1801282 with MetS under dominant, recessive and co-dominant model

Gene	Model	Case		Control			Odds Ratio	95% CI	Z-statistics	Significance
PPARG	Dominant model	Genotype	N	Genotype	N	test				
		CC	75	CC	84	CC vs CG+GG	1.30	0.60-2.82	0.68	0.50
		CG+GG	13	CG+GG	19					
	Co-dominant model (heterogenous)	CC	75	CC	83	CG vs CC	0.72	0.33-1.55	0.84	0.40
		CG	13	CG	20					
		GG	0	GG	0					

PPARG = Peroxisome proliferator-activated receptor gamma, CI = confidence interval

Table 5.17: Anthropometric and biochemical characteristics by genotype groups of rs1801282 under dominant genetic model.							
Coding of PPARG genotypes	CC (n = 159)			CG+GG (n = 32)			p-value
	Median	Minimum	Maximum	Median	Minimum	Maximum	
Height (cm)	163	144	186	163	140	176	0.67
Weight (Kg)	61.2	41.75	94.7	61.375	37	95.05	0.71
WC (cm)	87.5	66	118	86.75	67.5	110	0.37
HC (cm)	90	67	122	90	68.5	109	0.18
HR (beat per minute)	83	58	130	84	64	111	0.77
Systolic blood pressure (mm Hg)	119	105	149	118	93	140	0.50
Diastolic blood pressure (mm Hg)	80	64	105	79.5	62	100	0.90
fasting glucose (mmol/L)	5.5	3.7	10	5.5	4.4	8.2	0.40
TC(mmol/L)	4.47	2.59	12.93	4.57	2.64	6.88	0.53
HDL-C (mmol/L)	1.11	0.41	2.25	1.02	0.49	2.35	0.13
TG (mmol/L)	1.62	0.51	3.51	1.555	0.74	3.78	0.92
LDL-C (mmol/L)	2.64	0.96	9.9	2.86	1.58	4.6	0.65
BMI (kg/m2)	23.3	16.9	35.7	23.55	15.8	33.5	0.94
Waist-to-hip ratio	0.97	0.81	1.14	0.97	0.87	1.15	0.67

WC = waist circumference; HC = hip circumference; HR = heart rate; BMI = Body mass index; TC = Total cholesterol; HDL-C = High-density lipoprotein; TG = Triglycerides; LDL-C = Low-density lipoprotein; *statistically significant (p-value ≤ 0.05, Mann-Whitney U test).

Table 5.17 shows the difference in anthropometric and biochemical parameters under dominant genetic model. The data were not normally distributed and Mann-Whitney U test was used for the analysis. None of the variables were significantly different under the dominant genetic model of rs1801282.

5.4 Discussion

The allele frequency of the minor allele (T) was analyzed from the genotypes of the variant rs7903146 of TCF7L2 gene. The overall minor allele frequency (MAF) was found to be 0.21 and further it was noted lower in the control group (0.20) when compared with the cases (0.22).

The overall MAF of the rs7903146 in this study was lower (0.21) than MAF found in 100 genome project (0.30) (https://www.ncbi.nlm.nih.gov). The study carried out in South Asian population residing in the United Kingdom found MAF of the variant 0.36 in subjects with type 2 diabetes and 0.29 in subject without type 2 diabetes (Rees et al, 2008). Similarly, the other studies carried out in South Asian population, especially in the Indian population (Bodhini et al, 2007; Chandak et al, 2007; Gupta et al, 2010; Hussain et al, 2014) showed a higher prevalence of MAF in cases (subjects with type 2 diabetes) (0.33, 0.37, 0.47, 0.37) in comparison to control (with out type 2 diabetes) (0.18, 0.29, 0.37, 0.26) respectively and this is in contrary to the result that we found in this study.

The MAF found in our study was higher in comparison to the MAF found in East Asian population (2%) ((https://www.ncbi.nlm.nih.gov). The MAF of the variant ranged from 0.03-0.05 in the Japanese population, which was also markedly lower when compared to our study (Horikoshi et al, 2007).
The difference in the MAF of the allele in different populations might be due to the population specific selective pressure (Hancock et al, 2010) operating with different intensities. In our study, MAF was found to be in Hardy-Weinberg equilibrium (HWE) in both cases (subjects with MetS) and control (subjects without MetS) group which, in addition to the Hardy-Weinberg assumption, might suggest the accuracy of the genotyping methodology.

The minor allele of rs7903146 of TCF7L2 gene has been analzyed for an association with metabolic syndrome under allelic, dominant and recessive genetic model. No significant association was noted under any of the models studied. Under the allelic model, the OR of 1.14 (95% CI = 0.69-1.88) was obtained but did not reach statistical significance. Similarly, the OR under co-dominant model (CT vs CC, CT vs TT) was found (1.19 and 1.16) respectively but without reaching statistical significance. The study showed that the OR of the

heterozygote was relatively higher compared to other homozygotes under co-dominant model. This pattern was also noted when analyzed under an additive genetic model where the odds ratio for CT vs CC was found to be 1.26 when compared with TT vs CC (OR = 1.05).

