ASSOCIATION BETWEEN CHINESE MEDICINE BODY CONSTITUTION AND VARIANTS IN METABOLIC GENE (rs1501299 & rs1801282)

By

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ABSTRACT

ASSOCIATION BETWEEN CHINESE MEDICINE BODY CONSTITUTION AND VARIANTS IN METABOLIC GENE (rs1501299 & rs1801282)

Fong Yu Qi

Chinese Medicine Body Constitution (CMBC) classifies people into nine types based on physical, mental features and different disease susceptibility. Early classification of CMBC may prevent disease onset by providing prophylactic treatment, especially for metabolic disorder which is highly associated with lifestyle factors. Metabolic disorders are a group of diseases with abnormal biochemical pathway. There is a high risk of metabolic disorder development among Malaysian population, which evaluated by prevalence of metabolic syndrome (43.4%). This study was to investigate the association between specific CMBC types and risk of metabolic disorder development, which measured using metabolic risk factors (MRFs) and metabolic gene variants. A total of 85 subjects had been recruited and classification of CMBC type was assessed using CMBC questionnaire. Blood sampling had been carried out and DNA was extracted for genotyping of metabolic gene variant rs1501299 and rs1801282. MRFs including fasting blood glucose (FBG), total cholesterol level, blood pressure and waist circumference were measured. SPSS 22.0 was used for data analysis. Gentleness showed as the highest prevalence while Yin deficiency

and Qi deficiency were the predominant biased CMBC type in population studied. The prevalence of MRFs found to be 8.3% (prediabetes), 15.3% (central obesity), 12.9% (hypertension) and 41.2% (hypercholesterolemia). The rs1501299 was a significant variant while rs1801282 was an insignificant variant among Malaysian population. Hypertension and Log_{10}FBG were found associated with CMBC type at 90% significant level. Hypertension was positively associated with Yin deficiency while Yang deficiency showed no correlation with prediabetes. There was no significant difference among genotypes of rs1501299 and rs1801282 with CMBC types, which may not be a conclusive finding due to small sample size. Larger sample size and involvement of more MRFs are suggested in further study. In conclusion, CMBC may be used as a significant tool in assessing the predisposition towards certain diseases.

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Lastly, I am extremely grateful to my beloved family members for their unlimited love, understanding and caring to me throughout this study.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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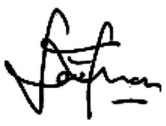
Fong Yu Qi

APPROVAL SHEET

This project report entitled "**ASSOCIATION BETWEEN CHINESE MEDICINE BODY CONSTITUTION AND VARIANTS IN METABOLIC GENE (rs1501299 & rs1801282)**" was prepared by FONG YU QI and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:



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PERMISSION SHEET

It is hereby certified that FONG YU QI (ID No: 19ADB01092) has completed this final year project report entitled "ASSOCIATION BETWEEN CHINESE MEDICINE BODY CONSTITUTION AND VARIANTS IN METABOLIC GENE (rs1501299 & rs1801282)" under the supervision of Dr. Teh Lai Kuan from the Department of Allied Health Sciences, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project report in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(FONG YU QI)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ARMS	Amplification refractory mutation system
bp	Base pair
BP	Blood pressure
CMBC	Chinese body constitution
CCMQ	Constitution of Chinese Medicine Questionnaire
CDC	Centers for Disease Control and Prevention
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DNA	Deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EDTA	Ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
FBG	Fasting blood glucose
HDL-C	High density lipoprotein cholesterol
HTN	Hypertension
IDF	International Diabetes Federation
JIS	Joint Interim Statement
JIS	Joint interim statement
M	Molar
MAPK	Mitogen activated protein kinase
MetS	Metabolic syndrome
MRFs	Metabolic risk factors
NCBI	National Center for Biotechnology Information

NCEP-ATP III	National Cholesterol Education Program Adult Treatment Panel III
NO	Nitric oxide
PCR	Polymerase chain reaction
PDK1	3-phosphoinositide-dependent protein kinase 1
PI3K	phosphoinositide 3-kinase
PPAR*y*	Peroxisome proliferator-associated receptor gamma
SBP	Systolic blood pressure
sdH2O	Sterile distilled water
SNP	Single nucleotide polymorphism
TAE	Tris base-Acetic acid-EDTA
TC	Total cholesterol
TCM	Traditional chinese medicine
TE	Tris-EDTA
UV	ultravioloet
WC	Waist circumference
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Traditional Chinese Medicine (TCM) has been categorised as alternative and complementary medicine in which diagnosis is based on a syndrome presented by an individual (World Health Organization, 2014a). One of the fundamental concepts in TCM is Chinese medicine body constitution (CMBC) which classifies an individual based on various physical and mental features to assess overall health status. Based on the classification of CMBC, it is then applied as diagnostic and individualised treatment guidelines. In China, many studies have been conducted to study the associations between CMBC type and diseases including diabetes mellitus (DM), cardiovascular disease (CVD) as well as individual metabolic risk factors (MRFs). It had been proposed that MRFs can be relieved after CMBC evaluation, on condition that specific prevention measures designed based on CMBC types had been practiced in daily life. However, there is a lack of study being conducted in Malaysia (Chong et al., 2018; Yap et al., 2021).

Metabolic disorder has become a worldwide challenge in human health. It is defined as abnormality in performance of biochemical pathways (Thong and Yunus, 2008). Among various metabolic disorders, CVD with abnormalities in heart and blood vessels may progress to ischemic heart disease. This is one of the top diseases causing a high fatality rate of up to 17% of total death among the Malaysian population as reported in 2020 (Department of Statistics Malaysia,

2021). Meanwhile, DM with impairment in insulin secretion or responses has been ranked as 9th global leading cause of death and the prevalence rate in Malaysian adults is up to 18.3% (National Institute of Health, 2020; World Health Organization, 2014).

Apart from high fatality rate, chronic metabolic disorder may lead to various unignorable complications. Patients with chronic metabolic disorder may suffer long-term burdens physically due to chronic pain in different body parts; as well as mental burden due to uncontrollable emotions and social problems as the disease causes low working performance (Kalra et al., 2018). In 2019, MOH spent approximately RM 9 billion in chronic disease management, including multiple diagnostic tests and periodically medical examinations. This will bring a high economic burden to the country if the situation continuously gets worse (World Health Organization, 2020). Thus, there is indeed a measure to prevent this disease at an early stage.

