SINGLE NUCLEOTIDE POLYMORPHISM OF *PPARγ*, *ENPP1* AND *CAPN-10* GENES IN TYPE 2 DIABETES MELLITUS PATIENTS WITH AND WITHOUT CORONARY ARTERY DISEASE IN A MALAYSIAN TERTIARY HOSPITAL.

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DESSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF MEDICAL SCIENCE

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ORIGINAL LITERARY WORK DECLARATION

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Title of Dissertation: Single Nucleotide Polymorphism of *PPARγ*, *ENPP1* and *CAPN-10* Genes in Type 2 Diabetes Mellitus Patients with and without Coronary Artery Disease in a Malaysian Tertiary Hospital.

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ABSTRACT

Type 2 Diabetes mellitus (T2DM) and coronary artery disease (CAD) are two major lifestyle disorders in the world. T2DM and CAD share several common risk factors, mainly genetic and environmental. The Single Nucleotide Polymorphisms (SNPs) of the *PPARγ* (Pro12Ala), *ENPP1 (*K121Q) *and CAPN-10* (SNP-63), respectively have been identified as the key regulators of glucose and lipid metabolism. These polymorphisms control the protein synthesis in multiple metabolic, biochemical and molecular pathways. The aim of this study was to investigate the *PPAR-γ*, *ENPP1* and *CAPN-10* genes polymorphisms as genetic risk factors for T2DM and CAD in the Malaysian population. A total of 360 subjects between the age of 35 and 85 years were recruited in this study. Out of the 360 subjects, 120 were T2DM patients, recruited from the outpatient clinic at UMMC; 120 were T2DM patients with CAD (T2DM+CAD), recruited from cardiac clinic at UMMC and the other 120 were healthy hospital staff. DNA from the blood samples was extracted using QIAamp DNA Mini Kits. SNP of *PPARγ*, *ENPP1* and *CAPN-10* were determined by polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP). The analysis showed that the Pro allele of *PPARγ* (Pro12Ala) is risk factor for T2DM (OR: 2.11, 95% CI: 1.10-4.04, p=0.020) and T2DM+CAD (OR: 3.56, 95% CI: 1.74-7.29, p=0.001) among the Chinese but not among the Malays and Indians. The K allele of *ENPP1* (K121Q) and C allele of *CAPN-10* (SNP-63) do not increase the risk of T2DM or T2DM+CAD in all three ethnic groups. We conclude that, SNP of *PPARγ* (Pro12Ala) gene could be a genetic risk factor for T2DM and CAD in the Chinese population but not in Malay and Indian population.

ABSTRAK

Diabetes mellitus jenis 2 dan penyakit arteri koronari adalah dua penyakit gaya hidup yang utama di dunia. Diabetes mellitus jenis 2 dan penyakit arteri koronari berkongsi beberapa faktor berisiko, terutamanya genetik dan persekitaran. Single Nucleotide Polymorphisms (SNPs) bagi *PPARγ* (Pro12Ala), *ENPP1 (*K121Q) *dan CAPN-10* (SNP-63), masing-masing telah dikenalpasti sebagai kunci pengawalaturan dalam metabolisma glukosa dan lipid. Polimorfisme- polimorfisme ini juga mengawal sintesis protein di beberapa laluan metabolik, biokimia dan molekul. Tujuan kajian ini adalah untuk menyiasat polimorfisme bagi gen *PPARγ*, *ENPP1* dan *CAPN-10* sebagai faktor genetik bagi diabetes mellitus jenis 2 dan penyakit arteri koronari di kalangan penduduk Malaysia. Sejumlah 360 subjek antara usia 35 hingga 85 tahun telah menyertai kajian ini. Daripada 360 subjek, 120 adalah pesakit diabetes mellitus jenis 2, dipilih dari klinik pesakit luar di UMMC; 120 adalah pesakit diabetes mellitus jenis 2 dengan penyakit arteri koronari, dipilih dari klinik jantung di UMMC dan 120 subjek yang lain adalah kakitangan hospital yang sihat. DNA dari sampel darah diekstrak menggunakan QIAamp DNA Mini Kits. Polimorfisme *PPARγ*, *ENPP1* dan *CAPN-10* telah ditentukan oleh polymerase chain reaction (PCR) dan restriction fragment length polymorphisms (RFLP). Analisis menunjukkan bahawa alel Pro daripada *PPAR-γ* (Pro12Ala) adalah faktor risiko untuk diabetes mellitus jenis 2 (OR: 2.11, 95% CI: 1.10-4.04, p= 0.020) dan diabetes mellitus jenis 2 dengan penyakit arteri koronari (OR: 3.56, 95% CI: 1.74-7.29, p= 0.001) di kalangan masyarakat Cina tetapi tidak di kalangan masyarakat Melayu dan India. Alel K bagi *ENPP1* (K121Q) dan alel C bagi *CAPN-10* (SNP-63) tidak meningkatkan risiko untuk diabetes mellitus jenis 2 atau diabetes mellitus jenis 2 dengan penyakit arteri koronari dalam ketiga-tiga etnik. Kami menyimpulkan bahawa, gen polimorfisme bagi *PPAR-γ* (Pro12Ala) boleh menjadi satu faktor risiko genetik

untuk diabetes mellitus jenis 2 dan penyakit arteri koronari dikalangan masyarakat Cina tetapi tidak di kalangan masyarakat Melayu dan India.

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LIST OF SYMBOLS AND ABBREVIATIONS

9p21	:	Chromosome 9p21
ADA	:	American Diabetes Association
BMI	:	Body Mass Index
CAD	:	Coronary Artery Disease
CAPN-10	:	Caplain 10
CAPN-10 SNP-19	:	Caplain 10 Single Nucleotide Polymorphisms 19
CAPN-10 SNP-43	:	Caplain 10 Single Nucleotide Polymorphisms 43
CAPN-10 SNP-44	:	Caplain 10 Single Nucleotide Polymorphisms 44
CAPN-10 SNP-63	:	Caplain 10 Single Nucleotide Polymorphisms 63
CDKAL1	:	CDK5 regulatory subunit associated protein 1-like 1
CDKN2A/B	:	Cyclin-Dependent Kinase Inhibitor 2A/B
CIMT	:	Carotid Intima-Media Thickness
CRP	:	C-Reactive Protein
DALY	:	Disability-Adjusted Life Year
ENPP1	:	Ectonucleotide Pyrophosphatase/Phosphodiesterase 1
ENPP1 K121Q Polymorphism	:	Ectonucleotide Pyrophosphatase/Phosphodiesterase 1 Lysin (K) to Glutamine (Q) at codon 121 Polymorphism
FTO	:	Fat Mass and Obesity-associated Protein
GWAS	:	Genome-Wide Association Studies
HbA1c NGSP	:	Glycated Hemoglobin National Glycohemoglobin Standardization Program
HDL-C	:	High Density Lipoprotein Cholesterol
HHEX/IDE	:	Hematopoietically-expressed Homeobox Protein/ Insulin-Degrading Enzyme
HNF1B	:	Hepatocyte Nuclear Factor 1 Homeobox B

IGF2BP2	:	Insulin-Like Growth Factor 2 Mrna-Binding Protein 2
IMT	:	Intima-Media Thickness
JAZF1	:	Juxtaposed with another Zinc Finger Protein 1
KCNJ11	:	Potassium Voltage-Gated Channel Subfamily J Member 11
KCNQ1	:	Potassium Voltage-Gated Channel Subfamily Q Member 1
kDa	:	Kilodalton
LDL-C	:	Low Density Lipoprotein Cholesterol
NIDDM	:	Noninsulin-Dependent Diabetes Mellitus
OR	:	Odd Ratio
PAD	:	Peripheral Artery Disease
PPAR Alpha	:	Peroxisome proliferator-activated receptor Alpha
PPAR Beta	:	Peroxisome proliferator-activated receptor Beta
PPARγ Pro12Ala Polymorphism	:	Peroxisome proliferator-activated receptor gamma Proline (Pro) to Alanine (Ala) at codon 12 polymorphism
PPARγ/ PPAR Gamma	:	Peroxisome proliferator-activated receptor gamma
*PPARγ1/ PPAR Gamma*1	:	Peroxisome proliferator-activated receptor gamma γ1
*PPARγ2/ PPAR Gamma*2	:	Peroxisome proliferator-activated receptor gamma γ2
*PPARγ3/ PPAR Gamma*3	:	Peroxisome proliferator-activated receptor gamma γ3
*PPARγ4/ PPAR Gamma*4	:	Peroxisome proliferator-activated receptor gamma γ4
SLC30A8	:	Solute Carrier Family 30 (zinc transporter), Member 8
SNP	:	Single Nucleotide Polymorphisms
T2DM	:	Type 2 Diabetes Mellitus
T2DM+CAD	:	Type 2 Diabetes Mellitus patients with Coronary Artery Disease

T2DM+CAD	:	Type 2 Diabetes Mellitus patients with Coronary Artery Disease
TC		Total Cholesterol
TCF7L2	:	Transcription Factor 7-Like 2
TG	:	Triglyceride
WBG	:	Whole Blood Glucose
WHO	:	World Health Organization
SBP	:	Systolic Blood Pressure
DBP	:	Diastolic Blood Pressure

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Type 2 diabetes mellitus (T2DM) is highly prevalent complex disease which is a major public health problem. T2DM is a complex heterogeneous metabolic disorder characterized by impaired insulin secretion and insulin resistance which also highly influenced by both genetic and environmental factors (Ho, Germer, Tam, So, Martin, Ma, Chan et al., 2012). T2DM associated with socioeconomic lifestyle factors both in developed and developing countries, like Malaysia. Besides that, the problem is no longer restricted to the ageing population, as young adults and children are also being diagnosed with T2DM. The International Diabetes Federation has estimated that the number of T2DM patients is expected to rise from 366 million in 2011 to 552 million by 2030, if no drastic actions are taken. Approximately 183 million people are unaware that they are suffering from diabetes (Aguiree, Brown, Cho, Dahlquist, Dodd, Dunning, Hirst et al., 2013). Therefore, the identification of individuals at high risk of developing diabetes is vital for investigators and health care providers.

T2DM shares several common risk factors with coronary artery disease (CAD) as both are multifactorial diseases caused by genetic and environmental factors. T2DM is directly associated with CAD (Mohan, Venkatraman, & Pradeepa, 2010). Risk for CAD is high among T2DM subjects is twice as much compared to non-diabetic subjects (Deepa, Arvind, & Mohan, 2002) and CAD has been reported to occur two to three decades earlier in the diabetic subjects as opposed to their nondiabetic counterparts (Haffner, Lehto, Rönnemaa, Pyörälä, & Laakso, 1998). The association between CAD and T2DM is strong despite the fact that there are wide ethnic and geographic variations. Women with diabetes are possibly more prone to develop CAD compared men with diabetes (Mohan et al., 2010).

In this study it was tested whether single nucleotide polymorphisms (SNPs) affects T2DM risk as well as increasing the risk of an individual developing CAD. The increased risk is expected to be independent of exogenous environmental factors, since these confounders are expected to be distributed evenly in the respective genotype groups (Martín-Timón, Sevillano-Collantes, Segura-Galindo, & del Cañizo-Gómez, 2014). Hence, if T2DM SNPs were also found to be associated with increased CAD risk, this analysis based on genetic information would support the evidence of T2DM as a causal risk factor for CAD (Mariana Murea, Lijun Ma, & Barry I. Freedman, 2012).

In recent years, there has been a surge in the number of genetic studies of T2DM and CAD, attempting to identify some of the underlying risk factors employing genome-wide association studies (GWAS) (Seo, Kim, & Kwon, 2008). GWAS has been conducted to identify the SNPs which are common to human genome and to determine how these polymorphisms are distributed across different populations. These studies have helped scientists to uncover associations between individual SNPs and disorders that are passed from one generation to the next in Mendelian fashion. GWAS have successfully identified and replicated nearly 75 susceptibility loci associated with T2DM and related metabolic traits, mostly in Europeans, but not much in Africans and South Asian populations (Sanghera & Blackett, 2012).

Studies have discovered and confirmed numerous novel and candidate genes that affect insulin secretion such TCF7L2, SLC30A8, HHEX/IDE, CDKAL1, CDKN2A/B, IGF2BP2, KCNJ11, HNF1B, JAZF1, and KCNQ1. Genes that affect insulin sensitivity are *PPAR*γ and FTO (Burton, Clayton, Cardon, Craddock, Deloukas, Duncanson, Kwiatkowski et al., 2007). Individual risk alleles only confer a small increase in risk, suggesting that these variants may act accumulatively in vivo to affect T2DM susceptibility. Epidemiology studies have identified T2DM as a major risk factor for CAD (Ramachandran, Ma, & Snehalatha, 2010). In 2003, a USA national health survey

showed that CAD was much more prevalent among diabetic patients with metabolic syndrome (19.2%) compared to those in the general population (11.7%) (Alexander, Landsman, Teutsch, & Haffner, 2003). The frequent coexistence of T2DM and CAD suggests that these two conditions may share common pathogenic mechanisms and common susceptibility genes.

Peroxisome Proliferator-Activated Receptors (PPARs) are a group of three nuclear receptor isoforms (*PPAR Alpha*, *PPAR Beta*, and *PPAR Gamma*) encoded by three different genes. *PPARγ* plays an important role in controlling lipid and glucose metabolism and is currently implicated with various metabolic diseases such as hyperlipidaemia, diabetes mellitus and CAD (Kliewer & Willson, 1998; Wan, Xiong, Chao, Xiao, Ma, Wang, & Roy, 2010). *PPARγ* Pro12Ala polymorphism is located near NH2-terminus region of the protein, in the ligand-independent activation domain. *PPARγ* activation develops insulin sensitivity and glucose, adiponectin, and fatty acid uptake (Savkur & Miller, 2006). Besides that, it also modulates the transcriptional activity of *PPAR-γ* which is enhanced through phosphorylation by insulin (Deeb, Fajas, Nemoto, Pihlajamäki, Mykkänen, Kuusisto, Laakso et al., 1998; Werman, Hollenberg, Solanes, Bjørbæk, Vidal-Puig, & Flier, 1997). The expression of genes mediates adipocyte differentiation, energy metabolism, and insulin action are highly expressed in adipocytes, skeletal muscle, liver, and kidney also shown to regulate (Fajas, Auboeuf, Raspé, Schoonjans, Lefebvre, Saladin, Najib et al., 1997).