The explanatory power of the regression of the genotypes against the metabolic variables was poor. The metabolic variables were not normally distributed, even after the log transformation of the data and therefore non-parametric Kruskal-Wallis test was used for the statistical difference of the variables of three genotypes. Except the heart rate none of the genotype variables were significantly different., However, this was not the case when the p-values were adjusted for multiple comparison with post-hoc test. The heart rate was also found to be significantly different under dominant genetic model (CC vs CT + TT) (p-value = 0.01) but not under recessive genetic model (TT vs CC + CT) (p-value = 0.06). Some studies have suggested the genetic components of heart rate and heart rate variability (Howden et al, 2007). Abnormal heart rate might indicate a cardiovascular problem (Korshunov et al, 2013) but this has not been reported in TCF7L2 gene. It is important to bear in mind that the measurement error, confounding factors and varability of other factors beyond the control of the researcher for example, anxiety or nervousness during the data measurement might also have influenced the results.

An association of the rs9939609 of FTO gene was analysed under allelic, dominant, recessive, co-dominant and additive genetic model and no statistical significance was noted in any of the models. Ethnic differences in individual genotypes in different populations might change the susceptibility for MetS which might have contributed along with other above mentioned factors in our study. Under the dominant model the OR was 1.31 but it was not statiscially significant, however, under the dominant model OR was 0.65 indicating the protective effect but it was not statistically significant.

The three genotype groups (TT, AT and AA) of rs9939609 with the metabolic variables were analysed using Krusal-Wallis test and it was found that weight, waist circumference and BMI were significantly different between these genotype groups. In the post hoc test, BMI was found significantly different in (AA vs TT) genotype groups (p-value = 0.05). The BMI was found significantly higher in the AA group in comparison to the TT genotype group. It is clear that the minor allele is responsible for higher BMI in the Nepalese population. The BMI cut off points for the South Asians as recommended by WHO are: overweight = 23–27.5 kg/m^2

and obesity = ≥ 27.5 kg/m^2 (WHO, E.C., 2004). The median BMI found in the AA genotype group was 25.3 kg/m^2 and according to WHO, 2004 guidelines, this genotype group might be considered as overweight group. The result of this test suggests the AA could be a risk genotype for overweight. The results obtained from this study match those observed in earlier studies as risk allele of rs9939609 has been consistently associated with obesity in various populations. The result seems to be consistent with those of other studies conducted in Asian populations (Song et al, 2008; Tabara et al, 2009; Chang et al, 2008; Hotta et al, 2008; Karasawa et al, 2010). A separate meta-analysis conducted in Indian population involving 28, 394 subjects found a significant association of the minor allele with obesity indices (BMI, waist circumference and waist-hip-ratio) (Vasan et al, 2014).

The rs9939609 SNP of FTO gene and its association with obesity was noted in Genome wide association study (Frayling et al, 2007). An association of the minor allele of rs9939609 with BMI in a study involving 177,330 individuals was a clear indication that the SNP could be a potential marker for obesity. Despite the fact that this SNP has been significantly associated with obesity, there are also studies where no association with obesity was observed (Li et al, 2008 and Fawwad et al, 2016).

In our study under multinomial logistic regression, both BMI and weight were significantly associated with the risk genotype (AA). A unit increase in BMI was significantly associated with increased odds for being AA genotype by 20%. Similarly, a unit increase in weight was significantly associated with increased odds for being AA genotype by 7%. In our study the minor allele was also associated with systolic blood pressure, but the statistical significance was not achieved after adjusting by BMI and age, which seem to be consistent with the result obtained by Prakash et al, 2016. When the metabolic variables were analyzed under dominant genetic model (TT vs AT+AA), weight, waist circumference, BMI and diastolic blood pressure were found to be significantly higher in genotypes with the risk allele (AT and AA) which was again observed in Indian population (Prakash et al, 2016). Surprisingly, a significantly higher level of diastolic blood pressure observed in genotypes with the risk allele in our study under a dominant model was also noted in a study conducted at French Canadians (Pausova et al, 2009) and Brazilian population Marcadenti et al, 2013). Under the recessive model (AA vs AT + TT) of study only BMI was found to be higher in the AA genotype

group in comparison with TT and AT genotype group. The result obtained from the recessive model should be interpreted with caution as the number of AA group is very low.

It is yet to understand the link between the risk variant of FTO gene and the risk of obesity. Some studies have suggested a common link of the FTO gene by which it could regulate both weight and blood pressure. A possible explanation could be that FTO might be responsible for regulating both obesity and blood pressure mediated through hypothalamus.

The allele frequency of PPARG was found to be 0.09 in the overall population and 0.07 in cases (subjects with MetS) and 0.10 in the control (without MetS) group. The genotype frequency of the overall population as well as case and control group were under Hardy-Weinberg equilibrium.

Under the allelic model, no significant association of the minor allele with metabolic syndrome was found. The odds ratio found was 0.79 which might indicate the protective effect of the allele against metabolic syndrome, but the results are not statistically significant. No association of the minor allele was observed under dominant or co-dominant model. The recessive model was not possible because because the GG genotype was not found in our study. No significant association of any of the variables was with the minor allele was observed in any of the statistical models in our study. It might be due to the small sample size and the population stratification cannot be ruled out.