Metabolic disorders can be resulted by unhealthy lifestyle, dietary uptake as well as genetic factors. Recently, studies on genetic susceptibility toward disease development of metabolic disorders emerged after completion of the Human Genome Project in 2013. Earlier study had been focussed into the genetic variant in adiponectin (rs1501299) and peroxisome proliferator-associated receptor gamma (PPARγ) (rs1801282) which found to be correlated with chronic metabolic disorders and MRFs, in which the group of dysmetabolic phenotypes was found carry interlinked pathogenesis toward common metabolic disorders including CVD, DM and stroke (American Heart Association, n.d.)

1.1 Problem Statement

There are 35.8% of Malaysian adults are reported to have metabolic syndrome, based on the average definition of National Cholesterol Education Programme Adult Treatment Panel III (NCEP-ATP III), International Diabetes Federation (IDF) and Joint Interim Statement (JIS). However, the complete measurement of multiple MRFs is not elucidated as this is a time-consuming and costly procedure. CMBC classification can be an alternative measurement as well as free and rapid tool for assessing the predisposition of an individual in the development of metabolic disorder. An early recognition of metabolic disorder development will be able to provide prophylactic treatment and thus prevent disease development, as chronic metabolic disorders such as T2DM are found to be preventable or manageable via proper lifestyle management (Asif, 2014). Hence, this has driven to the attention of this study to investigate the problem statement on how the association between CMBC types and MRFs and metabolic gene variants would be as well as to evaluate the ability of CMBC type classification in assessing the risk of metabolic disorder development.

1.2 Significance of the Study

CMBC classification has been used in screening and diagnostic of sub-health state before biological disease onset as being widely applied in China. This would be important in providing prophylactic treatment before disease onset, especially in prevention of chronic metabolic disorders. In this epidemiology study, the susceptibility of metabolic disorder in different CMBC types has been assessed according to metabolic gene variants (rs1501299 and rs1801282) and

four metabolic risks factors, which are fasting blood glucose (FBG), blood pressure (BP), total cholesterol level (TCO) and waist circumference (WC).

1.3 Objective of the Study

The general objective of this study was:

To investigate the association of all CMBC types with MRFs and the variants in metabolic genes (rs1501299 & rs1801282) among Malaysian adults.

The specific objectives of this study were:

- To investigate the prevalence of each CMBC types among Malaysian adults between 18-35 years old.
- To study the prevalence of metabolic risk factors among Malaysian adults between 18-35 years old.
- To assess the association of CMBC types with the metabolic risk factors (FBG, BP, WC, TCO).
- To investigate the association of CMBC types and adiponectin gene variants rs1501299.
- To study the association of CMBC types and PPAR*y* gene variants rs1801282.

CHAPTER 2

LITERATURE REVIEW

2.1 Chinese Medicine Body Constitution (CMBC)

TCM proposed that a harmony relationship between human and natural is essential for health maintenance, which represents the adaptability of a human toward external environment mentally and physically (Chan and Chien, 2013). *The Yellow Emperor's Inner Classic*, the oldest Chinese medical texts that build up the origin of TCM proposed concept of human classification based on different characteristics and adaptability toward environmental factors like Yin Yang (阴阳) and Wu Xing (五行) around 2600 BC (Marshall, 2020).

Through various TCM research which involves interdisciplinary between epidemiology, molecular biology, genetics, immunology, and mathematics statistic, "Constitution Rule of Nine" had been proposed by Professor Wang Qi and published by China Association of Chinese Medicine in 2009, together with Constitution in Chinese Medicine Questionnaire (CCMQ) for systematic CMBC classification (Wang, 2009). Besides gentleness (平和), there are eight unbalanced or biased body constitutions among "Constitution Rule of Nine", which are Yin-deficiency (阴虚 Yin Xu), Yang-deficiency (阳虚 Yang Xu), Qi-deficiency (气虚 Qi Xu), phlegm-dampness (痰湿 Tan Shi), dampness-heat (湿

热 Shi Re), blood stasis (血瘀 Xue Yu), Qi-stagnation (气郁 Qi Yu), and special diathesis (特禀 Te Bing).

According to Professor Wang, comprehensive definition of CMBC is an integrated specialty of morphology, physiological and psychological conditions of individuals. Different types of body constitution were shaped on ground of congenital and acquired endowments during life, which in turn define the predisposition of an individual towards specific pathogenic factors. Among congenital endowment, ethnicity, gender, and genetic factors had been involved. Meanwhile, acquired factors such as dietary habits, climate and stress may lead to transformation of CMBC in an individual, which becomes the target of prophylactic treatment in TCM to alleviate disease susceptibility (Sun et al., 2018).

2.1.1 Characteristic of each CMBC Type

Among nine CMBC types, people with gentleness type show Yin Yang balance which indicate a well adaptability of physiological and psychological conditions towards external environment. The features of gentleness CMBC type are energetic and have a moderate body conformation. Meanwhile, people with Yang deficiency and Yin-deficiency will show a low adaptability as well as high sensitivity towards cold and hot weather, respectively (Sun et al., 2018). According to TCM theory, Yang deficiency imply dysfunction of heat-

producing organ while Yin deficiency show a retention in fluid which lead to accumulation of internal heat within body (Chan and Chien, 2013).

One of the unique components in TCM concept is Qi (气), which is defined as a vital energy to sustain life activity by maintaining proper functioning of body organs (Yao et al., 2013). Qi is closely related to circulation of Xue (血, equivalent as blood in Western medicine) in transportation of nutritious substances within body (Yao et al., 2013). People with Qi deficiency is characterized by a low vitality, frequent lethargic and shortness of breath due to lack of energy in performing physical activity (Sun et al., 2018). On the other hand, people with Qi stagnation indicate an impaired Qi circulation among organ and visceral (Zang and Fu, 脏腑) under condition of abnormal essence-spirit and emotion, such as high level of stress and anxiety (Wang et al., 2012). Similarly, blood stasis shows an obstructed blood circulation within body and presented with the morphology of dark lip colour and dark complexion (Lin et al., 2012).

In other words, phlegm dampness is formed by defect in Qi, Xue and Yin which contribute to formation and accumulation of phlegm and dampness (Ma et al., 2021). People with phlegm dampness often show abnormal lipid metabolism with prevalence of overweight (Ma et al., 2021). Meanwhile, people who have excessive water intake or humid environments in long term may lead to abnormal water metabolism, which lead to dampness and heat accumulated inside body, thus contribute to formation of dampness heat constitution (You et al., 2017). Feature of people with dampness-heat including heavy feeling and

impatient. Meanwhile, special diathesis types indicate people presented with allergic or physical defect in congenital (Sun et al., 2018).