The Ecto-Nucleotide Pyrophosphatase/Phosphodiesterase 1 (*ENPP1*) encodes a type II protein transmembrane glycoprotein comprising two identical disulfide-bonded subunits which reduce sensitivity to insulin action by inhibiting the signal of the insulin tyrosine-kinase receptor (National Center for Biotechnology Information, 2016). In addition to plasma cells, *ENPP1* is also expressed in the liver, renal tubules, muscle, epididymis, chondrocytes, adipose tissue, pancreas, salivary duct epithelium, capillary

endothelium in the brain and the kidneys (Miao P. C., Fu M. C., Dao M. C., Jack C.R. T., Han F. H., & L., 2006). Many studies have identified 121 codon as a missense mutation which is significantly associated with increased diabetes risk and the mutant allele is also associated with obesity and many other related metabolic disorders (Barna, Matharoo, & Bhanwer, 2014; Prakash, Mittal, Awasthi, Agarwal, & Srivastava, 2013; Tang, Shen, Tang, Wang, Wei, Zhang, & Wang, 2014; Tripathi, Shukla, Dwivedi, Tripathi, Chauhan, Indurkar, & Singh, 2013). The 121Q (risk allele) variant binds to the insulin receptor with greater affinity compared with its wild allele (121K), resulting in less auto phosphorylation of the receptor. In a meta-analysis study, involving 15,801 T2DM patients and 26,241 control subjects, the 121Q allele was identified to be associated with an increased risk for T2DM , which is modulated by body mass index (BMI) (McAteer, Prudente, Bacci, Lyon, Hirschhorn, Trischitta, Florez et al., 2008).

The Calpain-10 gene (*CAPN-10*) on chromosome 2q37.3 is the first candidate gene for T2DM identified through a genome wide screening and positional cloning (Song, Niu, Manson, Kwiatkowski, & Liu, 2004). Calpains are hetero-dimers (80 kDa and 30 kDa), catalytic and regulatory sub-units respectively within the range of I to VI domains with calcium binding sites. *CAPN-10* is a cytoplasmic cysteine protease which requires calcium and phospholipids for its activity. It is an atypical Calpain and expressed ubiquitously in all adult and fetal human tissues as well as similar expression has been confirmed in rat and mouse (Garant, Kao, Brancati, Coresh, Rami, Hanis, Boerwinkle et al., 2002). *CAPN-10* is highly expressed in the heart, followed by the brain, liver, kidney, and pancreas. *CAPN-10* has 15 exons which span around 31 kb with twelve SNP's located in different intron regions out of which only four have been studied in detail with respect to T2DM. All the SNP's are situated in the non-coding regions such as SNP-44; T/C and SNP-43; G/A in the third intron region, SNP-19; 2R/3R in the sixth

intron region and SNP-63; C/T in the thirteenth intron region (Evans, Frayling, Cassell, Saker, Hitman, Walker, Levy et al., 2001). *CAPN-10* increases susceptibility to T2DM due to the high expression of SNP-43, -44, -19 and -63. Studies in different populations have identified different SNPs as associated with T2DM. For example, in a study among Tunisian Arab population, SNP-19 genotype was reported to be risk genotype to T2DM (Ezzidi, Turki, Messaoudi, Chaieb, Kacem, Al-Khateeb, Mahjoub et al., 2010; Pihlajamäki, Salmenniemi, Vänttinen, Ruotsalainen, Kuusisto, Vauhkonen, Kainulainen et al., 2006). However in another study that conducted among Finnish population has been reported that SNP-43 affects intra-abdominal obesity and insulin sensitivity in offspring of patients with T2DM. This too differs between men and women after an adjustment for insulin sensitivity (Pihlajamäki et al., 2006).

To date, there is still no data available on the association of *ENPP1* K121Q, *PPARγ* Pro12Ala and *CAPN-10* SNP-43 polymorphism with the risk of T2DM and CAD among Malaysians. The present study was designed to investigate whether a SNP of the *ENPP1*, *PPARγ* and *CAPN-10* gene could be associated with T2DM and CAD in Malaysian population.

1.1 Hypothesis

1. There is a significant relationship between Single Nucleotide Polymorphism of PPARγ, ENPP1 and CAPN-10 in type 2 diabetes mellitus and coronary artery disease.
2. There is a difference in biochemical parameters between diabetic patients, diabetic patients with coronary artery event and the control group
3. There is a relationship between SNP candidate's genes and ethnic groups in Malaysia.

1.2 Objective

1.2.1 General Objective

The aim of this study is to assess the genetic susceptibility of *PPARγ*, *ENPP1* and *CAPN-10* and risk factors for T2DM and coronary artery disease (CAD) in the Malaysian population.

1.2.2 Specific Objective

1. To determine single nucleotide polymorphism of *PPARγ*, *ENPP1* and *CAPN-10* in diabetic patients with coronary artery event.
2. To determine the biochemical parameter in diabetic patients with coronary artery event.
3. To determine the relationship between candidate's genes and ethnic groups Malaysia.

CHAPTER 2: LITERATURE REVIEW

2.1 Type 2 diabetes mellitus (T2DM)

2.1.1 Definition

Type 2 Diabetes mellitus is a metabolic disorder which is commonly known as noninsulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes since it is diagnosed later in life. It is a worldwide chronic health problem which is no longer restricted to the ageing population, as young adults and children are also being diagnosed with it (Barroso, 2005). T2DM occurs when the body does not produce enough insulin or when the cells are unable to use insulin properly, that is insulin resistance.

2.1.2 Epidemiology

The number of people with T2DM has doubled worldwide over the past three decades, particularly in developing countries. In 1985, the estimated number of people diagnosed with diabetes in the world was 30 million which has drastically increased to 135 million by 1995 and 284.6 million by 2010 (L. Chen, Magliano, & Zimmet, 2012). This increase is primarily due to genetic, life style changes, food intake, environmental factors and also obesity (World Health Organization, 2004). Based on the 2009 International Diabetes Federation Diabetes Atlas, the top ten countries with the largest number of T2DM patients were India (50.8 million), China (43.2 million), United States of America (26.8 million), Russian Federation (9.6 million), Brazil (7.6 million), Germany (7.5 million), Pakistan (7.1 million), Japan (7.1 million), Indonesia (7.0 million) and Mexico (6.8 million). As of 2013, the highest number of people with T2DM, aged 20-79 years, was from Western Pacific region, with 132 million out of the 381 million people across the globe (Aguiree et al., 2013). The number of people with T2DM in the world is projected to rise to 592 million by 2035, 347 million people

living in urban areas and145 million in the rural areas (Aguiree et al., 2013). The number represents 10.1% of the total adult population in the age group of 20 to 79 years. Even though the prevalence of T2DM is higher in the urban population, the number in the rural areas is on the rise too (Aguiree et al., 2013). One of the reasons cited for the sharp increase is reduced physical activity levels as countries become more industrialized. Six out of top 10 countries with the highest number of people with T2DM are countries in Asia (Mubarak, 2008).

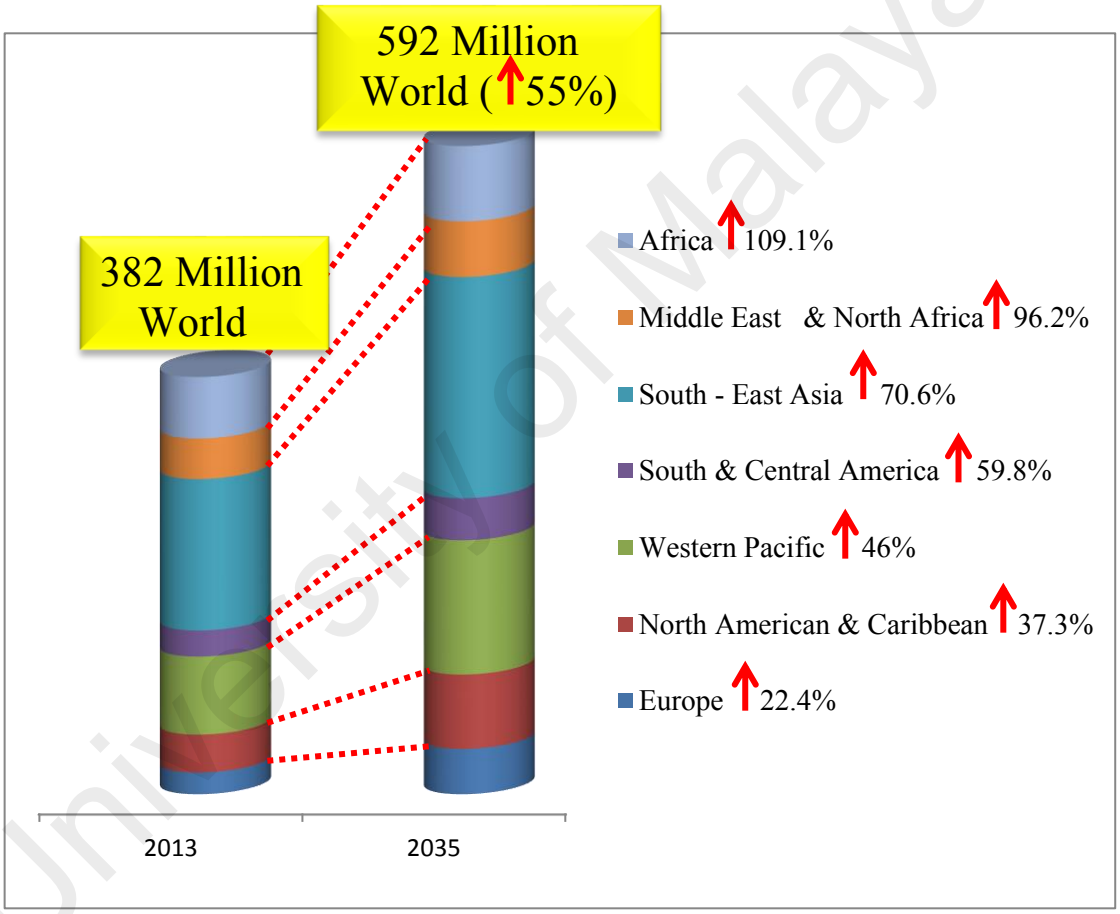

Figure 2.1: Worldwide extrapolation of 2013 diabetes mellitus to a future prediction of 2035 are shown by geographical region (Aguiree et al., 2013).

COUNTRY/ TERRITORY	2013 MILLIONS
China	98.4
India	65.1
United States of America	24.4
Brazil	11.9
Russian Federation	10.9
Mexico	8.7
Indonesia	8.5
Germany	7.6
Egypt	7.5
Japan	7.2

COUNTRY/ TERRITORY	2035 MILLIONS
India	142.7
China	109.0
United States of America	29.7
Brazil	19.2
Mexico	15.7
Indonesia	14.1
Egypt	13.1
Pakistan	12.8
Turkey	11.8
Russian Federation	11.2

Figure 2.2: Top 10 countries for number of people with diabetes mellitus (20-79 years). The number of people with diabetes mellitus in 2013 and the projected number in 2035. (Aguiree et al., 2013)

2.1.3 Causes of T2DM

The rapid increase in T2DM is mainly caused by defect in insulin secretion and insulin resistance which are influenced by both genetic and environmental factors (Ripsin, Kang, & Urban, 2009). Genome-wide association studies (GWAS) have confirmed that several candidate genes were associated with high risk for T2DM (Ho et al., 2012). The environmental factors that contribute to the development of T2DM include lack of physical activity, obesity, stress, unhealthy meals and urbanization (Mariana Murea, Lijun Ma, & Barry I Freedman, 2012). In addition smoking, hypertension and high alcohol consumption may increase risk of T2DM (S. Carlsson, Hammar, Grill, & Kaprio, 2003; Kilpelainen, 2009). Mothers with gestational diabetes are at higher risk of developing T2DM and their babies also have a higher risk of obesity and developing of T2DM when they become adults (Colagiuri, Brown, & Dain, 2011).

2.1.4 Signs and symptoms of T2DM

The symptoms for development of T2DM may not be so obvious, because the condition usually develops slowly over the years. The common symptoms of T2DM are urinating very often, feeling very thirsty and hungry, excessive fatigue, blurry vision, extreme weight loss, slow-healing sores or cuts, itching of the skin usually around the vaginal or groin area, acanthosis nigricans (velvety dark skin changes of the neck, armpit, and groin) and tingling, pain, or numbness in the hands or feet (American Diabetes Association, 2015; Vijan, 2010).

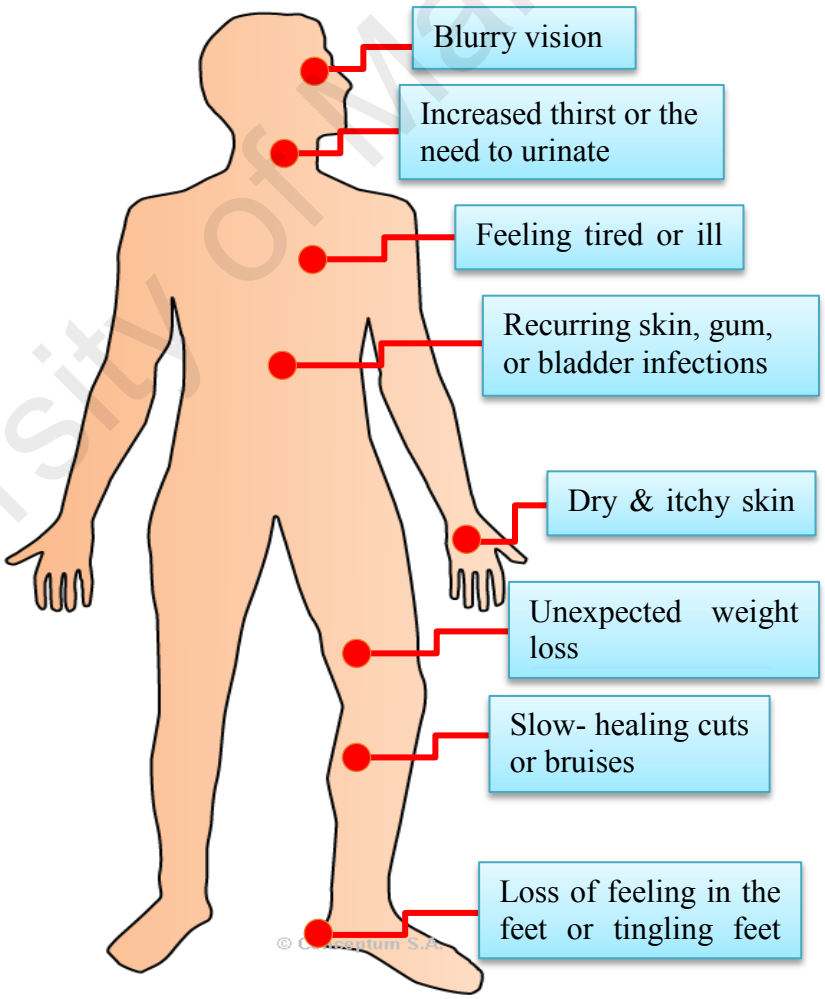

Figure 2.3: Most common signs and symptoms of type 2 diabetes mellitus. Adapted from (Vijan, 2010)

2.1.5 Complication

T2DM is highly associated with an increased risk of developing a number of serious health problems (Cade, 2008). The consistent high levels of blood glucose would lead to both microvascular and macrovascular complications which includes retinopathy, nephropathy, and neuropathy (microvascular) and ischemic heart disease, peripheral vascular disease, and cerebrovascular disease (macrovascular), resulting in organ and tissue damage (Cade, 2008). Poor wound healing, heart disease, retinopathy, hypoglycaemia, poor blood circulation in the extremities, joint disease, gangrene, nephropathy, skin ulcer, amputation, nerve damage, blindness and periodontal diseases are frequent complications which occur due to uncontrolled T2DM (Fowler, 2008).