The lack of association of the variant with metabolic syndrome might be due to the size of the sample studied. Even though the genotypes were under Hardy-Weinberg equilibrium and the allele frequency were also comparable within subpopulations, the possibility of population stratification cannot be ignored as most of the ethnicity were self reported. Another possible explanation would be that the variants are population specific and this might be absent in the Nepalese population.

In conclusion, to the best of our knowledge, the nature of this study has been conducted for the first time in the Nepalese population. None of the SNPs studied were significantly associated with metabolic syndrome under allelic, dominant, recessive or additive genetic model. However, the minor allele of some of the SNPs was significantly associated with some of the components of metabolic syndrome. Further study with increased sample size and homogenous population is warranted.

Chapter 6.

6.1 Overall discussion

The present study analyzed an association of single nucleotide polymorphisms (SNPs) and their relation to risk of type 2 diabetes (T2D), metabolic syndrome (MetS) and the components of metabolic syndrome. In chapter 2, the SNP rs57829442 of PPARGC1A gene has been analyzed its association with type 2 diabetes in the United Kingdom population. No statistically significant association (OR = 1.19 with 95% CI = 0.62-2.29) of the SNP with type 2 diabetes was observed in the United Kingdom population in this study. Even though the genome-wide association studies (GWAS) have been carried out in the United Kingdom population (Wellcome Trust Case Control Consortium, 2007) and the SNPs of this gene was not found to be associated with T2D. One of the drawbacks of the GWAS is that the individual SNPs might not be able to withstand the stringent multiple correction (Genome-wide significance level p-value ≤ $5X\ 10^{-8}$) (Lehne et al, 2011). In the GWAS the SNPs have to pass through rigorous quality control check and there is also a possibility that causal SNPs might be missed out.

The limitation of this study is that the case and control groups were not perfectly age matched. The control groups were relatively younger than the case group and the age of the most of control group was missing. In addition to that, the other metabolic variables of both case and control groups were not supplied. T2D susceptibility is also related to other metabolic phenotypes (McCarthy et al, 2009), for example, BMI, adiposity, lipid levels which were missing in this study.

In chapter 3, the common variant rs8192678 (Gly482Ser) of PPARGC1A gene was selected to analyse its association with various components of metabolic syndrome in a form of meta-analysis. The weighted mean difference of the components of metabolic syndrome between the genotype group under dominant and recessive model was analysed. Under the dominant genetic model (GG versus GA+AA), BMI in GG genotype was significantly higher in comparison to GA+AA genotype in Asian subgroups. The Asian group is ethnically homogeneous as most of the studies were conducted in South East Asian population. This was also the basis why the variant of this gene has to be looked at in Asian populations and this prompted us to look at the association of the SNP (rs8192678) (Gly482Ser) in the

Nepalese population. Similarly, The fasting plasma glucose was found to be significantly higher in the GA+AA group in overall and non-Asian population.. In addition to that, the heterogeneity was not significantly different in both and overall population. This indicates that AA genotype might offer the protective effect against glucose levels, however, the effect could be ethnic specific. In a meta-analysis conducted by Yang et al, 2011 found an ethnic specific association of the variant where the Indian sub-group were significantly associated with Type 2 diabetes which was not the case in Caucacians and South East Asians. Higher glucose levels than the normal counterparts is one of the salient features of T2D and this might partly explain why this variant was found to be associated with T2D in some while not in other populations. It is not the case that the genetic variants do act in isolation, but rather a complex interaction exists between the gene and environmental factors. It would have been more interesting to analyze interaction between various components of metabolic syndrome, which was not conducted in our study. It is not always the interaction between the gene and the environment, but also between the variants which could be done in future studies if robust statistical methods are in place.

The other interesting result found was that, the total cholesterol levels were significantly lower in "AA" genotype groups in non-Asian groups and not in the overall population (p-value = 0.07) however, the trend was similar. A study conducted by Verschuren et al, 1995 suggests that there is a notable relation between total cholesterol and coronary heart disease. This might indicate that there could be a possible link between lipid metabolism and coronary heart disease which might be mediated through this gene. But at the variant level, there might be variation due to the difference in ethnicity. For example, in Chinese population GA+AA genotype was significantly associated with Coronary artery disease (Zhang et al, 2008), however, it was not the case in European populations (Iglseder et al, 2006) which supports what was found in this meta-analysis.

The further study was conducted on the Nepalese population, especially the association of rs8192678 (Gly482Ser) with the metabolic syndrome and its components. The minor allele was not found to be associated with metabolic syndrome under various genetic models. However, the minor allele was found to have a protective effect against systolic blood pressure and had been supported by various studies which has already been explained in the discussion section of Chapter 4. The population specific cardioprotective effect of the minor

allele is clearly evident in the study, which is mediated through total cholesterol and systolic blood pressure. It was interesting to note that the protective effect against blood pressure was further modified by gender, where the minor allele was found more protective in females in comparison to males.

The SNP rs7903146 of TCF7L2 gene was not found to be associated with MetS and its components except heart rate in the Nepalese population. Similarly, the minor allele of rs1801282 (Pro12Ala) was not associated with MetS or its components.

In the Nepalese population, which was conducted for the first time, the study suggests that the minor allele of rs9939609 of FTO gene has been found to be significantly associated with obesity indices (weight, waist circumference, hip circumference and BMI) which is consistent with other studies conducted in various populations.