2.1.2 Application of CMBC and its Scientific Development

Completion of the Human Genome Project in 2003 had instigated a remarkable transformation in medical genetics by providing an exhaustive insight in human genetic variations (de Vargas, 2002). Instead of existing "one-drug-fits-all" medicine, biomedical scientists shifted their focuses on individualized or personalized medicine which is prescribed based on actionable gene variants (de Vargas, 2002). Concept of individualized medicine coincided with "patient-based models" in CMBC theory in TCM. In TCM, different treatments may be provided to the patients with the same disease which are designed according to their physique (Li et al., 2019). Besides, lots of studies had been carried out in determination of genetic profile in each CMBC type to provide scientific evidence for application of TCM in complementary to western medicine including biomarker, DNA methylation and single nucleotide polymorphism (SNP) (Chen et al., 2016; Wu et al., 2010; Yao et al., 2018). These scientific evidences had increased the reliability of CMBC in the involvement of current medical diagnosis and treatment.

Other than that, reductionism virtue in current western medicine with concept of tracing root of disease and focused on treating signs and symptoms said to be highly effective in treating acute disease but incompetent for prevention of disease recurrence and chronic metabolic disorders which are multifactorial with

maladaptive symptoms (van der Greef et al., 2010). In contrast, TCM is defined as a holistic medicine that formulate health and disease condition as a whole instead of particular symptoms, which may be more effective for chronic disease prevention and management in compared with western medicine (van der Greef et al., 2010). For example, metabolic syndrome (MetS) had been classified as sub-health state, which characterized as declined in some physiological characteristics without definite pathogenic appearance and attention in medical examination (Li et al., 2013).

2.1.3 CMBC Types in relation to Risk of Metabolic Disorder Development

Until 2012, 429 diseases had been linked with different TCM patterns by various studies (Lu et al., 2012). Among these diseases, association between metabolic disorders and various types of CMBC had been discovered without consistent conclusion. For example, T2DM was found to be biased in Yin deficiency, Yang deficiency, phlegm dampness and dampness-heat CMBC pattern in different studies (Dang et al., 2020; You et al., 2017). Meanwhile, Qi-deficiency found to be associated with cardiovascular disease among all CMBC types, which are contradictory to concept that individual with blood stasis constitution may experience higher risk of blood stasis syndrome that are closely related to cardiovascular disease (Huang et al., 2014; Zhu et al., 2017). Nevertheless, the susceptible SNPs paraoxonase 2 (PON2*)* found in people with blood stasis constitution may associate with development of atherosclerosis, as reported by Huang et al. in 2005.

It is worth mentioning that the implication of HTN had been linked with different CMBC patterns in different populations which implied the ethnic or geographical issue on disease-linked CMBC types (Dang et al., 2018). For instance, kidney Yin-deficiency found to be related to prevalence of menopause-related symptoms among Han Chinese population while same symptoms found to be correlated with kidney Yang-deficiency among German population (Rampp et al., 2008).

2.1.4 Gene Predisposition of Metabolic Disorder in CMBC Types

Study carried out by Wu et al. in 2010 showed that several metabolic gene variants become an independent factor of four investigated CMBC types. The PPARy ProAla studied in this project had been found significantly associated with Yin deficiency while APM1 rs1501299 failed to be associated with gentleness, Yin deficiency, Yang deficiency and phlegm dampness CMBC (Wu et al., 2010). Creation of specific genetic profiles for each CMBC type is said to be one of translational medicine which provides an alternative pathway between research findings into the clinical health care system (Wang et al., 2015).

2.2 Constitution in Chinese medicine questionnaire (CCMQ)

CCMQ is a set of self-evaluated questionnaire, consisting of a total of 60 physical and emotional conditions which are specific for each CMBC type. In contrast with the traditional impression of TCM as subjective clinical practice, implementation of CCMQ become an objective, consistent and standardized evaluation system conformed to essential requirements in scientific research

(Lin et al., 2012). The reliability and validity of CCBQ had been verified through a large-scale survey with 2,500 participants from five districts of China (Zhu, 2007). Thus, CCMQ was adopted into this study to examine the body constitution type of participants.

2.3 Metabolic Syndrome (MetS)

Metabolic Syndrome has been defined as a clustering of clinical manifestations which indicate an increased risk of chronic metabolic disorders development, such as T2DM, atherosclerotic and CVD (Huang, 2009). Insulin resistance has been considered as a central role in progression of CVD development. FBG, WC, BP, triglyceride, and high-density lipoprotein-cholesterol have been used in evaluation of MetS (Huang, 2009). Each clinical manifestation has been linked with different dysmetabolic phenotypes which may contribute to development of metabolic disorder. However, there are some limitations in evaluating MetS as an overall and comprehensive risk assessment. For example, hereditary factors, family history and some other significant risks factors including TC and smoking habitat are not included in MetS (Huang, 2009).

2.3.1 Prevalence of MetS

Until 2013, the latest prevalence rates of MetS in Malaysian were 26.5%, 37.4% and 43.4% with definition in NCEP-ATP III, IDF and JIS, respectively (Ramli et al., 2013). In this project, the prevalence rate of JIS had been applied due to higher levels of agreement with the Kappa index of 0.867 among all definitions as well as a modified threshold for Asian in WC measurements (Ramli et al.,

2013). The epidemiology data reported by Ramli and co-workers in 2013 showed an average of 35.7% prevalence in MetS, indicating high risk of CVD development among Malaysian adults. Individuals with MetS were found to be experiencing a 2-fold increase in CVD development, a 5-fold increase in T2DM development as well as a 3-fold increase in stroke developments.

2.4 Pathogenesis of Metabolic Risk Factors

2.4.1 Hyperglycaemia and Insulin Resistance

Insulin is an enzyme secreted by β-cell of islets of Langerhans in pancreas upon stimulation of hyperglycaemia and lower blood sugar level via several actions such as cellular glucose uptake and glycogenesis (Wilcox, 2005). Insulin resistance is described as insensitive in pancreatic cells towards insulin and consequently lead to high blood sugar level (Wilcox, 2005).