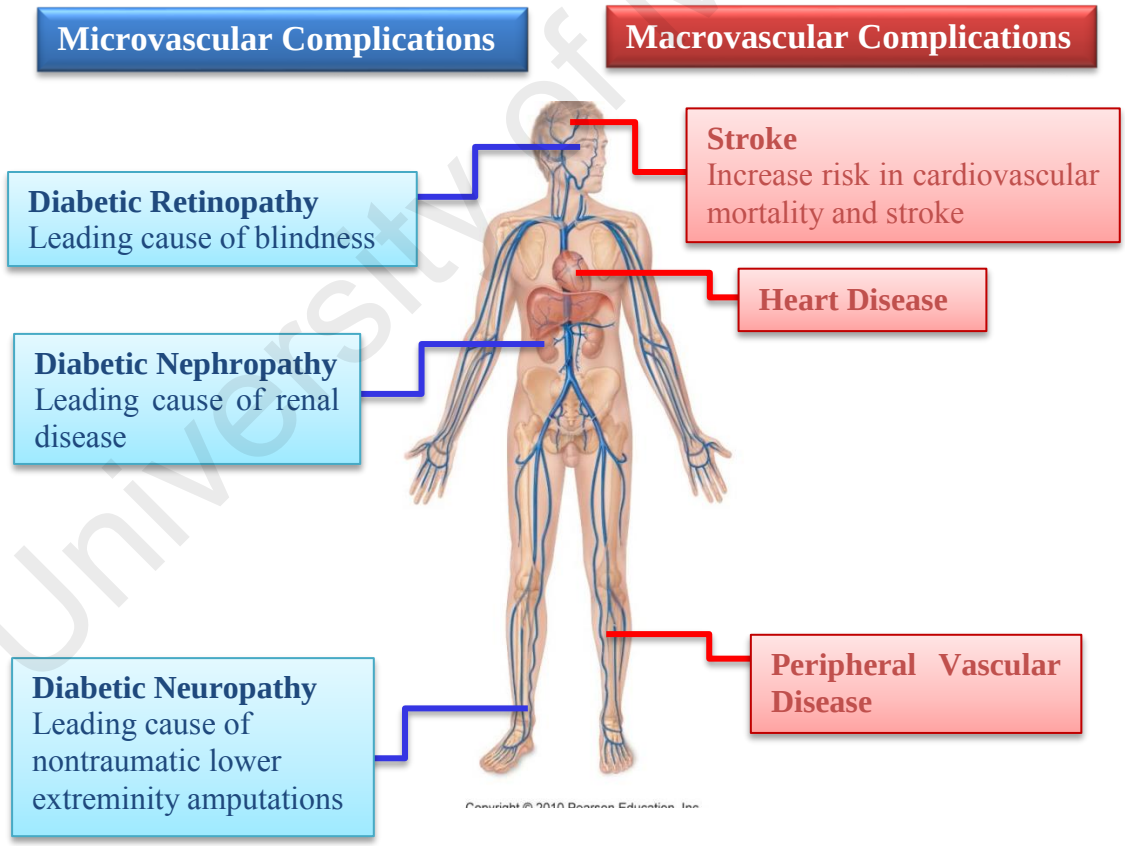

Figure 2.4: Major T2DM complications. Adapted from (Olokoba, Obateru, & Olokoba, 2012)

2.2 Coronary Artery Disease (CAD)

2.2.1 Definition

Coronary artery disease (CAD) is the narrowing or blockage of the arteries and major blood vessels that provide oxygen and nutrients to the heart. This is caused by atherosclerosis, an accumulation of fatty materials on the inner linings of arteries which leads to restricted blood flow to the heart (American Heart Association, 2014). Reduced or obstructed blood flow and oxygen supply to the heart muscles can lead to heart failure and arrhythmias.

2.2.2 Epidemiology

CAD is a widespread burden of morbidity and mortality worldwide, in both developed and developing countries. Each year more than 17 million people die due to CAD which represents 30% of all deaths, and the number is expected to increase drastically to 23.6 million by 2030 (Mendis, Puska, & Norrving, 2011). About 80% of people with CAD are from the low-income and middle-income countries (World Health Organization, 2011). In 2008, the World Health Organization (WHO) reported that 17.3 million deaths, worldwide, were due to CAD and the highest number was from Western Pacific (4.7 million) followed by Europe (4.6 million), South East Asia (3.6 Million), America (1.9 Million), Africa (1.9 Million) and East Mediterranean (1.2Million) (Sheikh, 2008).

About 80% of the global cardiovascular disease burden is expected to be borne by developing nations. Based on projections, from 1990 to 2020 the mortality of CAD dramatically would increase in developing country and projected to be 120% in women and 137% in men (Leeder, 2004). However, 80% of CAD, stroke, and T2DM can be prevented by eliminating obesity, unhealthy diets, and physical inactivity (Wong, 2012).

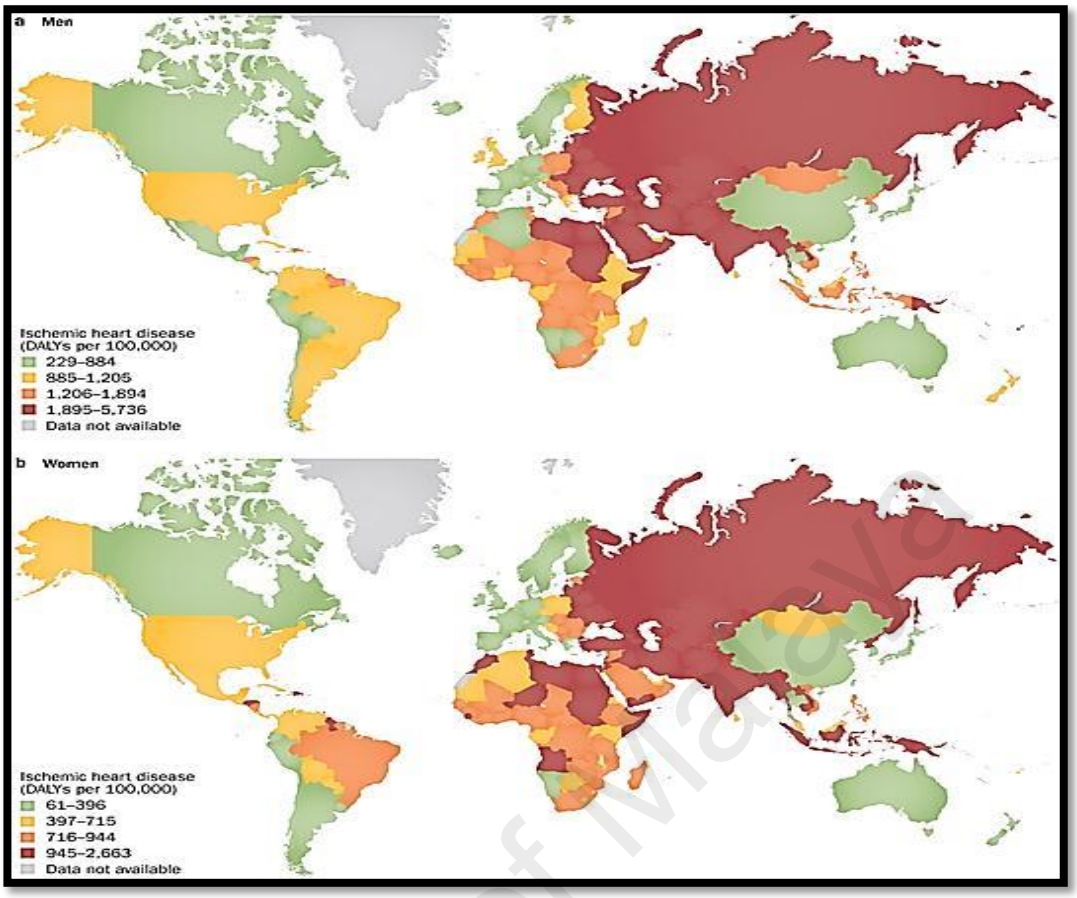

Figure 2.5: The global distribution of ischaemic heart disease burden in DALY (2011) (Wong, 2014)

2.2.3 Causes of CAD

The aetiology of CAD is multifactorial. The development of CAD is mainly caused by the combination of interaction between genetic, lifestyle and environmental factors (Sayols-Baixeras, Lluís-Ganella, Lucas, & Elosua, 2014). Although each risk factor is partly under genetic control, a family history of CAD is also an independent risk factor. In addition, researchers have also identified that more than 250 susceptibility genes play important role in CAD (Ellsworth, Sholinsky, Jaquish, Fabsitz, & Manolio, 1999). The most important behavioral risk factors of CAD are unhealthy diet, physical inactivity, smoking and harmful use of alcohol. The effects of these behavioral risk factors are raised blood pressure (hypertension), raised blood glucose (diabetes mellitus), raised blood lipids (hypercholesterolemia) and overweight (obesity)

(Hirashiki, Yamada, Murase, Suzuki, Kataoka, Morimoto, Tajika et al., 2003). These are the intermediate risk factors which lead to the increased risk of developing heart attack, stroke, heart failure and other complications (Townsend, Wickramasinghe, Bhatnagar, Smolina, Nichols, Leal, Luengo-Fernandez et al., 2012).

There are also a number of underlying causes of CAD that are reflection of the major forces driving social, economic and cultural change around globalization, urbanization and population ageing (World Health Organization, 2014). Other determinants of CAD include poverty, stress and hereditary factors (Yamada, Izawa, Ichihara, Takatsu, Ishihara, Hirayama, Sone et al., 2002).

2.2.4 Signs and symptoms

Often, there are no symptoms shown in the development of CAD in patients. Normally the first warning of CAD will be through heart attack or stroke which also comes with the feeling of pain or discomfort of the chest, arms, left shoulder, elbows, jaw or back. In addition, the patients also experience difficulty in breathing, sick or vomiting, light headed or fainted, breaking into cold sweat and become pale. Women are more likely to have shortness of breath, nausea, vomiting, and back or jaw pain (Kontos, Diercks, & Kirk, 2010; World Health Organization, 2011).

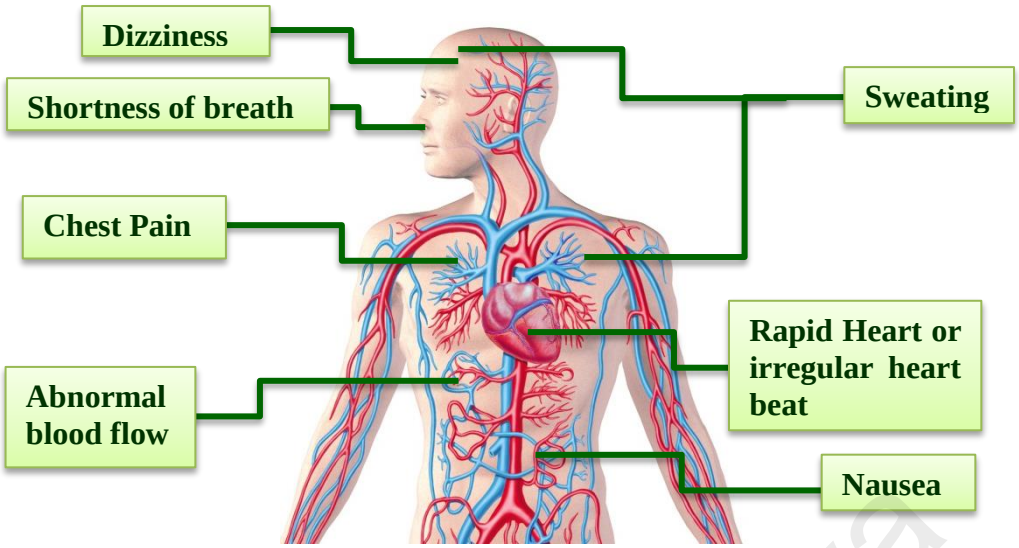

Figure 2.6: Most common signs and symptoms of coronary artery disease. Adapted from (Olokoba et al., 2012)

2.2.5 Complication

The ischemia of CAD can lead to serious heart damage. Acute ischemia can damage heart muscles, thereby weakening the heart and reducing its efficiency. Acute ischemia can also initiate a fatal arrhythmia and sudden cardiac death (Mayo Foundation for Medical Education and Research, 2015). Chronic ischemia, accumulating damages from even small ischemic episodes, can lead to heart failure. Frequent complications that occur are heart failure, heart attack, stroke, aneurysm, peripheral artery disease and sudden cardiac arrest**.**

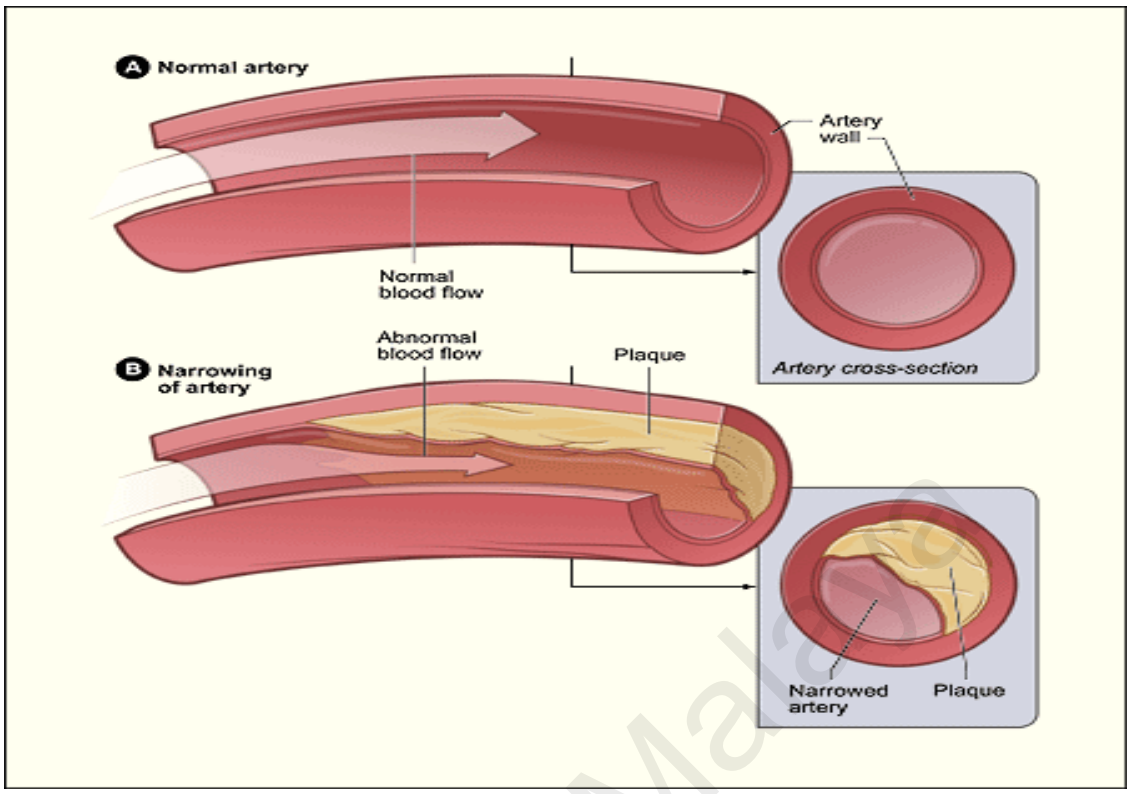

Figure 2.7: Normal coronary artery versus blocked coronary artery.
Adapted from (National Heart, Lung, & Blood Institute, 2016)

2.3 Genetic background

2.3.1 Genetic background of T2DM

Despite the recognition of strong inherited factors of T2DM, susceptibility genes that are involved in T2DM are still poorly defined. It is understood that T2DM is a multigenic disease where many genes are thought to be involved and the combinations of genes defects exist among diabetic patients. Furthermore, the multiple genetic variants interact with various environmental factors and the net effect becomes multifold (Kilpelainen, 2009). Common gene variations, known as single nucleotide polymorphisms (SNPs), are associated with increased risk of developing T2DM (Bush & Moore, 2012). Whole-genome linkage studies are ongoing to find the diabetes susceptibility genes (Wheeler & Barroso, 2011). The entire genome of affected family

members is scanned and the families are followed over several generations or large numbers of affected sibling-pairs are studied (Lyssenko & Laakso, 2013).