The strength of the study lies in the fact that meta-analysis has offered more power to the study because of the large sample size. The study conducted in the Nepalese population presents a novel and unique information regarding that population. The weakness of the study is that the sample size selected for the Nepalese population was relatively small. The meta-analysis of only one SNP (rs8192678) (Gly482Ser) has been conducted. In the Nepalese study, the ethnicity was self reported and mixed ethnicity might contribute to population stratification, however, these issues have been addressed by using various statistical models.

In conclusion, The SNP rs57829442 has not been associated with T2D in the United Kingdom population. A meta-analysis suggest the population specific association of the variant with different components of metabolic syndrome. In the Nepalese population, rs8192678 of PPARGC1A gene has a protective effect against systolic blood pressure and rs9939609 of FTO has been found to be associated with obesity indices.

Follow up studies are warranted in both United Kingdom and the Nepalese population. Genome wide association studies with larger sample size is recommended for better understanding the role of the SNPs in the Nepalese population. In vitro studies in Human cell lines by creating the mutation of interest, single or multiple, could be conducted in the future for better understanding the specific role of the SNPs in various metabolic pathways.

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Appendix A

Questionnaire :

"Prevalence of Gly482Ser variant of peroxisome proliferator-activated receptor gamma coactivator-1alpha (PPARGC1A) gene and its association with metabolic syndrome in the Nepalese population."

Layman title: "Understanding the importance of DNA sequence in the Nepalese population and to investigate how these changes may affect metabolism".

Subject ID:			

Date: D/M/Y			

Category (Please tick) To be completed at the end	
Case	**Control**

	In A.D. D/M/Y			In B.S. D/M/Y		
1.Date of birth:						

2. Sex:	Male	Female
Please tick		

7. Presence and history of disease:	Please tick below		8. Medication intake:	Please tick below	
Have you been diagnosed with any medical condition? If yes, please specify below	Yes	No	Do you take any medication? If yes, please specify below	Yes	No
Have you been diagnosed with type 2 diabetes?	Yes	No	Do you take statins or lipid lowering drugs?	Yes	No
Have you been diagnosed with high blood pressure?	Yes	No	Do you take drug for high blood pressure?	Yes	No
Do any of your parents have type 2 diabetes?	Yes	No	Do you take oral glucose lowering medicine?	Yes	No
Do any of your parents have high blood pressure?	Yes	No			

5.Occupation:	Please tick below	6.Smoking habit:	Please tick below
Farmer		Daily smoker	
Businessperson		Occasional smoker	
Student		Ex-smoker, How long you have stopped smoking? Please specify below.	
Labourer			
Employed			
Unemployed			
House-wife			
If other, please specify below			

9. Anthropometric measurement:	1st measurement	2nd measurement	Reference
Height (cm)			
Weight (Kg)			
Body fat (%)			
Waist circumference (cm)			≥90 cm (men) or ≥80 cm (women)
Hip circumference (cm)			
Heart rate (beats per minute)			
Blood pressure (mmHg)			
Systolic blood pressure (mmHg)			≥130mmHg
Diastolic blood pressure			≥85 mmHg

3. Ethnicity:	Please tick below	4. Marital status:	Please tick below
Bramhan		Single	
Chetri		Married	
Tharu		Divorced	
Madhesi		Widow/Widower	
Newar		Separate	
Gurung/Magar/Tamang		If other, please specific below	
Dalit			
If other, Please specify below			

10. Fasting glucose and lipid levels measurements	1st measurement	2nd measurement	Reference
Fasting glucose (mmol/L)			≥5.6 mmol/L
Fasting HDL-C (mmol/L)			< 1.0 mmol/L in men and < 1.30 mmol/L in women
Fasting LDL-C (mmol/L)			
Fasting triglyceride (TG) (mmol/L)			TG ≥1.70 mmol/L (Both men and women)
Fasting total cholesterol (TC) (mmol/L)			

APPENDIX B

ROBERT GORDON UNIVERSITY ABERDEEN

Participant Information Sheet

Understanding the importance of DNA sequence in the Nepalese population and to investigate how these changes may affect metabolism.

We would like to invite you to take part in a research study. If you think you are eligible to take part in this study, it is important that you take time (at least a day) to read through and understand the information provided. You can discuss this with your relatives, friends or even your General practitioner, if you would like to do so. If you then decide to take part in this study we would like to invite you to attend and meet with some members of the team at the following time and place:

Venue: Shree J K Higher Secondary School, Attaria, Kailali, Farwestern Nepal.

Time: 8am

Date: to be confirmed.

The purpose and the detail of the study will be explained and you will be given the opportunity to ask questions. Only when you are completely satisfied will you then be asked to sign a consent form indicating your willingness to take part in this study. A copy of the signed consent form will be given to you as well. If you would like to withdraw from the study at any time it is possible to do so without even giving any reason.

What is the purpose of the study?

The purpose of this study is to find out if there is any association between changes in the sequence of DNA within a specific gene and metabolism of the Nepalese population. These changes in metabolism may identify the presence of a condition called metabolic syndrome which may include high blood pressure, impaired glucose tolerance, abdominal obesity and abnormal lipid levels. Your participation will be an important part of this case-control study and may help in early detection, intervention and management of the condition.

Who can take part?