Insulin action is started from binding of insulin on α-subunit in the extracellular domain of glycoprotein receptors, which in turn undergo conformational changes and allow binding of ATP to β-subunit. This action will trigger phosphorylation of β-subunit in the intracellular domain of the glycoprotein receptor. Subsequently, downstream signalling of insulin action including phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signalling pathways will be activated. Through phosphorylation of serine and threonine kinase, activation of PI3K leads to endothelial nitric oxide synthase **(**eNOS) activation in endothelial cells while GLUT4, a glucose transporter, has been activated and recruited to cell surfaces in skeletal muscle

and adipose tissue. In the MAPK pathway, mediation of mitogenic effects on smooth muscle cells in vascular wall via ET-1 production had been carried out after activation of transcription factor (Petersen and Shulman, 2018).

Defects on insulin receptor expression and post-receptor signalling pathway had been accounted for by the onset of insulin resistance, which caused a right shift in insulin dose-response curve and decreased maximal response, respectively (Petersen and Shulman, 2018). Inhibition of PI3K pathway contributes to reduced production of NO and GLUT4 translocation which in turn lead to endothelial dysfunction and decreased glucose uptake, respectively (Choi et al., 2014). Moreover, affected PI3K and uninfluenced MAPK pathway lead to imbalance in vascular homeostasis due to reduce in production of vasodilator nitric oxide with normal level of vasoconstrictor ET-1, as shown in Figure 2.1 (Choi et al., 2014). Consequently, vasoconstriction and proliferation in vascular smooth muscle are manifested in insulin resistance patients (Janus et al., 2016).

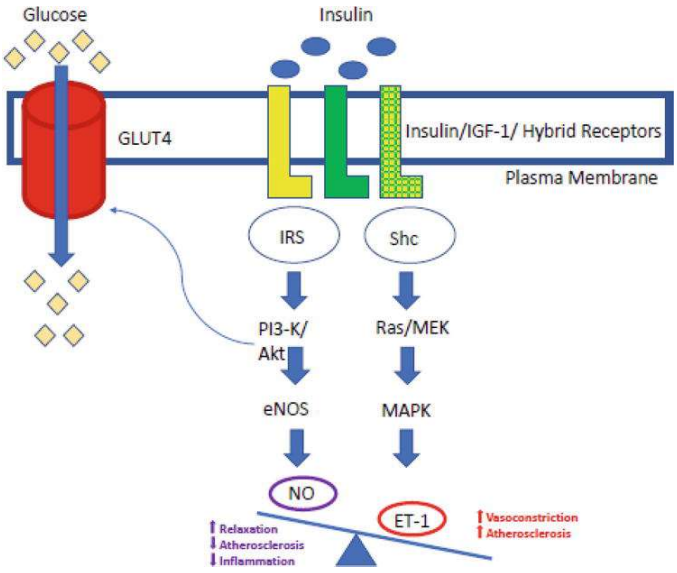


Figure 2.1: Insulin-mediated vasoconstriction and vasodilation (Gandhi et al., 2019).

2.4.2 Visceral Obesity

Among various types of obesity, visceral obesity indicates greater mass in intra-abdominal adipose tissue compared with lower extremities and hips. Visceral obesity has been proved to be an independent risk factor and better predictor for systemic inflammation and cardio-metabolic disorders (Owolabi et al., 2017; Purnell, 2018).

Dysfunction of visceral adipose tissue had been resulted by abnormality in lipid metabolism and adipokine secretion. In enlarged and dysfunctional visceral adipose tissue, there is an increasing secretion of proinflammatory biomarkers and reduced levels of adiponectin which act as anti-atherosclerosis cytokine (Ouchi and Walsh, 2007). As a result, systemic inflammation will be induced by proinflammatory molecules which in turn lead to endothelial dysfunction in CVD development, especially atherosclerosis (Marsland et al., 2010).

Meanwhile, decreased adiponectin also leads to insulin resistance by lowering the rate of lipid catabolism as well as insulin sensitivity in skeletal muscle through inhibition of insulin receptor (Vázquez-Vela et al., 2018).

2.4.3 Hypertension

Hypertension is always presented with abnormal systolic blood pressure (SBP) and diastolic blood pressure (DBP). The pathogenesis of hypertension could be due to insulin resistance via endothelial dysfunction, which is characterised as disturbance of metabolism in NO or imbalance between endothelium-derived constricting and relaxing factors (Spieker et al., 2000).

During mechanical stress, endothelial cells play various key functions in hemodynamic and mitogenic signalling pathway (Huang, 2009). Persistent high systemic pressure level in microvasculature could lead to structural alterations in microcirculatory beds and generate damage in endothelial cells (Levy et al., 2001). As a result, impaired endothelial cells will secrete less NO and eventually generate endothelial dysfunction via reduced vasodilation (Levy et al., 2001). Besides, disturbance of trans-endothelial transportation including endothelial cells with insulin dysfunction may contribute to insulin action delay in leading to insulin resistance (Richards et al., 2010).

2.4.4 Hypercholesterolaemia

Hypercholesterolaemia is also known as hyperlipidaemia. It is a lipoprotein disorder closely associated with formation of blood plaque in blood vessels, termed thrombosis. Hypercholesterolaemia has been proved as a significant factor in leading to coronary and ischaemic heart disease, especially when total cholesterol level showed positive correlation with CVD progression (Benfante et al., 1994).

High total cholesterol level increases risk of cholesterol accumulation in the extracellular matrix of tunica intima in the artery, especially the curvature and bifurcation region with consistent impaired endothelial cells (Wengrofsky et al., 2019). Subendothelial accumulation of LDL and VLDL remnants leads to increase in adhesion protein expression which in turn promotes entry of macrophage and plasmin formation. As a result, leukocyte adhesion on the foam cell will trigger an immune response via phagocytosis of the foam cell by macrophages. Abnormal continuous phagocytosis will lead to dendritic foam cell formation which further increases thrombus size. Extra coating of plaque by lipid necrotic core composed of cell debris will eventually progress into a thick collagenous fibrous cap via secretion of proinflammatory cytokines which contribute to additional foam cell destruction. Presence of stable or vulnerable plaque had led to development of ischemic heart disease by impeding oxygen supply and atherosclerotic cardiovascular disease.

2.5 Laboratories Findings of Metabolic Syndrome

2.5.1 Fasting Plasma Glucose Level in Detection of Diabetes Mellitus

According to WHO, the normal range of FBG falls between 3.9 mmol/L - 5.6 mmol/L while FBG above 6.9 mmol/L has been defined as a diabetes condition (World Health Organization, n.d.). FBG at the range of 5.6 mmol/L - 6.9 mmol/L demonstrate impaired fasting glucose, indicating prediabetes condition and insulin resistance (IR) (Centers for Disease Control and Prevention, 2021; World Health Organization, n.d.). There are 25% insulin resistance patients who will develop into T2DM within 3-5 years after diagnosis while 70% of patients with IR will progress to T2DM within lifetime (Tabák et al., 2012; Hostalek, 2019).