2.3.2 Genetic background of CAD

CAD may be caused by single-gene mutation and the interaction of multiple genes. The understanding of genotype has improved through experiments on genetic polymorphism and phenotypic effect. Recent genetic studies have confirmed that several genetic factors are associated with CAD, which has led to much excitement around the possibility (Roberts & Stewart, 2012b) for risk prediction. In 2007, the first genetic risk variant, 9p21, was discovered (Roberts & Stewart, 2012a). Since then 36 genes as genetic risk variant for CAD have been discovered through genome wide association studies using large number of samples (Roberts & Stewart, 2012a). These genetic risk factors occur more commonly in the population than expected, with over half of them occurring in more than 50% of the population, and 10 of them occurring in at least 75% of the population.

2.3.3 Genome–wide association studies

Genome wide association studies (GWAS) are a new way of experimentation designed to identify the genes that are involved in human disease (Burton et al., 2007). These studies typically focus on the associations between single-nucleotide polymorphisms (SNPs) which more frequently occurs in people with certain disease compare to others without disease. It measures 100,000 of SNPs from a single DNA by a single experiment (Billings & Florez, 2010). The first microarray done by 500,000 SNPs as DNA markers became available and within 5 years the results have been nothing short of remarkable. In total, over 1,319 genetic variants have been identified to be associated with increased risk for 160 diseases (Roberts & Stewart, 2012a).

The results from GWAS are indications of genetic variants that confer susceptibility to multifactorial disorders (Steinthorsdottir, Thorleifsson, Reynisdottir, Benediktsson, Jonsdottir, Walters, Styrkarsdottir et al., 2007). However, the effect of those variants or other alleles of the gene is still just one of many factors influencing disease risk. GWAS examines the SNPs across the whole genome and identify SNPs related to several complex conditions including diabetes, heart abnormalities, Parkinson disease, and Crohn disease (Steinthorsdottir et al., 2007).

2.4 PPAR Gamma

2.4.1 Chromosomal location and function

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes (Michalik, Auwerx, Berger, Chatterjee, Glass, Gonzalez, Grimaldi et al., 2006). PPARs play essential roles in the regulation of cellular differentiation, development, and metabolism of exogenous carbohydrate, lipid, and protein (Belfiore, Genua, & Malaguarnera, 2009). Three types of PPARs have been identified and they are classified as alpha, gamma, and beta (Berger & Moller, 2002). Peroxisome proliferator-activated receptor gamma (PPARG) is a nuclear hormone receptor which plays a critical role in regulating adipocyte differentiation and the transcription of genes that are important for lipogenesis. The PPARG gene is located on chromosome 3p25-24 (Yen, Beamer, Negri, Silver, Brown, Yarnall, Burns et al., 1997) and contains 9 exons and spans more than 100 kb. PPARG also exist in two major protein isoforms which are PPARG1 and PPARG2 and two minor forms PPARG3 and PPARG4 created by alternate promoter usage and alternative splicing at the 5′ end of the gene (Savkur & Miller, 2006). PPARG has been implicated in the pathology of numerous diseases including obesity, diabetes, atherosclerosis, and cancer.

2.4.2 PPAR Gamma and T2DM

The PPRG gene is known as a ligand-activated transcription factor that regulates the transcription of several genes which involved in glucose metabolism, angiogenesis, lipid oxidation, adipocyte differentiation and inflammation (Francis, Fayard, Picard, & Auwerx, 2003). The expression of genes involved in glucose and lipid metabolism is activated by PPARG which helps to convert nutritional signals into metabolic consequences. PPARG is the first gene that was identified as complex late onset form of T2DM via candidate gene approach. The SNP of PPARG, known as Pro12Ala variant, shows that different protein isoforms generated by different promoters and alternative splicing in a transcript and associated with the risk factor of T2DM and its intermediate traits (Mukherjee, Jow, Croson, & Paterniti, 1997). In 1997, Yen et al (1997) the first to discover that PPARG Pro12Ala variant plays a role of risk factor in T2DM and nearly 60 association studies have confirmed their finding. These studies were conducted in a variety of populations Caucasians, North Indian and Japanese population (Kawasaki, Tahara, Emoto, Shoji, Shioji, Okuno, Inaba et al., 2002; Sanghera, Ortega, Han, Singh, Ralhan, Wander, Mehra et al., 2008; Yen et al., 1997). However, in some studies PPARG in certain populations, such as African Americans (Palmer, McDonough, Hicks, Roh, Wing, An, Hester et al., 2012), East Asians (Cho, Chen, Hu, Long, Ong, Sim, Takeuchi et al., 2011) and South Asians (Kooner, Saleheen, Sim, Sehmi, Zhang, Frossard, Been et al., 2011) the association was not seen to be significant.

2.4.3 PPAR Gamma and CAD

PPARG belongs to a family of transcription factor which plays a major role by regulate fat cell development and glucose metabolism. PPARG is currently implicated with various vascular diseases such as CAD, hyperlipidemia, peripheral arterial disease (PAD) and stroke (Vidal-Puig, Considine, Jimenez-Liñan, Werman, Pories, Caro, &

Flier, 1997). It is also expressed through macrophage foam cell, endothelial cell, smooth muscle cells and mononuclear cells and it may affect the atherosclerogenic processes in atherosclerotic plaques (Sueyoshi, Yamada, Niihasi, Kusumi, Oinuma, Esumi, Tsuru et al., 2001). PPARG also promotes atherosclerosis process by stimulates the uptake of oxidized low density lipoprotein (Ox-LDL) known to be one of the critical events in foam cell formation (Tontonoz, Nagy, Alvarez, Thomazy, & Evans, 1998). Atherogenic oxidised LDL particles uptake may be induced by themselves through the activation of PPARG and expression of CD36 which leads to foam cell formation and atherosclerosis (Nagy, Tontonoz, Alvarez, Chen, & Evans, 1998). Various studies show that Pro12Ala gene variant of PPARG is associated with basic vascular disease risk factors (Altshuler, Hirschhorn, Klannemark, Lindgren, Vohl, Nemesh, Lane et al., 2000; Deeb et al., 1998; Meirhaeghe, Fajas, Helbecque, Cottel, Auwerx, Deeb, & Amouyel, 2000). Some clinical and epidemiological studies have reported that Ala allele of Pro12Ala to be significantly associated with lower insulin resistance (Doney, Fischer, Cecil, Boylan, McGuigan, Ralston, Morris et al., 2004), higher body mass index (BMI) (Masud & Ye, 2003) and decreased blood pressure, regulated macrophage lipid homeostasis (Marx, Schonbeck, Lazar, Libby, & Plutzky, 1998), attenuate intimal and medial complex thickening (Koshiyama, Shimono, Kuwamura, Minamikawa, & Nakamura, 2001; Minamikawa, Tanaka, Yamauchi, Inoue, & Koshiyama, 1998) and narrowing of the coronary lumen (Takagi, Akasaka, Yamamuro, Honda, Hozumi, Morioka, & Yoshida, 2000). Collectively, these findings strongly suggest that Pro12Ala gene variant of PPARG plays a possible role in the development of CAD. However, there are some clinical findings that provided inconsistent results; with some studies showing reduce risk of CAD (Doney, Fischer, Leese, Morris, & Palmer, 2004; Ridker, Cook, Cheng, Erlich, Lindpaintner, Plutzky, & Zee, 2003) and others reporting no association between PPARG and CAD (Gallicchio, Kalesan, Huang, Strickland, Hoffman, & Helzlsouer,

2008; Pischon, Pai, Manson, Hu, Rexrode, Hunter, & Rimm, 2005; Vogel, Segel, Dethlefsen, Tjonneland, Saber, Wallin, Jensen et al., 2009; Zafarmand, van der Schouw, Grobbee, de Leeuw, & Bots, 2008).

2.5 *ENPP1*

2.5.1 Chromosomal location and function

ENPP1 belongs to Ectonucleotide pyrophosphatase/phosphodiesterase (ENPP) family member 1 which downregulates insulin signaling by inhibiting insulin-receptor tyrosine kinase activity (Betty A. Maddux, Sbraccia, Kumakura, Sasson, Youngren, Fisher, Spencer et al., 1995). *ENPP1* known to be involved in regulation of adipocyte maturation and highly regulated during adipogenesis, thus suggesting a role of insulin resistance in the absence of obesity (Liang, Fu, Ciociola, Chandalia, & Abate, 2007). The gene that codes *ENPP1* spans over 83 kb and is located on chromosome 6q22-23. Previous studies of variant rs1044498, located in exon 4, which causes an amino acid change from lysine to glutamine at codon 121 (K121Q) (Pizzuti, Frittitta, Argiolas, Baratta, Goldfine, Bozzali, Ercolino et al., 1999), has shown positive associations with diabetes (Chandalia, Grundy, Adams-Huet, & Abate, 2007; Keene, Mychaleckyj, Smith, Leak, Perlegas, Langefeld, Freedman et al., 2008; Meyre & Froguel, 2006) insulin resistance , glucose homeostasis (Baratta, Rossetti, Prudente, Barbetti, Sudano, Nigro, Farina et al., 2008), metabolic syndrome , obesity, coronary artery disease (Bottcher, Korner, Reinehr, Enigk, Kiess, Stumvoll, & Kovacs, 2006; Meyre, Bouatia-Naji, Tounian, Samson, Lecoeur, Vatin, Ghoussaini et al., 2005) and diabetic nephropathy (L. S. Wu, Hsieh, Pei, Hung, Kuo, & Lin, 2009). In support of a potential role of ENPP1 and diabetes risk, functional studies have demonstrated that mutations in the gene result in overexpression of the glycoprotein leading to decreased insulin receptor tyrosine kinase activity (Goldfine, Maddux, Youngren, Frittitta, Trischitta, & Dohm, 1998).

ENPP1 is known to play a central role not only in orthotopic, but also in heterotopic mineral deposition. Inactivating mutations of ENPP1 are associated with generalized arterial calcification of infancy, a spontaneous and frequently lethal form of widely disseminated arterial media calcification (Rutsch, Ruf, Vaingankar, Toliat, Suk, Hohne, Schauer et al., 2003).

2.5.2 ENPP1 and T2DM

ENPP1 is a class II transmembrane glycoprotein, that encodes for a protein that inhibits insulin receptor (IR) signaling and which has been, proposed as a candidate for insulin resistance (Prudente, Morini, & Trischitta, 2009). In 2000, Maddux and Goldfine found that *ENPP1* interacts directly with α-subunit of the insulin receptor, thereby decreasing insulin-mediated activation of the tyrosine phosphorylation in ß-subunit of the insulin receptor and downstream insulin signaling activation (B. A. Maddux & Goldfine, 2000). Several polymorphisms are located in the *ENPP1* gene, the one most frequently analysed being located in exon 4 and causing an amino acid change. This variation results in a lysine (K) to glutamine (Q) at codon 121. The minor Q allele (risk allele) of K121Q variant is known to be associated with a stronger interaction with protein by inhibiting insulin receptor function and insulin signaling more effectively than the major K allele (wild allele) which resulting in a reduction of insulin receptor autophosphorylation (Jacobsen, Grarup, Tarnow, Parving, & Pedersen, 2002). Many studies have been conducted in different ethnic groups in investigating the association of K121Q variant with insulin resistance and related phenotypes (Barna et al., 2014; Keshavarz, Inoue, Sakamoto, Kunika, Tanahashi, Nakamura, Yoshikawa et al., 2006; Prakash et al., 2013; Tang et al., 2014). However, the impact of this allelic polymorphism, which has been widely investigated the association with features of insulin resistance and T2DM susceptibility, is still under debate, with contradictory

findings (Abate, Carulli, Alberto Cabo-Chan, Chandalia, Snell, & Grundy, 2003; Gonzalez-Sanchez, Martinez-Larrad, Fernandez-Perez, Kubaszek, Laakso, & Serrano-Rios, 2003; Gu, Almgren, Lindholm, Frittitta, Pizzuti, Trischitta, & Groop, 2000; Rasmussen, Urhammer, Pizzuti, Echwald, Ekstrøm, Hansen, Hansen et al., 2000). Likewise, overexpression of the *ENPP1* Q allele in mouse muscle and liver tissues causes insulin resistance and glucose intolerance in vivo (B. A. Maddux, Chang, Accili, McGuinness, Youngren, & Goldfine, 2006). In humans, the *ENPP1* K121Q gene have been considered to be a strong candidate gene for insulin resistance and associated with T2DM. Pizzuti et al. (1999) reported an association of K121Q variant with insulin resistance in a Sicilian Caucasian population (Pizzuti et al., 1999). In 2003 and 2005, researches by Abate and his members have proved that type 2 diabetes is associate with *ENPP1* among South Asians in India and US (Abate et al., 2003; Nicola Abate, Manisha Chandalia, Pankaj Satija, Beverley Adams-Huet, Scott M. Grundy, Sreedharan Sandeep, Venkatesan Radha et al., 2005) and also Caucasians who live in US and Finland (Kubaszek, Markkanen, Eriksson, Forsen, Osmond, Barker, & Laakso, 2004). Subsequently, several other studies conducted in French, Chinese and Polish populations have indicated that K121Q variant significantly increase the risk of T2DM (Bochenski, Placha, Wanic, Malecki, Sieradzki, Warram, & Krolewski, 2006; Meyre et al., 2005; Xu M, 2003). .