We are looking for men and women aged 18-40, with and without metabolic syndrome. You don't have to worry about which group you are in as our trained facilitators will do that. If you have a chronic or acute illness, or if you are taking lipid lowering drugs, such as statins or you are pregnant, you will not be able to take part in the study.

Do I have to take part?

No, your participation in this project is entirely voluntary. If you decide not to take part in this study you do not have to give any reason. You are free to withdraw from the study at any time, even if you agree to take part in the study. If you wish to withdraw from the study the

information gathered from you and the data along with blood samples taken from you will be destroyed immediately.

What will you have to do if you agree to take part?

If you agree to take part in the study, you will be asked to fast overnight, about 10 hours, and attend a clinic that will be set up for this purpose. At the clinic, the study will be explained to you, you will sign a consent form and then you will be asked about your age, gender, occupation and ethnicity. We will measure your height, weight, hip circumference and waist circumference, blood pressure and heart rate during which we might ask you to take off your shoes or slippers and heavy clothes. The tip of your finger will be pricked to obtain a few drops of blood and some blood (equivalent to a teaspoon) will be drawn from your vein. It might take around 30—50 minutes for the completion of this process. You might expect some minimal pain and bruising after pricking your fingertip and drawing blood from your vein. If we attain the required sample size for control group and after the examination if you are not found to be in MetS group then the data collected from you will be destroyed immediately.

Will your participation in the project remain confidential?

If you agree to take part in the study, the information gathered from you and subsequent data gathered from blood samples will be kept confidential and will be used for this project and for projects of similar nature in the future. We can assure you that you will remain anonymous in any publication about any of the findings from this study.

What are the advantages of taking part?

The research will determine the genetic risk factor associated with metabolic syndrome in Nepalese population for this particular change in DNA sequence. This study will enhance our understanding of the genetic basis of the syndrome, which might help in early detection, intervention and management of the condition.

At a personal level, you would be able to know your height, weight and blood pressure. You would also know your blood sugar levels and lipid levels and any abnormality will be communicated to you and will be advised to seek the help of the Medical staff.

Are there any disadvantages of taking part?

There is no serious risk involved in taking part in this research project. You might feel some discomfort while pricking your finger. Even though taking blood is a safe procedure, it may sometimes cause bruising, pain and discomfort. Fully trained medical staff will be involved for blood collection purposes which will help to minimize bruising.

Payments?

No payment will be made for your participation in this project.

What if there is a problem?

If you experience any problems with the study, you can contact Dr. Rachel M Knott on +44 (0) 1224 262524 or Robert Gordon University Ethics Committee, Robert Gordon University, Garthdee Road, Aberdeen, AB10 7QG. If you have any concern in relation to your health as a

consequence of taking part in the study, you can contact Dr. Shiva Raj Paneru on 00977-9842052220. You may also find further contact details at the end of this information sheet.

What will happen to the samples in the future?

The blood sample collected from you might be used to determine if there are any differences in the sequence of DNA within a specific gene. We also hope to use the sample for similar research in the future. If you wish not to use your sample for similar future research study we will record this at the time of sampling and it will be destroyed immediately after use for this study. The data collected from you will be retained for at least 3 years and after that it will be securely disposed.

How do I find out what was learned from this study?

The data from the study will be analysed and disseminated in journal publications, thesis and conferences; however, your identity will remain anonymous.

Further information and contact details.

If you would like to know more information or discuss the project with the research team or in case you may have any questions in the future, please don't hesitate to contact us:

Mr. Prabhakar Bhatta
Email: p.bhatta@rgu.ac.uk
Telephone: 0044(0)7599257444

Dr. Rachel M Knott
Email: r.knott@rgu.ac.uk
Telephone: 0044 (0)1224 262524

Thank you for reading this information sheet and for considering taking part in the study.

APPENDIX C

ROBERT GORDON UNIVERSITY ABERDEEN

सहभागी जानकारी पत्र

नेपाली जनसंख्यामा डी.एन.ए सिकवेन्स र यसको परिवर्तन सँगै मेटाबोलीजममा पर्ने असर

आदरणीय सहभागी

नमस्कार,

हामी तपाईलाई यस अनुसन्धानमा सहभागीताको लागि आवहान गर्दछौं । यो अध्ययनमा भाग लीनु अगाडी तपाईले यसको बारेमा जानकारी लीनु जरुरी छ । तपाई यस बारेमा आफ्ना नातेदार, साथीभाई , अथवा आफ्नो डाक्टर सँग परामर्श लिन सक्नु हुनेछ । यो अध्ययन सहभागीताको पूर्ण जानकारी पश्चात तपाई यसमा सहभागी हुन चाहानु हुन्छ भने तल उल्लेखीत समय र स्थानमा उपस्थीतीको लागि हार्दीक अनुरोध गर्दछु ।

स्थान: श्री. जे.के हाइयर सेकेन्डरी स्कूल, अत्तरीया , कैलाली, सुदुरपश्चिम नेपाल

समय: विहान ८ बजे

मिति: तय हुन बाकीं

यो अध्ययनको बारेमा विस्तृत रुपमा जानकारी तपाईलाई गरिने छ र प्रश्न राख्ने अवसर दिइनेछ । यदि तपाई यस अध्ययनमा सहभागी हुन इच्छुक हुनु भएमा सहमति पत्रमा सहीछाप गर्नु पर्ने छ र यसको एउटा प्रतिलिपी तपाईलाई दिइनेछ । यो अध्ययनमा भाग लिने वा नलिने सम्पूर्ण अधिकार तपाईमा निहीत छ र यदि भाग लिनु भएको खण्डमा जुनसुकै बेलामा पनि बिना इस्पष्टिकरण अध्ययन छाडेर जान सक्नु हुनेछ ।

यो अध्ययनको उद्देश्य के हो ?