2.5.2 Blood Pressure in Detection of Hypertension

Different ranges in SBP and DBP had been defined deliberately for each category of hypertension and showed in Figure 2.3. AHA guidelines have been adopted in defining the status of hypertension (Whelton et al., 2018).

The seriousness of hypertension is characterised with higher values in SBP and DBP (Whelton et al., 2018). Among people within the age of 40 - 89, an increase of every 20 mmHg in SBP or 10 mmHg in DBP lead to a two-fold risk of death due to ischemic heart disease and stroke (Whelton et al., 2018).

BLOOD PRESSURE CATEGORY	SYSTOLIC mm Hg (upper number)	and/or	DIASTOLIC mm Hg (lower number)
NORMAL	LESS THAN 120	and	LESS THAN 80
ELEVATED	120 – 129	and	LESS THAN 80
HIGH BLOOD PRESSURE (HYPERTENSION) STAGE 1	130 – 139	or	80 – 89
HIGH BLOOD PRESSURE (HYPERTENSION) STAGE 2	140 OR HIGHER	or	90 OR HIGHER
HYPERTENSIVE CRISIS (consult your doctor immediately)	HIGHER THAN 180	and/or	HIGHER THAN 120

Figure 2.2: Blood pressure category under AHA guideline (Whelton et al., 2018).

2.5.3 Waist Circumference for Detection of Visceral Obesity

Waist circumference found to be a better biomarker for visceral obesity via measurement of abdominal fat mass in comparison with body mass index (BMI) (Ross et al., 2020). In the Malaysian population, WC also indicates a better risk factor for visceral obesity in terms of accuracy and simple operation (Ahmad et al., 2016).

There are different diagnostic criteria for visceral obesity in different genders due to physiological differences in body mass and fat deposition as result of different sex hormone action (World Health Organization, 2011). For example, a relatively higher central fat distribution and total lean mass was found in male while females had more peripheral fat distribution and higher fat mass (World Health Organization, 2011). For the Malaysian population, the diagnostic criteria was < 90 cm in men and < 80 cm in women (Ministry of Health, 2004).

2.5.4 Total Serum Cholesterol Level in Detection of Hypercholesterolaemia

There are three board categories for TC, including desirable (160-200 mg/dL), borderline high (201-239 mg/dL) and high (>240 mg/dL) (NCEP, 2005). Each category represented a different risk for chronic metabolic disorder development via Framinghan point score (National Cholesterol Education Program, 2005).

2.6 Clinical Manifestation for Metabolic Disorders

Metabolic disorders such as CVD, T2DM present with different signs and symptoms. The most common signs and symptoms for CVD are shortness of breath, irregular heartbeat, chest pain, fatigue and dizziness which may not be notifiable by the patients (Mayo Clinic, 2021a). Additionally, the early stage of CVDs always shows asymptomatic. It can only be diagnosed in a late stage that usually requires long-term medication and medical monitoring. For example, 53% of T2DM patients experienced asymptomatic coronary artery disease while cardiomyopathy will only possess symptoms of irregular heartbeat in the late stage (MayoClinic, 2021b.; Tsujimoto et al., 2011).

T2DM patients will experience nocturia, high frequency of thirst and fatigue, numbness in hands and feet and dry skin (Mayo Clinic, 2021b). Untreated T2DM may lead to severe complications caused by other impaired metabolisms due to hyperglycaemia. There are three main categories for severe T2DM, one of them is acute complications like diabetic ketoacidosis and hyperglycaemic hyperosmolar state (Ministry of Health, 2015). Besides, chronic complications are often related to vascular abnormality, which is also classified into

microvascular-like nephropathy and retinopathy (Ministry of Health, 2015). In macrovascular complications, coronary artery and cerebrovascular disease may be manifested (Ministry of Health, 2015).

2.7 apM1 Gene Variants Related with Metabolic Disorders

The adiponectin (apM1) gene is encoded for adiponectin. It is one of the cytokines secreted from adipose tissue in humans (Hannah et al., 2016). The apM1 has been involved in maintenance of normal glucose and fatty acid metabolism in playing a protective role against hyperglycaemia and atherogenic disease (Sheng and Yang, 2018). In regulation of lipid metabolism, apM1 enhances fatty acid oxidation in skeletal muscle through activation of the MAPK pathway with involvement of p38 mitogen-activated kinase and peroxisome proliferation-acrivated receptor α (PPARα) (Sheng and Yang, 2018).

The apM1 is also devoted to insulin sensitivity enhancement through increased glucose uptake in skeletal muscle and inhibition of gluconeogenesis in liver cells (Sheng and Yang, 2018). The action of apM1 decreases mRNA expression of gluconeogenic enzymes phosphoenolpyruvate kinase (PEPCK) and glucose-6-phosphatase (G6Pase) through the AMPK signalling pathway, the signalling pathway responsible for hepatocyte gluconeogenesis. Moreover, apM1 promotes NO synthesis in endothelial cells, exerting an anti-atherogenic effect (Ouchi and Walsh, 2007).

Suppressed apM1 level or hypoadiponectinaemia have been proven to cause metabolic dysregulation in insulin resistance and T2DM (Sheng and Yang, 2018). Other than that, hypoadiponectinaemia is also found in a series of chronic metabolic disorders such as CVD, essential hypertension, and obesity (Fan et al., 2017).

2.7.1 rs1501299 and Metabolic Disorders

Single nucleotide polymorphism (SNP) in apM1 gene has been reported in association with the predisposition of MetS. One of the SNPs, rs1501299 with G to T polymorphism has been found in +276 position in intron 1 of apM1 gene (de Luis et al, 2016). Each allele of this SNP shows a contradictory relationship with prevalence in metabolic syndrome among different populations. The mutant T allele, acting as a protective role against insulin resistance and HTN in Asiatic and Caucasian populations, respectively (Caramori et al, 2002; Fan et al, 2017). Meanwhile, wildtype G allele showed higher prevalence in T2DM patients in both Japanese and Han Chinese (Hara et al., 2002; Dong et al., 2020).

In contrast, T allele is found to be associated with predisposition of CVD and increased serum TC in South Indian populations (Ramya et al., 2013). Moreover, the codominant GT genotype showed a protective role in CVD and dyslipidaemia compared with the TT recessive model (Ramya et al., 2013). This polymorphism was included into this study to elucidate its association with CMBC among young adults.