2.5.3 *ENPP1* and CAD

The *ENPP1* gene is well known as a candidate gene for insulin resistance since Maddux and his colleague first proposed that K121Q polymorphism of *ENPP1* is significantly associated with insulin resistance (Betty A. Maddux et al., 1995). Most of the evidence show that insulin resistance is a major component of pathogenic for and an important risk factor for CAD (Alexander et al., 2003; Rutter, Meigs, Sullivan,

D'Agostino, & Wilson, 2005). The pathogenic mechanism which relates both insulin resistance and CAD alike to be a consequence of systemic abnormalities which are strictly associated to insulin resistance, such as arterial hypertension, obesity and dyslipidemia (Howard, O'Leary, Zaccaro, Haffner, Rewers, Hamman, Selby et al., 1996). A direct deleterious effect of impaired insulin signaling on the endothelium (Federici, Pandolfi, De Filippis, Pellegrini, Menghini, Lauro, Cardellini et al., 2004) is that it leads to reduced insulin-stimulated nitric oxide release and decreased vasodilatation. In addition, both insulin resistance (Almind, Doria, & Kahn, 2001) and atherosclerosis (S. Bacci, Rizza, Prudente, Spoto, Powers, Facciorusso, Pacilli et al., 2011) widely demonstrate a clear genetic background. Insulin resistance genes are likely to play a modulating role in the development or severity of both T2DM and CAD. A study conducted in Austrian general population showed that, among myocardial infarction (MI) patients, those carrying the ENNP1 Q121 variant had an approximately 2.5-fold increased risk of early coronary event (Endler, Mannhalter, Sunder-Plassmann, Schillinger, Klimesch, Exner, Kapiotis et al., 2002). Similar study, replicated among people from central Germany (Endler et al., 2002), observed that the association of the variant was independent from other known CAD risk factors. This suggests a direct effect of the variant on wall arterial homeostasis may accelerate the atherosclerotic process.

More recently, a collaborative study has reported that the prevalence of Q121 variant progressively and significantly increased CAD in T2DM (Simonetta Bacci, Ludovico, Prudente, Zhang, Di Paola, Mangiacotti, Rauseo et al., 2005). The Q121 variant has been also associated with atherosclerosis related phenotypes in European (S. Bacci, Di Paola, Menzaghi, Di Fulvio, Di Silvestre, Pellegrini, Baratta et al., 2009; Simonetta Bacci et al., 2005; Endler et al., 2002) but not in Brazilian (Moehlecke, Kramer, Leitao,

Krahe, Balbosco, Azevedo, Gross et al., 2010) or in Chinese (Miao P. C. et al., 2006) populations.

2.6 *CAPN- 10*

2.6.1 Chromosomal location and function

Calpains are heterodimers composed of 80 KDa and 30 KDa, catalytic and regulatory subunits respectively within the range of I to VI domains probably with calcium binding sites (Bukowska, Lendeckel, Bode-Böger, & Goette, 2012). Calpain 10 (*CAPN-10*) is a well-conserved phospholipids and calcium-sensitive cysteine proteases (Ma, Fukiage, Kim, Duncan, Reed, Shih, Azuma et al., 2001). It requires 14 genes from 80K family and 2 genes from 30K family are ubiquitously expressed in humans, which located on chromosome 2q37.3 (Song et al., 2004). Probably its main function is in protease activity and cellular signaling. Although *CAPN-10* is highly expressed in heart, brain, liver, kidney and pancreas, the main role of it is in the tissues such as skeletal muscles. Besides that, *CAPN-10* protein also regulates insulin secretion (S. Carlsson et al., 2003; Orho-Melander, Klannemark, Svensson, Ridderstråle, Lindgren, & Groop, 2002; Weedon, Schwarz, Horikawa, Iwasaki, Illig, Holle, Rathmann et al., 2003) and insulin-mediated glucose metabolism. Besides that, *CAPN-10* comprises 15 exons spanning 31 kb of genomic sequence that encodes a 672-amino-acid intracellular protease but probably only 12 SNP's are spanning around 31 Kb which are located in different intron region (Goll, Thompson, Li, Wei, & Cong, 2003). However, only four SNP's have undergone detailed study with respect to T2DM. All the four SNP's are located in the non-coding regions SNP-44 and SNP-43 in the 3rd intron region, SNP-19 in the 6th intron region and SNP-63 in the 13th intron region. The strong involvement of SNP-43, SNP-44, SNP-19 and SNP-63 of *CAPN-10* increases susceptibility to T2DM (Song et al., 2004).

2.6.2 *CAPN-10* and T2DM

The *CAPN-10* is considered to be one of the candidate genes for T2DM which regulates insulin secretion and insulin-mediated glucose metabolism (E. Carlsson, Poulsen, Storgaard, Almgren, Ling, Jensen, Madsbad et al., 2005; Weedon et al., 2003). In 2000 a GWAS study on 330 Mexican-American concluded (Horikawa, Oda, Cox, Li, Orho-Melander, Hara, Hinokio et al., 2000) that *CAPN-10* gene is the first positionally cloned as a putative diabetes predisposing gene within T2DM susceptibility. *CAPN-10* is expressed at mRNA and protein levels by several tissues, with different mRNA isoforms being reported (E. Carlsson et al., 2005; Ling, Groop, Del Guerra, & Lupi, 2009; Pihlajamäki et al., 2006). In addition, the level of mRNA is elevated from T2DM patient's pancreatic islets and shows a positive correlation between *CAPN-10* expression and insulin release in response to arginine in non-diabetic but not in diabetic donors (Ling et al., 2009). The SNP-43 of *CAPN-10* is involved in many phenotypes that associated with T2DM, for example insulin resistance, lipogenesis, insulin secretion and microvascular function. SNP-44 of *CAPN-10* has also been associated with increased risk for T2DM (Shore, Evans, Frayling, Clark, Lee, Horikawa, Hattersley et al., 2002; Tsuchiya, Schwarz, del Bosque-Plata, Hayes, Dina, Froguel, Towers et al., 2006). In addition, beta-cell function was found lower in subjects without T2DM that carried the risk genotypes G/G at SNP-43 and T/C at SNP-44 (Johnson, Otani, Bell, & Polonsky, 2009). However, previous studies also proved that susceptibility to T2DM is not associated with homozygosity of G alleles at SNP-43, but rather with a specific high-risk haplotype defined not only by SNP-43 but also by polymorphisms at SNP-19 and SNP-63 of *CAPN-10*. This haplotype was found to be associated with T2DM in a number of ethnic group studies, including Mexican Americans, Finns and Germans (Horikawa et al., 2000) and with a predisposition towards increased glucose and decreased insulin response in a British population (Lynn, Evans, White, Frayling,

Hattersley, Turnbull, Horikawa et al., 2002). The polymorphism SNP-19 is also associated with T2DM in Mexican Americans. In Mexican Americans, Finns and Germans haplogenotype 112/121, based on SNP-43, SNP-19 and SNP-63 is associated with an increased risk of T2DM (Y. Chen, Kittles, Zhou, Chen, Adeyemo, Panguluri, Chen et al., 2005). A study on a population in South India indicated that haplogenotype 112/121 was associated with increased risk of both T2DM and impaired glucose tolerance though the haplogenotype had a low frequency in the population (Cassell, Jackson, North, Evans, Syndercombe-Court, Phillips, Ramachandran et al., 2002).

2.6.3 *CAPN-10* and CAD

CAPN-10 can be expressed in many major tissues in glucose metabolism, such as pancreatic islets, skeletal muscle and liver which might affect insulin secretion, insulin action and hepatic glucose production (Horikawa et al., 2000). This gene is regarded as one of the candidate genes for T2DM in human. The main principal point of metabolic syndrome, better known as insulin resistance, characterized by hyperglycemia, hypertension, dyslipidemia, often leads to serious micro and macrovascular complications, including CAD (Haffner et al., 1998). *CAPN-10* has been associated with various risk factors of metabolic syndrome, such as elevated body mass index (BMI) (Shima, Nakanishi, Odawara, Kobayashi, & Ohta, 2003), plasma cholesterol concentration (B. Wu, Takahashi, Fu, Cheng, Matsumura, & Taniguchi, 2005), hypertension (S. f. Chen, Lu, Yan, Huang, & Gu, 2007) and hypertriglyceridemia (E. Carlsson, Fredriksson, Groop, & Ridderstråle, 2004). SNP's of *CAPN-10* gene are also associated with elevated triglyceride levels and reduced expression of *CAPN-10* in the adipose tissue of obese subjects (E. Carlsson et al., 2004). However, the allele combination in the *CAPN-10*, SNP-43, SNP-19, and SNP-63 is known to increase the risk of T2DM in many populations (Cassell et al., 2002; Lynn et al., 2002). The

prevalence of CAD is known to be much higher in patients with T2DM than in general population (Alexander et al., 2003). A study documented that transgenic overexpression of *CAPN-10* may reduce myocardial hypertrophy in a mouse model of angiotensin-II–induced hypertension, suggesting that *CAPN-10* may be a contributing factor to cardiac hypertrophy (Letavernier, Perez, Bellocq, Mesnard, de Castro Keller, Haymann, & Baud, 2008).

CHAPTER 3: METHODOLOGY

3.1 Patients and Samples

This study was conducted at University Malaya Medical Centre (UMMC) between 2012 and 2014. There were three groups of subjects in this study: (1) patients with T2DM only, (2) patients with T2DM and CAD and (3) subjects free of T2DM and CAD (the control group). For this study, 120 T2DM patients free of CAD were recruited at the outpatient clinic at UMMC by trained staff nurse. The selection of patients with T2DM and CAD was done at cardiac clinic at UMMC by Cardiologist where T2DM patients were scheduled for angiography. At the end angiogram, patients were as classified having coronary lesion coronary angiography with less than 75% luminal stenosis. The presence CAD was based on the diagnosis from two cardiologists based on coronary angiography. A total of 120 patients with T2DM and CAD were selected through this procedure. As the control group, 120 subjects free of T2DM and CAD were selected from the hospital staff. The patients comprised of Malay, Indian and Chinese ethnicity. All the subjects in this study were given explanation on the purpose and procedures of the study. In this study patients with other chronic diseases or complication from diabetes were excluded. This study was approved by an internal ethics committee, and written informed consent was obtained from each patient.

3.2 Inclusion and Exclusion Criteria of sample collection

Inclusion: > 30 years, T2DM, early stage of Diabetes and early stage of coronary artery disease.

Exclusion: Diabetic complications or other chronic diseases (neuropathy, nephropathy, retinopathy, Alzheimer's disease, cancer and etc.)

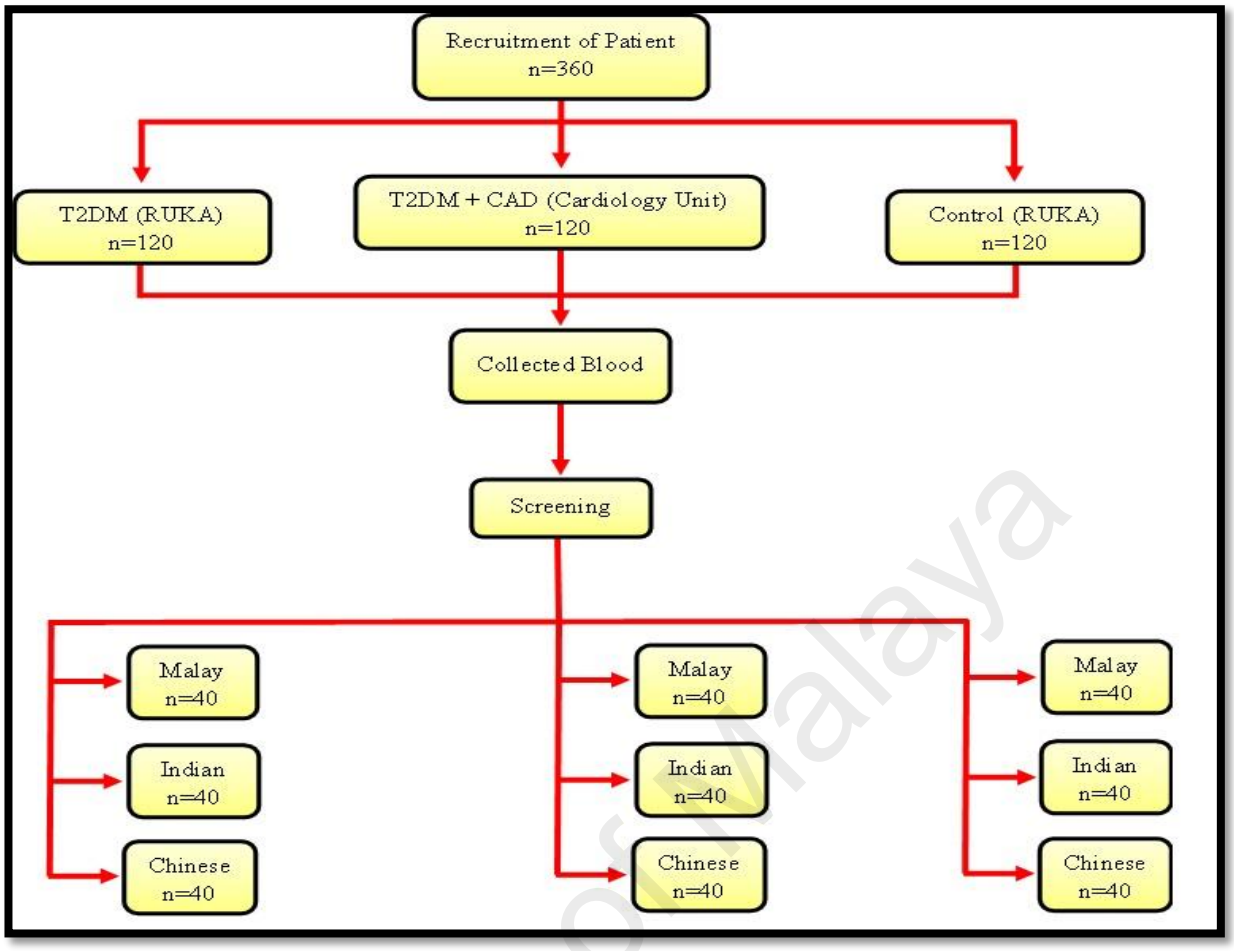

Figure 3.1: Flow chart of patient recruitment process

3.3 Anthropometry

Heights, weight, systolic and diastolic blood pressures (SBP&DBP) of the subjects' were taken from the medical folders. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (Centers for Disease Control and Prevention, 2016). Hypertension was defined as blood pressure > 140/90 mm Hg or current use of antihypertensive medications. Subjects who had smoked and drunk alcohol regularly in the previous year were considered as active smokers.