यो अध्ययनको उद्देश्य नेपाली जनसंख्यामा डी. एन. ए सिक्वेन्स परिवर्तन सँगै मेटाबोलीजममा पर्ने असर हो । यो मेटाबोलीजममा परिवर्तन हुने अवस्थालाई मेटाबोलीक सिन्ड्रोम भनिन्छ । जस अन्तर्गत उच्च रक्तचाप अधिकतम ग्लुकोज, पेटको मोटोपना र लिपीडको मात्रामा परिवर्तन पर्दछन । यस केस कन्ट्रोल अध्ययनमा तपाईको सहभागीताले यस रोगको पुर्व पहिचान , नियन्त्रण तथा व्यवस्थापन गर्न सहयोग प्रदान गर्नेछ ।

यसमा कस कसले भाग लिन सक्छन ?

यसमा १८ देखि ४० वर्ष सम्मका पुरुष तथा महिलाले सहभागी हुन सक्नु हुने छ । यदि तपाई कुनै ऐक्युट तथा पुरानो रोग बाट ग्रसित हुनु हुन्छ र लीपीड कम गर्ने औषधी जस्तै इस्सटयाटीन प्रयोग गर्नु हुन्छ भने तपाईले सहभागी हुन पर्दैन ।

यदि केहि समस्या भएमा के गर्ने ?

यदि तपाई लाई अध्ययन सम्बन्धी समस्या भएमा डा. रिचेल एम. नट लाई फोन नं:+४४ (०) १२२४२६२५२४ मा अथवा रोबर्ट गोर्डन युनिभरसिटी इथिक्स कमिटी, रोबर्टगोर्डन युनिभर सिटी गार्थरोड, ऐवरडीन मा सम्पर्क गर्न सक्नु हुनेछ । यसै क्रममा यस अध्ययनमा भाग लिएर तपाईको स्वास्थयमा कुनै असर परेमा , तपाईले डा.सिव राज पनेरु लाई फाने नं. ००५७७- ५८४२०५२२२० मा सम्पर्क गर्न सक्नु हुनेछ । विस्तृत जानकारीको लागि अन्तीम पृष्ठ हेर्नु होला ।

यस अध्ययनको क्रममा लिइएका नमुना (स्याम्पल) लाई भविष्यमा के गरिने छ ?

यस अध्ययनको क्रममा संकलित नमुनाहरु डि.एन.ए मा भएका परिवर्तनहरुको अध्ययनको लागि प्रयोग गरिने छ । यी नमुनाहरु भविष्यमा यस्तै खाले अनुसन्धानको लागि पनि प्रयोग गरिने छ । यदि तपाई यी नमुनाहरु भविष्यमा हुने अनुसन्धानको प्रयोग गर्न चाहानु हुदैन भने यस अध्ययन पस्चात त्यसलाई तत्काल नष्ट गरिनेछ ।

यस अध्ययन बाट के थाहा पाइयो भनेर कसरी थाहा पाउनु ?

यस अध्ययन बाट लिइएका आकडाहरुलाई विस्लेषण गरि वैज्ञानिक समाचार पत्र, शोधग्रन्थ, तथा वैज्ञानिक सम्मेलनमा प्रकाशित गरिने छ ।

सम्पर्क विवरण ?

यदि तपाई यस अध्ययनको बारेमा बृस्तीत जानकारी लिन चाहानु हुन्छ र यदि भविष्यमा कुनै प्रश्न सोध्न चाहानु हुन्छ भने, तल उल्लेखीत नाम र ठेगानामा सम्पर्क गर्नु होला

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इमेल: r.knott@rgu.ac.uk
टेलिफोन: ००४४(०) १२२४२६२५२५

"यो जानकारी पत्र पढनको लागि तथा यसमा सहभागीहुन इच्छुक हुनु भएकोमा धन्यवाद"

के मैले भाग लिन जरुरी छ ?

छैन, यो अध्ययनमा सहभागीता तपाईको नितान्त निर्णय हो । यदि तपाईले भाग लिन चाहानु भएन भने कुनै पनि कारण खुलाउनु पर्ने छैन र जुनसुकै बेलामा पनि अध्ययन छाडेर जान सक्नु हुनेछ ।

म अध्ययनमा सहभागी हुन मन्जुर भएमा के गर्नु पर्ने हुन्छ ?