2.8 Peroxisome Proliferator-activated Receptor γ (PPARγ) and Metabolic Disorders

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated transcription factor which plays a role in energy homeostasis including lipid and glucose metabolism (Picard and Auwerx, 2002). Among three subtypes, PPARγ functions as an intranuclear hormone receptor which is activated and dimerized with retinoid X receptor and acts as a transcription factor in particular gene expression after ligand binding (Picard and Auwerx, 2002).

The PPARγ act as master regulator of adipogenesis via control in various gene expression involved in lipid metabolism such as fatty acid transport protein 1 (FATP-1) that responsible for long chain fatty acid uptake in adipose tissue (Ahmadian et al., 2013; Tyagi et al., 2011). Initiation of lipid acid uptake prevents possible development of insulin resistance which is linked to high circulating free fatty acids level and accumulation of lipid in non-adipose tissue (Sears and Perry, 2015). Besides, PPARγ also represses the gene expression of inflammatory cytokines which in turn prevents inflammatory conditions in insulin resistance (Sears and Perry, 2015).

2.8.1 rs1801282 and Metabolic Disorders

PPAR*y* ProAla or rs1801282 revealed a point mutation in position 12 of B exon in NH2-terminal part of PPARγ 2, resulted transition of proline (CCA triple codon) to alanine (GCA triple codon) (Picard and Auwerx, 2002). Therefore, partial loss-of-function mutations make trans-activated responsive promoters

become less effective which in turn reduce transcriptional activity of PPAR*y* (Picard and Auwerx, 2002).

Results from meta-analysis indicate the presence of minor alleles had been associated with the decreased risk of T2DM, with particularly high significance in European, East Asian and Southeast Asian populations (Sarhangi et al., 2020). Controversial association between rs1801282 polymorphism with T2DM has been discovered in different populations. For instance, the protective role of Ala variants toward T2DM has been shown in East Asia while it shows no association with DM among the Han Chinese population (Hara et al., 2020; Tong et al., 2012). Other than T2DM, Ala variants are also considered as risk markers for hypertension in the Han population (Wang et al., 2015). Recessive allele also showed a positive association with central obesity risk compared with dominant allele in Caucasians and Asians population (Yao et al., 2015). In this study, this significant mutation was included to study the association of this mutation with CMBC among the Malaysian population.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals, reagents, and instruments

The chemicals, reagents, instruments, and consumables that are being used throughout the study has been listed in Table 3.1 and 3.2, respectively.

Table 3.1: Chemicals and reagents used in this study.

Chemicals / reagents	Manufacturer
DNA extraction kit	FAVORGEN Biotech Corp., Taiwan
Agarose powder	Sisco Research Laboratories, India
GelRed	Vivantis Technologies Sdn Bhd, Malaysia
DNA loading dye	NIPPON GENETICS, Japan
100 bp ladder	SMOBio Technology Inc., Taiwan
50 bp ladder	SMOBio Technology Inc., Taiwan
Primers	Integrated DNA Technologies, USA
PCR mix	GeneDirecX Inc., USA

Table 3.2: Instruments used in this study.

Instruments / consumables	Manufacturer
EDTA vacutainer	Chengdu Puth Medical Plastic Packaging, China
Syringes	Terumo Corporation, Japan
Needles	Nipro, Japan
Automated blood pressure monitor	Omron, Japan
Microcuvette	HemoCue, Sweden
HemoCue	HemoCue, Sweden
Glucose strip	Ascensia Diabetes Care, Switzerland
Glucometer	Ascensia Diabetes Care, Switzerland
Total cholesterol strip	Bioptik Technology, Taiwan
Total cholesterol meter	Bioptik Technology, Taiwan
Micropipette	Eppendorf, Germany
Centrifuge machine	Thermo Scientific, USA
Microcentrifuge machine	Thermo Fischer Scientific, USA
Nanodrop	Thermo Scientific, USA
Gel electrophoresis set	Major Science, USA
Thermal cycler	Bio-Rad Laboratories, USA Biometra, Germany
Heatblock shaker	Qinstuments, Germany
Vortex	Stuart, United Kingdom
Microwave	Panasonic, Japan
Freezer	Samsung, Korea
Fridge	Samsung, Korea
Gel documentation system	Bio-Rad Laboratories, USA
Measuring cylinder	Favorite. Malaysia
Schott bottle	Duran, Germany
Lancet	ACCU-Chek, Malaysia
Incubator oven	Memmert, Schwalbach

3.1.1 Reagents Preparation

The 50 x TAE buffer was prepared by adding 242 g of Tris Base powder in 600 mL of distilled water and mixed by using a magnetic stirrer until fully dissolved. After that, 100 mL of 0.5 M EDTA and 57.1 mL of acetic acid were added into the mixture. Distilled water was then added to the mixture until the final volume of 1 L and stored at room temperature.

3.2 Experimental Design

Figure 3.1 presents the experimental flow chart for this study.