3.4 Blood Collection and Plasma Separation

Venous blood samples were obtained from the subjects by trained nurse after 8 hours of overnight fasting in vacutainers blood collection tube with and without appropriate anticoagulants. Immediately, plasma and serum from the respective vacutainers blood

collection tube were separated by centrifuging the tubes at 1000 rpm for 10 min at 4°C. Later plasma was stored at -20°C for further biochemical analysis.

3.5 Biochemical Analysis

Biochemical parameters related to T2DM and CAD were obtained at University of Malaya Medical Center by medical laboratory technologist for all subjects. Measurement of serum levels of total cholesterol (TC), triglycerides (TG), HbA1c, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), C-reactive protein (CRP) and creatinine were measured based on spectrophotometric method using automated clinical chemistry analyzer, Cobas Integra 400 plus (Roche Diagnostics, Mannheim, Germany).

3.6 DNA Isolation from Blood

Genomic DNA was extracted from whole blood using standard method by Qiagen company (Qiagen, 2012).

3.6.1 Preparation of Reagent (Buffer AL)

Buffer AL was thoroughly mix by shaking before use. Buffer AL is stable for one year when stored at room temperature.

Figure 3.2: QIAamp DNA Blood Mini Kit

3.6.2 Preparation of Reagents (Buffer AW1 and AW2)

Buffer AW1 and AW2 were supplied as a concentrated form. Ethanol (96-100%) was added appropriately as indicated on the bottle. Buffer AW1 and AW2 were stored closed at room temperature for one year.

3.6.3 Protocol of DNA Isolation from Blood

First 20 µl QIAGEN Protease were pipetted into the bottom of a 1.5 ml microcentrifuge tube. Next, 200 µl of whole blood sample were added into the QIAGEN Protease contained microcentrifuge tube. Then, 200 µl of Buffer AL were added into the sample and mix by pulse-vortexing for 15 second. These mixtures were then incubated at 56°C for approximate 10 minutes. After that, the microcentrifuge tubes were briefly centrifuged to remove the drops from the inside of the lid. Later, 200 µl of ethanol (96–100%) were added into the sample and again mix by pulse-vortexing for 15 second. After mixing, the microcentrifuge tubes were again briefly centrifuged to remove the drops from the inside of the lid. Then, carefully the centrifuged mixture was transferred to the 2 ml QIAamp Mini spin column by fine-tip transfer pipet without wetting the rim. The tube caps were closed, and centrifuged at 6000 x g (8000 rpm) for 1 minute. QIAamp Mini column was placed into a clean 2ml collection tubes and the tube containing the filtrate was discarded. This step was repeated with the QIAamp Mini column open. QIAamp Mini column were opened carefully, and 500µl of buffer AW1 were added. The tube caps were closed, and centrifuged at 6000 x g (8000 rpm) for 1 minute. QIAamp Mini column was placed into a clean 2ml collection tube and the tube containing the filtrate was discarded. The QIAamp Mini column were opened carefully, 500µl of buffer AW2 was added and centrifuged at full speed for 3 minutes. QIAamp Mini column was placed in a new 2 ml collection tube, and was centrifuged at full speed for 1 minute. The QIAamp mini column was then placed in a clean 1.5 ml

microcentrifuge tube. QIAamp was opened carefully, and 200 µl buffer AE was added, at room temperature. Close the tube cap, and the solution was incubated at room temperature for 1 minute, centrifuge at 6000 x g (8000 rpm) for 1 minute. The DNA was then transferred to a clean 1.5 ml microcentrifuge tube. These purified DNA extraction was stored at -20ºC until the PCR was performed. It is known that these extractions are stable for up to one year if it is stored at -20ºC.

3.7 Genotyping

3.7.1 *PPARγ* (Pro12Ala Polymorphism)

The PCR was carried out in a final volume of 20µl containing 0.2µl of genomic DNA, 10 µl of 1 × PCR Master Mix (Gendirex, USA), 2µl of forward and reverse primers and 7.8 µl of dH2O. After an initial denaturation of 3 min at 94°C, the samples were subjected to 35 cycles at 94°C for 30 sec, at 64°C for 30 s, and 72°C for 1 min, with a final extension of 500 s at 72°C in a mega cycler (Edvotek 542, China). The PCR products were digested by enzyme restriction Bsh1236i (Thermo Scientific) and separated in agarose gel. The allele encoding variant wild homozygote appeared as a single fragment of 270bp, variant mutant homozygote appeared as a double fragment of 227bp and 43bp and variant heterozygote appeared as triple fragment of 270bp, 227bp and 43bp.

3.7.2 *ENPP1* (K121Q Polymorphism)

The PCR was carried out in a final volume of 20µl containing 0.2µl of genomic DNA, 10 µl of 1 × PCR Master Mix (Gendirex, USA), 2µl of forward and reverse primers and 7.8 µl of dH2O. After an initial denaturation of 3 min at 94°C, the samples were subjected to 35 cycles at 94°C for 30 sec, at 62°C for 30 s, and 72°C for 1 min, with a final extension of 500 s at 72°C in a mega cycler (Edvotek 542, China). The PCR products were digested by enzyme restriction AvaII (Eco471)* (Thermo Scientific) and

separated in agarose gel. The allele encoding variant KK appeared as a single fragment of 265bp, variant KQ appeared as a double fragment of 155bp and 110bp and variant QQ appeared as triple fragment of 265bp, 155bp and 110bp.

3.7.3 *CAPN-10* (SNP-63 Polymorphism)

The PCR was carried out in a final volume of 20µl containing 0.2µl of genomic DNA, 10 µl of 1 × PCR Master Mix (Gendirex, USA), 2µl of forward and reverse primers and 7.8 µl of dH2O. After an initial denaturation of 3 min at 94°C, the samples were subjected to 35 cycles at 94°C for 30 sec, at 58°C for 30 s, and 72°C for 1 min, with a final extension of 500 s at 72°C in a mega cycler (Edvotek 542, China). The PCR products were digested by enzyme restriction HhaI (Thermo Scientific) and separated in agarose gel. The allele encoding variant CC appeared as a single fragment of 162bp variant CT appeared as a double fragment of 162bp and 192bp and the allele encoding variant TT as one fragments of 192bp each.

3.8 Statistical Analyses

The SPSS version 20 computer software was used in data analysis. Continuous variables were described as mean ± standard deviation while, categorical values were presented as frequencies and percentages. Differences between groups were tested using one-way ANOVA procedure. The Chi-square test was used in making comparisons between categorical variables Chi-square test also was used to test for deviation of *PPARγ*, *ENPP1* and *CAPN-10* genotypes distribution from Hardy-Weinberg equilibrium (HWE). The association between the selected genes as well as T2DM and CAD risk was estimated by calculating odds ratios (ORs) and 95% confidence intervals (CIs) using the logistic regression analyses. For all tests the level of significance was set as 0.05.

CHAPTER 4: RESULTS

4.1 Study Population

In this study there were 120 patients with T2DM only, 120 patients with T2DM and CAD and 120 subjects free of T2DM and CAD.

4.2 Anthropometric Parameters and Clinical Characteristics

4.2.1 Anthropometric Parameters of the Study Population

The anthropometric parameters of the 360 subjects are shown in table 4.1. There was a significant difference in age, BMI, SBP and alcohol consumption between the groups. The mean age in the T2DM+CAD group is higher compared to the other two groups and the mean age in the T2DM group is higher compared to the control group. The mean BMI in the T2DM group is significantly higher compared to the T2DM+CAD group. The mean SBP in the T2DM+CAD group is significantly higher compared to the other two groups. In terms of alcohol consumption, more subjects in the T2DM+CAD group (21.7%) consumed alcohol compared to about 11% in the other two groups.

Table 4.1: Anthropometric parameters of the subjects in study by group

Parameters	**T2DM (N=120)**	**T2DM + CAD (N=120)**	**Control (N=120)**	***p value***
Age (Years)	55.2±8.8	61.0±8.3	47.9±12.3	0.001
Weight (kg)	70. 5±13.3	68.4±11.3	68.5±10.7	0.325
Height (cm)	1.6±0.1	1.6±0.1	1.6±0.1	0.001
BMI (kg/m^2)	28.1±5.2	25.7±3. 7	26. 9±4.2	0.000
SBP (mmHg)	132.9±16.1	137.8±18.2	128.9±12.8	0.001
DBP (mmHg)	76.0±10.0	77. 8±11.8	76.8±10.7	0.459
Smoking (%)	(13) 10.8%	(18) 15.0%	(13) 10.7%	0.515
Alcohol (%)	(13) 10.8%	(26) 21.7%	(13) 10.7%	0.021

Values are mean ± SD
Notes: *The mean difference is significant at the p= <0.05.

4.2.2 Clinical Characteristics of the Study Population

The clinical characteristics of the 360 subjects are presented in table 4.2. There were significant differences in TG, TC, LDL-C, HbA1c, WBG and creatinine between the groups. The mean TG in T2DM+CAD group was lower compared to the control and T2DM groups. The mean TC values in the three groups were significantly different from each other. The mean LDL-C in the control group was significantly higher compared to the T2DM+CAD and T2DM groups. The mean HbA1c value was significantly higher in the control group compared to the T2DM+CAD and T2DM groups. TC, HDL-C and LDL-C means are higher in control group compared to T2DM group and T2DM+CAD group, these due to medication intake by the patients. The mean WBG values in the three groups were significantly different from each other. The mean creatinine in T2DM+CAD group was higher compared to the control and T2DM groups.

Table 4.2: Clinical characteristics of the subjects in study by group

Variable	**T2DM (n=120)**	**T2DM + CAD (n=120)**	**Control (n=120)**	***p value***
TG (mmol/L)	1.7±0.8	1.2±0.9	1.7±0.8	<0.001
TC (mmol/L)	4.8±1.1	4.4±1.2	5.4±1.2	<0.001
HDL-C (mmol/L)	1.4±0.8	1.3±0.7	1.5±0.7	0.134
LDL-C (mmol/L)	2.7±1.0	2.7±1.0	3.3±1.0	<0.001
HbA1c NGSP (%)	7.6±1.8	7.2±1.9	5.4±0.5	<0.001
WBG (mmol/L)	9.7±3.2	8.2±3.5	5.7±1.0	<0.001
CRP (mg/dL)	0.6±0.7	0.7±0.7	0.5±0.7	0.110
Creatinine (µmol/L)	84.1±42.5	110.3±116.9	74.9±24.7	0.001

Values are mean ± SD
Notes: *The mean difference is significant at the p= <0.05.

4.3 *PPARγ, ENPP1* and *CAPN-10* gene's genotype distributions of the subjects in study by ethnicity

For the 360 subjects in the study, the *PPARγ*, *ENPP1* and *CAPN-10* genes were genotyped using multiple GWA studies (Mtiraoui, Turki, Nemr, Echtay, Izzidi, Al-Zaben, Irani-Hakime et al., 2012; Ridderstrale & Nilsson, 2008; Wheeler & Barroso, 2011). The test for HWE and comparison of genotype and allele frequencies in the T2DM, T2DM+CAD and controls were performed using the $\chi 2$ test.

4.3.1 *PPARγ* (Pro12Ala) genotype distributions of the subjects in study by ethnicity

In the T2DM group the distribution of the observed genotypes did not deviate from the HWE expectations in all three ethnic group (P=0.984, 0.313 and 0.071 for Indian, Malay and Chinese respectively). Similarly in control group there was no deviation either (P=0.416, 0.527 and 0.537 for Indian, Malay and Chinese respectively). However, in the T2DM+CAD group there was a significance deviation from the HWE expectations in all three ethnic groups (P=0.048, 0.009 and 0.000 for Indian, Malay and Chinese respectively) (refer to Appendix E1). Table 4.3 shows the genotype and allele frequencies of the Pro12Ala polymorphism by ethnicity. For the T2DM group, there were statistically significant differences in the genotype frequency of Pro12Ala polymorphism within each ethnic group, but no statistically significance difference was observed between ethnic groups. The highest frequencies were for ProPro genotype and the lowest was for AlaAla. For the T2DM+CAD group, there were statistically significant differences in the genotype frequency of Pro12Ala polymorphism within each ethnic group, but no statistically significance difference was observed between ethnic groups. The highest frequencies were for ProPro genotype and the lowest was for AlaAla. For the control group, there were statistically significant differences in the genotype frequency of Pro12Ala polymorphism for Indians and Malays only. The highest frequencies were for ProPro genotype and the lowest was for AlaAla. In this

group there was a statically significance difference between ethnic groups and genotype frequency. Among the Chinese the highest frequency were for ProAla genotype and almost similar frequencies for ProPro and AlaAla.

Table 4.3: Frequencies of Pro12Ala genotypes within races and between races in all study subjects

		***PPARγ* Genotype**			**Within Ethnicity**	**Between Ethnicity**
Group	**Race**	ProPro	ProAla	AlaAla	***p value***	***p value***
T2DM	Indian	21 (52.5%)	16 (40.0%)	3 (7.5%)	0.002	0.070
	Malay	29 (72.5%)	11 (27.5%)	0 (0.0%)	0.004	
	Chinese	22 (55.0%)	12 (30.0%)	6 (15.0%)	0.007	
T2DM+CAD	Indian	23 (60.5%)	10 (26.3%)	5 (13.2%)	0.001	0.600
	Malay	33 (73.3%)	8 (17.8%)	4 (8.9%)	0.000	
	Chinese	27 (73.0%)	5 (13.5%)	5 (13.5%)	0.000	
Control	Indian	20 (50.0%)	16 (40.0%)	4 (10.0%)	0.006	0.021
	Malay	26 (63.4%)	10 (24.4%)	5 (12.2%)	0.000	
	Chinese	11 (27.5%)	20 (50.0%)	9 (22.5%)	0.076	

Notes: *The mean difference is significant at the p= <0.05. Value "%" indicates genotype frequency as a percentage.

In Table 4.4 the results for the comparison between T2DM and controls for Pro12Ala polymorphism genotype and the allele frequencies by ethnicity are presented. There was a statistically significant association between T2DM and controls for the allele frequencies among the Chinese. The odds of Pro allele among the Chinese with T2DM was 2.11 (95% CI 1.10, 4.04) times more compared to the control group. Even though not statically significance, the odds of ProPro genotype among Malays with T2DM was 4.46 (95% CI 0.47, 42.51) times more compared to the control group. Similarly, the odds of ProAla genotype among Malays with T2DM was 4.40 (95% CI 0.42, 46.26)

times more compared to the control group. Again even though not statically significant the odds of ProPro genotype among Chinese with T2DM was 3.00 (95% CI 0.85, 10.59) times more compared to the control group.