यदि तपाई यस अध्ययनमा सहभागी हुन मन्जुर हुनुभएमा रातभरमा कम्तीमा १० घण्टा सम्म खाली पेट राख्नु पर्ने हुन्छ । अनि विहान पख माथि उल्लेखीत ठाँउमा आउनु पर्ने हुन्छ । अध्ययनको लागि खडा गरिएको क्लीनीकमा हामीले तपाईको सहमति पत्रमा सहिछाप गराउने छौ । यस क्रममा तपाईले आफ्नो उमेर, लिङ्ग , पेशा, र जात खुलाउनु पर्नेछ । हामीले तपाईको उचाई, तौल, पेटकोनाप, रगतचाप, मुटुकोगति नाप्ने छौ र यस क्रममा तपाइले लगाएका भारीलुगा, तथा, चप्पल फुकाल्नु पर्नेछ , यस अध्ययनको शिलशीलामा तपाइको औलाको टुप्पो बाट दुई थापा रगत निकालिन्छ र लीपीड तथा ग्लुकोजको जाच गरिने छ । अन्तमा तपाइको भेनबाट कम्तीमा एक चम्चा रगत निकालिने छ । रगत निकाल्ने क्रममा तपाई लाई अलिकति दुखाई र असजिलाको अनुभव हुन सक्छ । सबै प्रक्रिया पुरागर्न ३० देखि ५० मिनेट लाग्न सक्छ ।

के तपाईको सहभागीता गोप्य रहने छ ?

यस अध्ययनको क्रममा तपाइबाट संकलीत सम्पूर्ण जानकारीहरु गोप्य रहनेछन् । यो जानकारी यस अध्ययन र भविष्यमा हुने यस्तै खाले अध्ययनको लागि मात्र प्रयोग गरिने छ । हामी तपाई लाई यो विश्वस्त गराउन चाहान्छौ की यस अध्ययन बाट प्रकाशित प्रकाशनमा तपाई गुमनाम रहनु हुनेछ ।

यस अध्ययनमा सहभागी हुने फाइदाहरु ?

यस अध्ययनले नेपाली जनसंख्यामा डी.एन.ए मा भएको परिवर्तन र मेटाबोलीक सिनड्रोम हुनमा वंशानुगत खतराको सम्बन्धको बारेमा प्रकाश पार्ने छ । यसले गर्दा हाम्रो वंशानुगतरुपमा अधारित मेटाबोलीक सिनड्रोम सम्बन्धीजानकारी लाई पराकीलो पार्ने छ जसले गर्दा यस रोगको पुर्व पहिचान, नियन्त्रण तथा व्यवस्थापन मा सहयोग प्रदान गर्ने छ ।

यस अध्ययनमा सहभागी भएमा हुनेवेफाइदा के के हुन ?

यस अध्ययन् सहभागी भएमा तपाईलाई कुनै पनि किसिमको गम्भीर खतराहरु हुने छैनन् । तपाईको औलाको टुप्पो बाट दुई थोपा रगत निकालिन्छ । केहि दुखाई असजिलो हुन सक्ला, यसैगरि तपाईको नशाबाट रगत निकाल्दा केहि दुखाई र सामान्य घाउ हुनसक्ला जुन हामी सँग भएका अनुभवी चिकीत्शकले गर्दा न्युन हुने छ ।

व्यक्तीगत रुपमा यस अध्ययन बाट तपाइले आफ्नो उचाई, तौल , रगतचाप थाहा पाउनु हुने छ र साथ साथै तपाईको रगतमा ग्लुकोज र लिपिडको मात्राको बारेमा जानकारी पाउनु हुनेछ । यस क्रममा केही असामान्यता देखीएमा तपाईलाई जानकारी दिई , डाक्टरको परामर्शको लागि तपाइलाई अनुरोध गरिनेछ ।

भुक्तानी ?

यस अध्ययनमा सहभागी भए बापत तपाईले कुनैपनि किसिमको भुक्तानी पाउनु हुने छैन ।

ROBERT GORDON UNIVERSITY ABERDEEN

सहमति फारम

अध्ययन शीर्षक : नेपाली जनसंख्यामा पेरआक्सीसोम प्रोलिफिलेटर-ऐक्टीभेटेड रिसेप्टर गामाकोएक्टीभेटर-१ अल्फा (PPARGC1A) जीनको Gly482ser भेरिएन्टको व्यापकता र यसको मेटाबोलीक सिन्ड्रोम सँग सम्बन्ध ।

आई डी. नम्बर :

१. यस अध्ययनको बारेमा मिति .....(सस्करण .....) मा मैले विस्तृतरुपमा पढे वा सुने यसबारेमा मलाई बुझ्ने भाषामा विस्तृत जानकारी दिइयो र प्रश्न राख्ने मौका पनि दिइयो । छोटकरी नाम

२. म आफ्नै इच्छाले यसमा सहभागी भएको छु र यो अध्ययनको दौरान कुनैपनि समयमा अध्ययन छाडेर जानसक्छु भन्ने बारेमा म जानकार छु ।

३. म यो अध्ययनमा भाग लिन सहमत छु ।

४. भविष्यमा हुने अध्ययनका लागि पनि जैवीक नमुना तथा अन्य जानकारीको प्रयोग गर्न म सहमत छु ।

सहभागीको नाम	हस्ताक्षर	मिति

अथवा

सहभागीको औठा छाप	
बायाँ	दायाँ
मिति:	

साछीको नाम	हस्ताक्षर	मिति

सहमति लिने व्यक्तीको नाम	हस्ताक्षर	मिति

सस्करण 3 (24/02/2017)

APPENDIX D

ROBERT GORDON UNIVERSITY ABERDEEN

SCHOOL OF PHARMACY & LIFE SCIENCES
Robert Gordon University
Sir Ian Wood Building
Garthdee Road
Aberdeen
AB10 7GJ
United Kingdom
Tel: 01224 262500/2800
www.rgu.ac.uk

Ref: S70

Dear Prabhakar

Re.: Prevalence of Gly482Ser variant of peroxisome proliferator-activated receptor gamma coactivator-1alpha (PPARGC1A) gene and its association with metabolic syndrome in the Nepalese population.