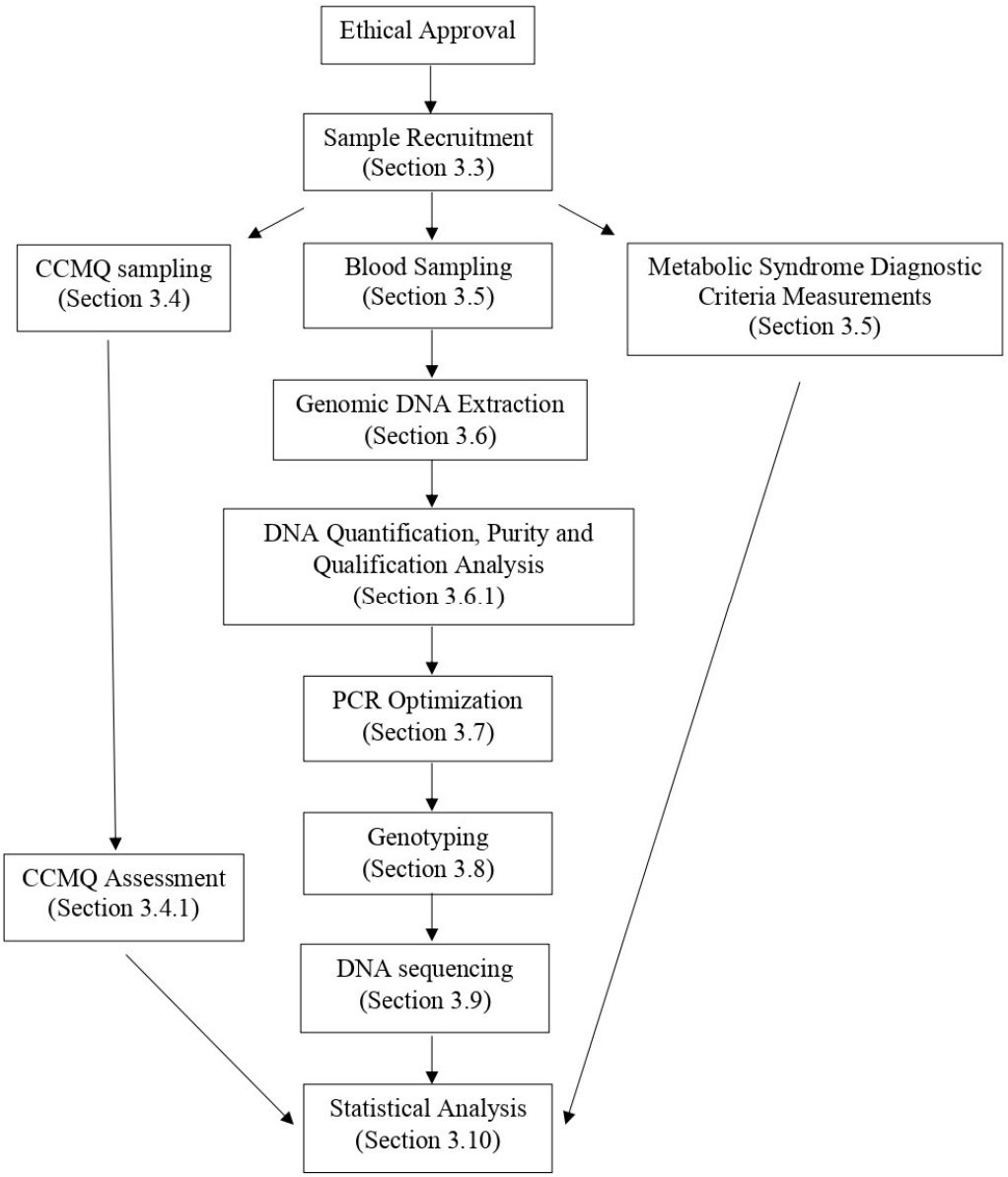


Figure 3.1: Experimental flowchart.

3.3 Sample recruitment

Ethical approval had been applied for and obtained from UTAR Scientific and Ethical Review Committee before commencement of study. Convenient sampling had been implemented and all subjects were recruited on a voluntary basis with detailed informed consent. The exclusion and inclusion criteria of subjects were:

Inclusion criteria:

- Age between 18 – 35 years old
- In fasting mode for fasting glucose level measurement

Exclusion criteria:

- Age below 18 years old or above 35 years old
- Pregnancy

Sample size of this study had been calculated according to the prevalence rate of metabolic syndrome in Malaysian adults in JIS which is 43.4% (Ramli et al., 2013). The calculated sample size was 81 subjects based on 90% confidence interval (CI) and 20% drop-off rate while a total of 85 subjects had been recruited in this study.

The calculation was presented as below:

Calculation of sample size:

$$n = \frac{(Z)^2 p(1-p)}{d^2}$$

$$n = \frac{(1.645)^2 0.434(1-0.434)}{0.01^2}$$

n = 67

Addition of 20% drop off rate:

n = 67 x 120%

n = 81

n = sample size

Z = statistical level for level of confidence

= 1.645

p = expected prevalence

d = allowable error

= 0.1

3.4 Constitution in Chinese Medicine Questionnaire (CCMQ) sampling

The CCMQ was published by the China Association of Chinese Medicine in 2009. This had been adopted in this study and translated from Chinese-to-English language through Cross-culture Compiling in Foreign Language (Jing et al., 2015). Questionnaire had been distributed to participants via Google Form. Content of the questionnaire consisted of basic demographic and 60 CCMQ questions, which had been categorised into 9 sections, according to specific physical and mental conditions in each CMBC type. Subjects were requested to recall the frequency of conditions that occurred near a month and rated via 5-point Likert-type scale.

3.4.1 Assessment of Constitution in Chinese Medicine Questionnaire (CCMQ)

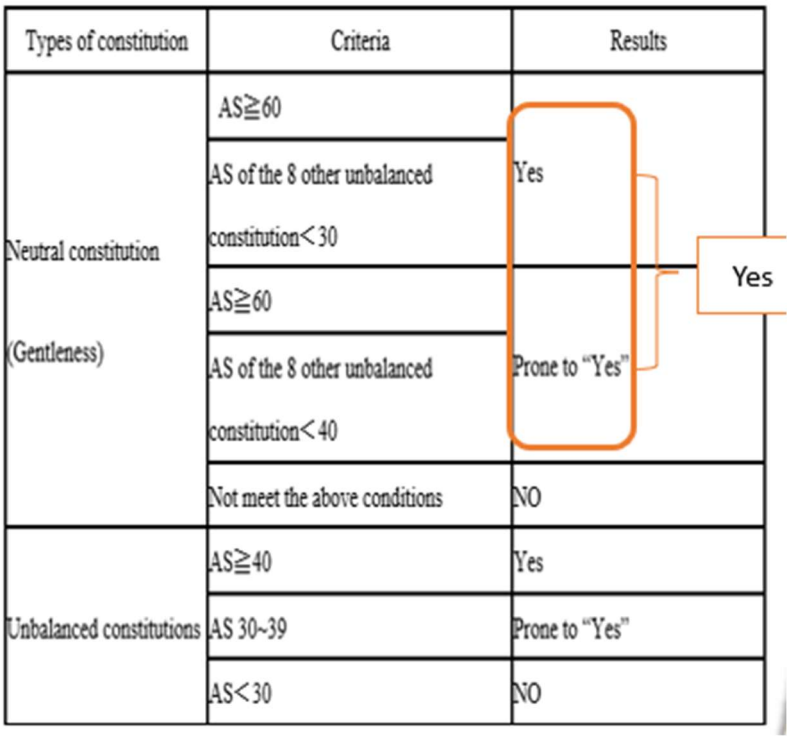
Scoring was given for each condition in CCMQ and converted from frequency of condition occurrence provided by subjects in the form of positive correlation (Table 3.3). There are exceptions for 5 questions which are required to be calculated in inverse form. Scores calculated for each CMBC type will be applied in a formula for CMBC differentiation. Assessing score for CMBC had been provided in Figure 3.2 . The CMBC score was calculated using the formula below. Result of "prone to Yes" and "Yes" will be summarized and reported as confirmed CBMC type.