Table 4.4: Comparisons between T2DM and controls for Pro12Ala polymorphism genotype and the allele frequencies by ethnicity

***PPARγ* genotype**	**T2DM (%)**	**Controls (%)**	***p* -value**	**Odds ratio (95% CI)**
Indian (*n* = 40)				
ProPro	29.2 (21)	35.1(20)	0.683	1.4 (0.28-7.06)
ProAla	41.0 (16)	34.8 (16)	0.732	1.3 (0.26-6.94)
AlaAla	33.3 (3)	22.2 (4)		1
Alleles				
Pro	72.5 (58)	70 (56)	0.730	1.13 (0.57-2.24)
Ala	27.5 (22)	30 (24)		1
Malay (*n* = 40)				
ProPro	40.3 (29)	45.6(26)	0.194	4.46 (0.47-42.51)
ProAla	28.2 (11)	21.7 (10)	0.217	4.40 (0.42-46.26)
AlaAla	0 (0)	27.8 (5)		1
Alleles				
Pro	86.25 (69)	79.73 (59)	0.283	1.60 (0.68-3.74)
Ala	13.75 (11)	20.27 (15)		1
Chinese (*n* = 40)				
ProPro	30.6 (22)	19.3 (11)	0.088	3.00 (0.85-10.59)
ProAla	30.8 (12)	43.5 (20)	0.869	0.90 (0.26-3.16)
AlaAla	66.7 (6)	50.0 (9)		1
Alleles				
Pro	70 (56)	52.5 (42)	0.020	2.11 (1.10-4.04)
Ala	30 (24)	47.5 (38)		1

Notes: *The mean difference is significant at the p= <0.05. Value "%" indicates genotype frequency as a percentage.
CI=Confidence Interval; OR=Odds Ratio

In Table 4.5 the results for the comparison between T2DM+CAD and controls for Pro12Ala polymorphism genotype and the allele frequencies by ethnicity are presented. There was a statistically significant association between T2DM+CAD and controls for the genotype as well as allele frequencies among the Chinese. The odds of ProPro genotype among the Chinese with T2DM was 4.42 (95% CI 1.21, 16.19) times more compared to the control group. Likewise, the odds of Pro allele among Malays with T2DM+CAD was 3.56 (95% CI 1.74, 7.29) times more compared to the control group. Even though not statically significance, the odds of ProPro genotype among Malays

with T2DM+CAD was 1.27 (95% CI 0.29, 5.57) times more compared to the control group. Similarly, the odds of ProPro allele among Indians with T2DM+CAD was 1.20 (95% CI 0.60, 2.42) times more compared to the control group.

Table 4.5: Comparisons between T2DM+CAD and controls for Pro12Ala polymorphism genotype and the allele frequencies by ethnicity

***PPARγ* genotype**	**T2DM+CAD (%)**	**Controls (%)**	***p* Value**	**Odds ratio (95% CI)**
Indian (*n* = 40)				
ProPro	27.7 (23)	35.1(20)	0.910	0.92 (0.22-3.90)
ProAla	43.5 (10)	34.8 (16)	0.376	0.50 (0.11-2.32)
AlaAla	35.7 (5)	22.2 (4)		1
Alleles				
Pro	73.68 (56)	70 (56)	0.609	1.20 (0.60-2.42)
Ala	26.32 (20)	30 (24)		1
Malay (*n* = 40)				
ProPro	39.8 (33)	45.6(26)	0.752	1.27 (0.29-5.57)
ProAla	34.8 (8)	21.7 (10)	0.793	0.80 (0.15-4.25)
AlaAla	28.6 (4)	27.8 (5)		1
Alleles				
Pro	82.22 (74)	79.73 (59)	0.685	1.18 (0.54-2.57)
Ala	17.78 (16)	20.27 (15)		1
Chinese (*n* = 40)				
ProPro	32.5 (27)	19.3 (11)	0.025	4.42 (1.21-16.19)
ProAla	21.7 (5)	43.5 (20)	0.286	0.45 (0.10-1.95)
AlaAla	35.7(5)	50.0 (9)		1
Alleles				
Pro	79.73 (59)	52.5 (42)	0.001	3.56 (1.74-7.29)
Ala	20.27 (15)	47.5 (38)		1

Notes: *The mean difference is significant at the p= <0.05. Value "%" indicates genotype frequency as a percentage.
CI=Confidence Interval; OR=Odds Ratio

4.3.2 *ENPP1* (K121Q) genotype distributions of the subjects in study by ethnicity

The genotype frequencies of the *ENPP1* (K121Q) variants in all ethnicity with T2DM group (P=0.181, 0.228 and 0.377 for Indian, Malay and Chinese respectively), T2DM+CAD group (P=0.936, 0.502 and 0.987 for Indian, Malay and Chinese respectively) and the control group (P=0.160, 0.969 and 0.109 for Indian, Malay and Chinese respectively) were in agreement with the HWE (refer to Appendix E2). Table 3.6 shows the genotype and allele frequencies of the K121Q polymorphism by ethnicity.

For the T2DM group, there were statistically significant differences in the genotype frequency of K121Q polymorphism within each ethnic group, but no statistically significance difference was observed between ethnic groups. The highest frequencies were for KQ genotype and the lowest was for QQ. For T2DM+CAD group, there were statistically significant differences in the genotype frequency of K121Q polymorphism within each ethnic group, but no statistically significance difference was observed between the ethnic groups. The highest frequencies were for KK genotype and the lowest was for QQ. For control group, there were statistically significant differences in the genotype frequency of K121Q polymorphism within each ethnic group, but no statistically significance difference was observed between the ethnic groups. Among the Indians and Chinese, the highest frequencies were for KQ genotype, whereas among the Malays the highest was KK genotype. The lowest frequencies were for QQ in all ethnic groups.

Table 4.6: Frequencies of K121Q genotypes within and between ethnic groups

		***ENPP1* Genotype**			**Within Ethnicity**	**Between Ethnicity**
Group	**Race**	KK	KQ	QQ	***p value***	***p value***
T2DM	Indian	13 (32.5%)	23 (57.5%)	4 (10.0%)	0.001	0.767
	Malay	18 (45.0%)	20 (50.0%)	2 (5.0%)	0.001	
	Chinese	17 (42.5%)	20 (50.0%)	3 (7.5%)	0.002	
T2DM+CAD	Indian	20 (52.6%)	15 (39.5%)	3 (7.9%)	0.002	0.914
	Malay	21 (46.7%)	18 (40.0%)	6 (13.3%)	0.015	
	Chinese	19 (51.4%)	15 (40.5%)	3 (8.1%)	0.004	
Control	Indian	17 (42.5%)	21 (52.5%)	2 (5.0%)	0.001	0.657
	Malay	22 (53.7%)	16 (39.0%)	3 (7.3%)	0.001	
	Chinese	16 (40.0%)	22 (55.0%)	2 (5.0%)	0.000	

Notes: *The mean difference is significant at the p= <0.05. Value "%" indicates genotype frequency as a percentage.

In Table 4.7 the results for the comparison between T2DM and controls for K121Q polymorphism genotype and the allele frequencies by ethnicity are presented. There was no statistically significance difference between the T2DM and controls groups in all allele and genotype frequencies in all ethnic groups.

Table 4.7: Comparisons between T2DM and controls for K121Q polymorphism genotype and the allele frequencies by ethnicity

***ENPP1* genotype**	**T2DM (%)**	**Controls (%)**	***p* Value**	**Odds ratio (95%CI)**
Indian (*n* = 40)				
KK	27.1 (13)	30.9 (17)	0.307	0.38 (0.60-2.42)
KQ	36.5 (23)	35.6 (21)	0.511	0.55 (0.09-3.31)
QQ	44.4 (4)	28.6 (2)		1
Alleles				
K	61.3 (49)	68.8 (55)	0.321	0.72 (0.37-1.38)
Q	38.8 (31)	32.25 (25)		1
Malay (*n* = 40)				
KK	37.5 (18)	40.0 (22)	0.752	1.36 (0.20-9.02)
KQ	31.7 (20)	27.1 (16)	0.518	1.88 (0.28-12.61)
QQ	22.2 (2)	42.9 (3)		1
Alleles				
K	70.0 (56)	73.2 (60)	0.655	0.86 (0.43-1.70)
Q	30.0 (24)	26.8 (22)		1
Chinese (*n* = 40)				
KK	35.4 (17)	29.1 (16)	0.724	0.71 (0.10-4.81)
KQ	31.7 (20)	37.3 (22)	0.603	0.61 (0.92-4.01)
QQ	33.3 (3)	28.6 (2)		1
Alleles				
K	67.5 (54)	67.5 (54)	1.00	1.00 (0.52-1.94)
Q	32.5 (26)	32.5 (26)		1

Notes: *The mean difference is significant at the p= <0.05. Value "%" indicates genotype frequency as a percentage.
CI=Confidence Interval; OR=Odds Ratio

In Table 4.8 the results for the comparison between T2DM+CAD and controls for K121Q polymorphism genotype and the allele frequencies by ethnicity are presented. There was no statistically significance difference between the T2DM and controls groups in all allele and genotype frequencies in all ethnic groups.

Table 4.8: Comparisons between T2DM+CAD and controls for K121Q polymorphism genotype and the allele frequencies by ethnicity

***ENPP1* genotype**	**T2DM+CAD (%)**	**Controls (%)**	***p* Value**	**Odds ratio (95% CI)**
Indian (*n* = 40)				
KK	33.3 (20)	30.9 (17)	0.802	0.78 (0.12-5.26)
KQ	31.2 (15)	35.6 (21)	0.446	0.48 (0.07-3.21)
QQ	25.0 (3)	28.6 (2)		1
Alleles				
K	72.4 (55)	68.8 (55)	0.620	1.19 (0.60-2.37)
Q	27.6 (21)	32.25 (25)		1
Malay (*n* = 40)				
KK	35.0 (21)	40.0 (22)	0.369	0.50 (0.11-2.27)
KQ	37.5 (18)	27.1 (16)	0.464	0.56 (0.12-2.63)
QQ	50.0 (6)	42.9 (3)		1
Alleles				
K	66.7 (60)	73.2 (60)	0.354	0.73 (0.38-1.41)
Q	33.3 (30)	26.8 (22)		1
Chinese (*n* = 40)				
KK	31.7 (19)	29.1 (16)	0.810	0.79 (0.12-5.34)
KQ	31.2 (15)	37.3 (22)	0.417	0.46 (0.07-3.06)
QQ	25 (3)	28.6 (2)		1
Alleles				
K	71.6 (53)	67.5 (54)	0.579	1.22 (0.61-2.42)
Q	28.4 (21)	32.5 (26)		1

Notes: *The mean difference is significant at the p= <0.05. Value "%" indicates genotype frequency as a percentage.
CI=Confidence Interval; OR=Odds Ratio

4.3.3 *CAPN-10* (SNP-63) genotype distributions of the subjects in study by ethnic

The genotype frequencies of the *CAPN-10* SNP-63 variants in all subjects according HWE with T2DM (P=0.592, 0.842 and 0.117 for Indian, Malay and Chinese respectively), T2DM+CAD (P=0.363, 0.112 and 0.987 for Indian, Malay and Chinese respectively) and control (P=0.416, 0.527 and 0.537 for Indian, Malay and Chinese respectively) (refer to Appendix E3). Genotype frequencies were in HWE in all groups. Table 3.9 shows the genotype and allele frequencies of the SNP-63 polymorphism by ethnicity. For the T2DM group, there were statistically significant differences in the genotype frequency of SNP-63 polymorphism within each ethnic group, but no statistically significance difference was observed between ethnic groups. The highest frequencies were for CC genotype and the lowest was for TT. For the T2DM+CAD

group, there were statistically significant differences in the genotype frequency of SNP-63 polymorphism within each ethnic group, but no statistically significant difference was observed between ethnic groups. The highest frequencies were for CC genotype and the lowest was for TT. For the control group, there were statistically significant differences in the genotype frequency of SNP-63 polymorphism within each ethnic group, but no statistically significance difference was observed between ethnic groups. The highest frequencies were for CC genotype and the lowest was for TT.

Table 4.9: Frequencies of SNP-63 genotypes within and between ethnic groups

		***CAPN-10* Genotype**			**Within Ethnicity**	**Between Ethnicity**
Group	**Race**	CC	CT	TT	***p value***	***p value***
T2DM	Indian	21 (52.5%)	15 (37.5%)	4 (10.0%)	0.004	0.907
	Malay	22 (55.0%)	15 (37.5%)	3 (7.5%)	0.001	
	Chinese	23 (57.5%)	12 (30.0%)	5 (12.5%)	0.002	
T2DM+CAD	Indian	19 (50.0%)	14 (36.8%)	5 (13.2%)	0.019	0.875
	Malay	23 (51.1%)	15 (40.5%)	7 (15.6%)	0.014	
	Chinese	19 (51.4%)	15 (40.5%)	3 (8.1%)	0.004	
Control	Indian	20 (50.0%)	18 (45.0%)	2 (5.0%)	0.001	0.714
	Malay	20 (48.8%)	16 (39.0%)	5 (12.2%)	0.012	
	Chinese	21 (52.5%)	17 (42.5%)	2 (5.0%)	0.001	

Notes: *The mean difference is significant at the p= <0.05. Value "%" indicates genotype frequency as a percentage.

The results for the comparison between T2DM and controls for SNP-63 polymorphism genotype and the allele frequencies by ethnicity are presented in Table 4.9. There was no statistically significance association between T2DM and controls for the allele and genotype frequencies among all the races.

Table 4.10: Comparisons between T2DM and controls for SNP-63 polymorphism genotype and the allele frequencies by ethnicity

***ENPP1* genotype**	**T2DM (%)**	**Controls (%)**	***p* Value**	**Odds ratio (95% CI)**
Indian (*n* = 40)				
CC	31.8 (21)	32.8 (20)	0.484	0.53 (0.09-3.19)
CT	35.7 (15)	35.3 (18)	0.349	0.42 (0.07-2.60)
TT	33.3 (4)	22.2 (2)		1
Alleles				
C	71.3 (57)	72.5 (58)	0.860	0.94 (0.47-1.87)
T	28.8 (23)	27.5 (22)		1
Malay (*n* = 40)				
CC	33.3 (22)	32.8 (20)	0.376	2.02 (0.43-9.55)
CT	35.7 (15)	31.4 (16)	0.583	1.56 (0.32-7.70)
TT	25.0 (3)	55.6 (5)		1
Alleles				
C	73.8 (59)	68.3 (56)	0.445	1.30 (0.66-2.58)
T	26.3 (21)	31.7 (26)		1
Chinese (*n* = 40)				
CC	34.8 (23)	34.4 (21)	0.353	0.44 (0.08-2.50)
CT	28.6 (12)	33.3 (17)	0.168	0.28 (0.05-1.71)
TT	41.7 (5)	22.2 (2)		1
Alleles				
C	72.5 (58)	73.8 (59)	0.858	0.94 (0.47-1.89)
T	27.5 (22)	26.3 (21)		1

Notes: *The mean difference is significant at the p= <0.05. Value "%" indicates genotype frequency as a percentage.
CI=Confidence Interval; OR=Odds Ratio

The results for the comparison between T2DM+CAD and controls for SNP-63 polymorphism genotype and the allele frequencies by ethnicity are presented in Table 4.10. There was no statistically significant association between T2DM+CAD and controls for the allele and genotype frequencies between races.