The School Research Ethics Committee has assessed your application and the overall decision is that there are no ethical issues with your project. However, they have provided some comments that you may find useful going forward.

I can now confirm that you are able to proceed with your research and any further ethics applications.

Should there be any amendments to this project during the research we would advise you to consult with the convener of the ethics committee as to whether a further ethical review would be required.

Please use the reference number above in any future correspondence.

We wish you success with your project.

Regards

Convener of the School Ethics Review Panel

Government of Nepal

Nepal Health Research Council (NHRC)

Estd. 1991

Ref. No.: 2269

13 June 2017

Dr. Rachel M. Knott
Principal Investigator
Robert Gordon University, UK

Subject: Approval of research proposal entitled **Prevalence of Gly482Ser variant of peroxisome proliferator-activated receptor gamma coactivator-1alpha (PPARGC1A) gene and its association with metabolic syndrome in the Nepalese population**

Dear Dr. Knott,

It is my pleasure to inform you that the above-mentioned proposal submitted on **20 March 2017** (**Reg.no. 75/2017** please use this Reg. No. during further correspondence) has been approved by NHRC Ethical Review Board on **17 May 2017**.

As per NHRC rules and regulations, the investigator has to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure, data management and budget that may be necessary in course of the implementation of the research proposal can only be made so and implemented after prior approval from this council. Thus, it is compulsory to submit the detail of such changes intended or desired with justification prior to actual change in the protocol before the expiration date of this approval. Expiration date of this study is **October 2017.**

If the researcher requires transfer of the bio samples to other countries, the investigator should apply to the NHRC for the permission. The researchers will not be allowed to ship any raw/crude human biomaterial outside the country; only extracted and amplified samples can be taken to labs outside of Nepal for further study, as per the protocol submitted and approved by the NHRC. The remaining samples of the lab should be destroyed as per standard operating procedure, the process documented, and the NHRC informed.
Further, the researchers are directed to strictly abide by the National Ethical Guidelines published by NHRC during the implementation of their research proposal and submit progress report and full or summary report upon completion.

As per your research proposal, the research amount is **USD 3346.31** and accordingly the processing fee amount to **USD 100.00**. It is acknowledged that the above-mentioned processing fee has been received at NHRC. If you have any queries, please feel free to contact the Ethical Review M & E section of NHRC.

Thanking you,

Prof. Dr. Anjani Kumar Jha
Executive Chairman

Tel: +977 1 4254220, Fax: +977 1 4262469, Ramshah Path, PO Box: 7626, Kathmandu, Nepal
Website: http://www.nhrc.org.np, E-mail: nhrc@nhrc.org.np

Government of Nepal

Nepal Health Research Council (NHRC)

Estd. 1991

Ref. No.: 339.

17 August 2017

Ms. Rachel M Knot
Principal Investigator, Robert Gordon University, UK
Mr. Prabhakar Bhatt
Principal Investigator, Robert Gordon University, UK

Subject: **Approval of requested amendment** for research proposal entitled **Prevalence of Gly482Ser variant of peroxisome proliferator-activated receptor gamma coactivator-1alpha (PPARGC1A) gene and its association with metabolic syndrome in the Nepalese population**

Dear Ms. Knot

The meeting of Ethical Review Board of Nepal Health Research Council held on 16 August 2017 discussed about the amendment requested on 01 August 2017. The meeting has approved the following requested amendments:

- *To increase sample size to 200* instead of previous 144.
- To send extracted DNA sample to Xcelris Labs Limited, India for further analysis.

If you have any queries, please feel free to contact the Ethical Review M & E section of NHRC.

Thanking you.

Prof. Dr. Anjani Kumar Jha
Executive Chairman

Appendix E

REDTaq (Sigma-aldrich)

REDTaq ReadyMix PCR Reaction Mix, with MgCl2

Catalog Number R2648

20 mM Tris-HCl, pH 8.3, with 100 mM KCl,

3 mM MgCl2, 0.002 % gelatin, 0.4 mM dNTP mix

(dATP, dCTP, dGTP, TTP), stabilizers, and

0.06 unit/mL of *Taq* DNA Polymerase

Restriction endonuclease Tsp451 (New England Biolabs)

Concentration: 5000 units/ml

Incubation time: 1 hours

Incubation temperature: 65 degree centigrade

Cut smart buffer (New England Biolabs)

1X Buffer Components

50mM Potassium Acetate
20mM Tris-acetate
10mM Magnesium Acetate
100µg/ml BSA
pH 7.9@25°

Taq DNA (Thermo Scientific)

Concentration: 5U/µl

Magnesium Chloride (Thermo Scientific)

Concentration: 25mM

dNTP Mix (Thermo Scientific)

Concentration: 10mM each