Table 4.11: Comparisons between T2DM+CAD and controls for SNP-63 polymorphism genotype and the allele frequencies by ethnicity

***ENPP1* genotype**	**T2DM+CAD (%)**	**Controls (%)**	***p* Value**	**Odds ratio (95% CI)**
Indian (*n* = 40)				
CC	31.1 (19)	32.8 (20)	0.280	0.38 (0.07-2.20)
CT	31.8 (14)	35.3 (18)	0.199	0.31 (0.05-1.85)
TT	33.3 (5)	22.2 (2)		1
Alleles				
C	68.4 (52)	72.5 (58)	0.577	0.82 (0.41-1.64)
T	31.6 (24)	27.5 (22)		1
Malay (*n* = 40)				
CC	37.7 (23)	32.8 (20)	0.826	0.87 (0.24-3.17)
CT	34.1 (15)	31.4 (16)	0.559	0.67 (0.17-2.57)
TT	46.7 (7)	55.6 (5)		1
Alleles				
C	67.8 (61)	68.3 (56)	0.233	0.51 (0.28-0.91)
T	32.2 (56)	31.7 (26)		1
Chinese (*n* = 40)				
CC	31.1 (19)	34.4 (21)	0.601	0.60 (0.09-4.00)
CT	34.1 (15)	33.3 (17)	0.588	0.59 (0.09-4.00)
TT	20.0 (3)	22.2 (2)		1
Alleles				
C	71.6 (53)	73.8 (59)	0.767	0.90 (0.44-1.83)
T	28.4 (21)	26.3 (21)		1

Notes: *The mean difference is significant at the p= <0.05. Value "%" indicates genotype frequency as a percentage.
CI=Confidence Interval; OR=Odds Ratio

In Table 4.11 the results for the comparison between T2DM+CAD and controls for SNP-63 polymorphism genotype and the allele frequencies by ethnicity are presented. There was no statistically significance association between T2DM+CAD and controls for the allele and genotype frequencies among all the races.

CHAPTER 5: DISCUSSION

Type 2 diabetes mellitus and coronary artery disease are well-established multifactorial disorders that are strongly associated with genetic as well as lifestyle and environmental factors. Both genetic and environmental factors affect T2DM along with CAD. *PPAR-γ* (Pro12Ala), *ENPP1* (K121Q) and *CAPN-10* (SNP-63) are three most common variants which are mostly expressed in adipose tissue. The active roles of *PPAR-γ*, *ENPP1* and *CAPN-10* in glucose and lipid are well documented. Previous studies have reported that Pro12Ala polymorphism of the *PPAR-γ*, K121Q polymorphism of *ENPP1* and SNP-63 polymorphism of *CAPN-10* genes are implicated in T2DM in various ethnic populations of the world (N. Abate, M. Chandalia, P. Satija, B. Adams-Huet, S. M. Grundy, S. Sandeep, V. Radha et al., 2005; Kifagi, Makni, Mnif, Boudawara, Hamza, Rekik, Abid et al., 2008; Radha, Vimaleswaran, Babu, Abate, Chandalia, Satija, Grundy et al., 2006).

This study is the first of this kind carried out in Malaysian population to determine the association between these three selected SNP in T2DM and CAD patients. Malaysia consists of multiple ethnic groups and the three largest groups are Indian, Malay and Chinese. This study was designed to improve the understanding of association between the three selected polymorphism with T2DM as well as CAD and its possible role in the progression of the disease using polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP).

The findings in this study showed high frequency of genotype ProPro in all compared groups except for the control group from the Chinese ethnicity. The AlaAla genotype appeared with low frequency in all three ethnic groups. In the control group, among the Chinese, the highest frequencies were observed for the ProAla genotype. In this study, the frequencies of the Pro allele in all races with both T2DM and

T2DM+CAD group were higher compared to control group. The odds of Pro allele among all races with T2DM and T2DM+CAD were higher compared to the control group. Among the Chinese, the odds of Pro allele among the T2DM was higher compared to the control group (OR=2.11, 95% CI 1.10, 4.04). Also among the Chinese, the odds of Pro allele among those with T2DM+CAD was higher compared to the control group (OR=3.56, 95% CI 1.74, 7.29). Ala allele of the *PPAR-γ* polymorphism was low compared to the control among all races with T2DM and T2DM+CAD groups. In these two groups the frequency was higher among the Chinese compared to Indians and Malays.

This study demonstrated that the Pro risk allele is a risk factor for T2DM and the development of CAD in Chinese population. The results from our study are in concordance with a previous study on 1417 T2DM Chinese population in Hong Kong, which reported that Pro allele increased risk of developing both T2DM and CAD (Ho et al., 2012). Even though T2DM and CAD have common genetic basis, most of the studies found that Pro allele is significantly associated with T2DM but not with CAD (Dallongeville, Iribarren, Ferrières, Lyon, Evans, Go, Arveiler et al., 2009; Pischon et al., 2005; Z. Wu, Lou, Jin, Liu, Lu, & Lu, 2012). In a 10 year follow up study, Regieli et al (Regieli, Jukema, Doevendans, Zwinderman, van der Graaf, Kastelein, & Grobbee, 2009) found an association between Pro allele and increased risk of cardiovascular mortality in 679 CAD patients. A study in Germany with 622 individuals showed that Pro allele was more susceptible to atherosclerosis by increasing the intima-media thickness (IMT) (Temelkova-Kurktschiev, Hanefeld, Chinetti, Zawadzki, Haulon, Kubaszek, Koehler et al., 2004). A cell culture study in 2009 showed that ligand-activated PPARG reduced inflammation in cardiovascular cells (Hamblin, Chang, Fan, Zhang, & Chen, 2008), and that treatment with PPAR agonists (TZDs) improved insulin

sensitivity. There is no risk association of the Pro allele in Indian and Malay populations.

Findings from this study also suggest that minor Ala allele, when high in frequency, may play a protective role in the pathophysiology of T2DM in Chinese population. This finding is similar to that of several studies conducted in different populations of the world (Douglas, Erdos, Watanabe, Braun, Johnston, Oeth, Mohlke et al., 2001; Kawasaki et al., 2002; Pattanayak, Bankura, Balmiki, Das, Chowdhury, & Das, 2014; Sanghera et al., 2008; Tripathi et al., 2013). Several studies conducted in different populations concluded that the Ala allele at codon 12 of *PPARG* gene plays a protective role in T2DM patients (Altshuler et al., 2000; Deeb et al., 1998; Frederiksen, Brødbæk, Fenger, Jørgensen, Borch-Johnsen, Madsbad, & Urhammer, 2002; Ridker et al., 2003; Yen et al., 1997). However, studies conducted in populations like South Indian and Canadian Oji-Cree reported that the Ala allele is associated with a higher risk of T2DM and indicated that the allele does not have any protective effects (Hegele, Cao, Harris, Zinman, Hanley, & Anderson, 2000; Radha et al., 2006). A meta-analysis study by Altshuler *et al* (2000) (Altshuler et al., 2000) reported that the presence of Ala allele reduces the onset of T2DM by as much as 20%. Our results demonstrate that the Ala allele of the *PPARγ* gene has no significant association with T2DM in Malay and Indian populations.

Findings from this study suggest that high frequency of minor Ala allele may play a protective role in the pathophysiology of CAD in Chinese population. A case-control study (Ridker et al., 2003) of incident myocardial infarction among 2615 healthy men followed for 13.2 years in the Physicians' Health Study observed a protective effect of the Ala allele (OR=0.79, 95% CI 0.63, 0.99). A cohort study in a Chinese population reported that Ala allele is protective against T2DM but increases the

risk to myocardial infarction (L. Li, Cheng, Nsenga, He, & Wu, 2006). There is no relationship between Ala allele and CAD in Malay and Indian population. The inconsistent findings on the role of Ala allele on T2DM and CAD risk may be due to genetic origins and/or environmental variations.

In this study we observed high frequency of KQ genotype among T2DM group in all ethnicity. On the other hand, in the T2DM+CAD group, KK genotype appeared with high frequency in all ethnicity compared to other genotypes. In the control group, among the Indians and Chinese KQ genotype was higher in frequencies while among the Malays KK genotype was higher. In the T2DM group, Malays had the highest frequency for K allele (70.0%) while in the T2DM+CAD group, Indians had the highest frequency for K allele (72.4%). Among the Indians and Malays the odds of K allele were lower in the T2DM group compared to control group. Similarly, among the Malays the odds of K allele were lower in the T2DM+CAD group compared to control group. Also among the Chinese, the odds of K allele among those with T2DM+CAD was higher compared to the control group (OR=1.22, 95% CI 0.61, 2.42).

ENPP1 gene is known to be a susceptible gene, because of its affinity to bind with alpha subunit of insulin receptor and may inhibit the tyrosine kinase activity which is essential for glucose metabolism (B. A. Maddux & Goldfine, 2000). The Q allele variant binds more strongly to the insulin receptor and inhibits its protein kinase activity more effectively than the K allele (Costanzo, Trischitta, Di Paola, Spampinato, Pizzuti, Vigneri, & Frittitta, 2001). Even though, the role of genetic polymorphism of *ENPP1* gene in susceptibility to T2DM has been widely studied, the results are inconsistent in different populations (Bottcher et al., 2006; El Achhab, Meyre, Bouatia-Naji, Berraho, Deweirder, Vatin, Delplanque et al., 2009; Grarup, Urhammer, Ek, Albrechtsen, Glümer, Borch-Johnsen, Jørgensen et al., 2006; Hamaguchi, Terao, Kusuda, Yamashita,

Hazoury Bahles, Cruz, Brugal et al., 2004; Jing, Xueyao, & Linong, 2012; Y.-y. Li, 2012; McAteer et al., 2008). However, the results in this study showed no association between minor Q allele and insulin resistance.

A study in Taiwan reported Q allele frequency of 18.8%, which is lower compared to our study (Miao P. C. et al., 2006). Another study in the South Asian Indian population reported Q allele frequency of 27.5 to 34.2%, similar to findings in our study (Abate et al., 2003; N. Abate et al., 2005).

High frequency of Q allele is likely to play a modulating role in the development of both T2DM and its cardiovascular complications compared to K allele (Moehlecke et al., 2010). In this study, it was shown that Q allele was not associated with T2DM+CAD group compared to control group, in all the three races. Our findings suggest that the common Q allele does not predispose to a rapid progression of atherosclerosis that leads to CAD. A study among Chinese population of Han origin concluded that Q allele does not increase the risk level of CAD or ischemic cerebrovascular disease in T2DM subjects (Miao P. C. et al., 2006). The study of racial and ethnic differences in diseases and the detection of risk factor levels must be based on the interaction of genetic attributes. Several studies conducted in different populations concluded that the K121Q of *ENPP1* gene is not associated with an earlier onset of myocardial infarction in diabetic subjects (Eller, Hochegger, Feuchtner, Zitt, Tancevski, Ritsch, Kronenberg et al., 2008; Jeong, Lee, Kim, Cho, & Kim, 2010; Miao P. C. et al., 2006; Moehlecke et al., 2010).

In our study, in the T2DM and T2DM+CAD groups, CC genotype was higher in frequencies while, TT genotype was lower in frequencies compared to control group, for all ethnicity. Frequencies of C allele among Malays in the T2DM and T2DM+CAD groups were higher compared to control group. On the other hand, among Indians and

Chinese with T2DM and T2DM+CAD groups the frequencies of C allele were lower compared to control group. In T2DM group the T allele frequencies were high among Indians and Chinese compared to control group. In T2DM+CAD group T allele frequency was high among Indians and Malays but lower among Chinese compared to control group. The odds of C allele among the Malays in T2DM group was higher while among the Indians and Chinese it was lower compared to the control group.

Our study demonstrated that the presence of the T allele was not associated with increased risk of T2DM, in all three ethnicities. This observation is similar with the results from Uma Jyothi et al (2013) and Dhanasekaran Bodhini et al (2010), where it was reported that T allele is not a risk factor for increased T2DM in South Indian population (Bodhini, Radha, Ghosh, Sanapala, Majumder, Rao, & Mohan; Kommoju, Maruda, Kadarkarai Samy, Irgam, Kotla, & Reddy, 2014). Another study by Kang et al (2006) reported that there is no association between T allele of SNP-63 and T2DM in the Korean population (Kang, Kim, Nam, Nam, Ahn, Cha, & Lee, 2006). Several other studies, conducted in different populations, also concluded that the single SNP-63 of *CAPN-10* gene is not associated with increased risk of T2DM (Orho-Melander et al., 2002; B. Wu et al., 2005; Zaharna, Abed, & Sharif, 2010). Frequencies of T allele in our study were very low compared to previous studies conducted in Asian populations (Song et al., 2004).

The high frequency of C allele that was observed in T2DM among Malays is similar to some previous studies conducted in several other populations like in Hyderabad (Evans et al., 2001), Tunisians (Kifagi et al., 2008), Japanese (Iwasaki, Horikawa, Tsuchiya, Kitamura, Nakamura, Tanizawa, Oka et al., 2005) and Oji-Cree Indians (Hegele, Harris, Zinman, Hanley, & Cao, 2001).

Findings in this study provide evidence of genetic link between atherosclerosis and diabetes. Previous studies have shown that T2DM and CAD share common genetic determinants that were identified in several populations (Roberts, 2014). Our result is similar to the study by Shu-feng et al (2009) conducted among Han Chinese population (Shu-feng, 2009). In their study Shu-feng et al concluded that T allele is a probable risk factor for increased dyslipidemia that can lead to CAD. Based on a study by Kang et al (2006), T allele is not associated with increased risk of metabolic syndrome such as cardiovascular disease, in Korean T2DM patients (Kang et al., 2006).

CHAPTER 6: CONCLUSION

The study of ethnic differences in T2DM with CAD and the detection of risk factors must consider lifestyle and possible genetic attributes and the interaction between the two. Differences in diet, physical activity, age, gender, nutrition status, and personal habits all have influence on the prevalence and susceptibility of both T2DM and CAD.

The findings in this study did not show any association between Pro12Ala of PPARG gene in CAD or T2DM among the Indians and Malays. However there was an association between Pro12Ala of PPARG gene and CAD as well as T2DM among the Chinese. The finding indicates that the *ENPP1* gene K121Q polymorphism is not a risk factor for T2DM and CAD, in all three ethnicity.

Based on the findings in this study, there is no association between *CAPN-10* of SNP-63 polymorphism and genetic susceptibility for T2DM as well as T2DM with CAD, in all three ethnicity.

Further studies on *PPARγ*, *ENPP1* and *CAPN-10* gene with large samples are needed to confirm the association as well as pathogenesis of T2DM and CAD.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

1. **Darishiani Paramasivam**, Sher Zaman Safi, Rajes Qvist, Imran Bin Zainal Abidin, Noran Naqiah Mohd Hairi, Karuthan Chinna. "Role of PPARG (Pro12Ala) in Malaysian type 2 diabetes mellitus patients" (*International Journal of Diabetes in Developing Countries*, ISI Indexed).
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