Table 3.3: Scoring system for frequency of CCMQ questionnaire.

Scales	Frequencies (/week)	Scores
Never	0	1
Seldom	1-2	2
Sometimes	3-4	3
Often	5-6	4
Always	7	5

Formula for CMBC score calculation:

$$\frac{\text{Total score in each CMBC} - \text{number of questions in each CMBC}}{\text{Number of questions in each CMBC} \times 4} \times 100\ \%$$



Types of constitution	Criteria	Results
Neutral constitution (Gentleness)	AS≧60	Yes
	AS of the 8 other unbalanced constitution＜30	
	AS≧60	Prone to "Yes"
	AS of the 8 other unbalanced constitution＜40	
	Not meet the above conditions	NO
Unbalanced constitutions	AS≧40	Yes
	AS 30~39	Prone to "Yes"
	AS＜30	NO

Figure 3.2: Assessing score for CDC classification.
*AS: Assessing score

3.5 Blood Sample Collection

A volume of 6 mL venous blood had been collected via venipuncture by supervisor, Dr. Teh Lai Kuan into two ethylenediaminetetraacetate (EDTA) vacutainers equally to prevent blood cell coagulation via calcium chelation and allow buffy coat collection (Banfi et al, 2007). EDTA vacutainers were centrifuged at 3,300 rpm for 15 minutes to separate the whole blood into three distinct layers: plasma, buffy coat, and packed red cell. A volume of 200 µL buffy coat was transferred into a 1.5 mL microcentrifuge tube and stored in a 4 °C freezer.

3.5.1 Waist Circumference Measurement

Subjects were asked to point out the position of the navel which is located around the top of hip bones. Sewing tape was placed and rounded with the same horizontal level slightly above navel position and circumference had been recorded (World Health Organization, 2011).

3.5.2 Blood Pressure Measurement

Subjects were only allowed for blood pressure measurement if they did not undergo vigorous exercise 30 mins before sampling. After clothing on upper arm was removed, subject's palm was turned upward and sit on chair to make cuff on upper arm in same level with heart. Next, the cuff was applied to the upper arm with a position about 1 inch above the elbow and space for two fingers was left between the cuff and the upper arm of the subject. Air tube of the blood pressure monitor was aligned with the middle finger. Subject was required to keep calm and quiet throughout blood pressure measurement and displayed SBP as well as DBP was recorded.

3.5.3 Fasting Glucose Level Measurement

Subjects were requested to be fasted for eight hours before fasting glucose measurement. Finger pricking had been applied on the middle finger or ring finger of the non-dominant hand of subjects. Disinfection was carried out by wiping the fingertips of subjects with an alcohol swab in one direction. Afterwards, fingertips of subjects were punctured by lancets across the fingerprint and first capillary blood was cleaned using cotton wool to prevent contamination. Capillary blood was squeezed out and loaded into a sample tip

which grey square end of the test strip was already inserted into glucometer. Fasting glucose level displayed on monitor was recorded.

3.5.4 Total Cholesterol Measurement

EasyTouch GCU metre had been used in total cholesterol measurement where a control test was required. Cholesterol code key was inserted into the key slot and the test strip was inserted into the meter. After the control test, capillary blood obtained in the same finger pricking for plasma glucose level had been loaded into the sample target area and the total cholesterol value displayed was recorded for each subject.

3.6 Genomic DNA extraction

Genomic DNA extraction had been carried out using FavorPrep^{TM} Blood Genomic DNA Extraction Mini Kit Protocol. 20 µL of proteinase K and 200 µL of FABG buffer were added into 200 µL buffy coat layer, followed by vortex for 1 min and spun. Mixture was then heated in a preheated heating block shaker for 15 mins. In each 5-minute interval, the mixture was vortexed for 10 secs and spun slightly. Afterwards, 200 µL of absolute ethanol was added into the mixture and the mixture was transferred into FABG mini column which was inserted onto a collection tube.

After the FABG mini column with the collection tube centrifuged at 6,000 g for 1 min, the collection tube was discarded and replaced by a new collection tube. 400 µL of W1 buffer was added into the FABG mini column and sent to the centrifuge at 17,000 x g for 40 secs followed by discarding filtrate in the

collection tube. Hereafter, the mixture was added with a 750 µL Wash buffer and centrifuged at 17,000 x g for 40 secs. After filtrate in the collection tube was discarded, the mixture was centrifuged again at 17,000 x g, 3 mins for drying purpose.

Next, the FABG mini column was transferred onto the elution tube. 30 µL of pre-heated elution buffer was added into the FABG mini column and stood for 30 min at room temperature which repeated twice. Mixture was centrifuged at 17,000 x g, 1 min. The FABG mini column was discarded and DNA in the elution tube was sent to analysis and stored in -20 °C.

3.6.1 DNA Quantification, Purity, and Qualification Analysis

DNA samples were measured using Nanodrop for determination of concentration and purity with an elution buffer as blank. A volume of 1 µL DNA was placed onto the sensor pedestal of the nanodrop. Nanodrop arm was lowered after ensuring that there was no bubble on the mixture. DNA concentration and purity at a 260/280 ratio had been recorded while the Nanodrop curve had been observed for any abnormal fluctuation. The ideal range of DNA at 260/280 ratio is 1.8 - 2.0.

In preparation of genomic DNA gel electrophoresis for DNA integrity evaluation, 20 mL, and 30 mL of 1 x TAE buffer with 2% (w/v) agarose powder were used for small and big gel trays, respectively. In application of a small gel tray, 20 mL 1 x TAE buffer was measured using a measuring cylinder and 2% (w/v) agarose powder (0.4 g) was weighted by analytical balance. Mixture was

heated in the microwave until agarose powder was dissolved completely. Afterwards, the mixture was added with 1 µL GelRed and poured into a gel tray with gel comb. Gel tray was stored in a light avoidance place and stood for 30 mins at room temperature.

Gel tray with solidified gel was placed into the buffer tank and 1 x TAE buffer are soaked into gel, with confirmation of fully covered agarose gel. 1 µL of 3 x DNA loading dye and 1 µL of 100 bp ladder will be mixed and loaded into the first gel lane. Meanwhile, 1 µL of DNA and 1 µL of 3 x DNA loading dye were mixed and resuspended before being added into the gel lane. Afterwards, the buffer tank was connected to the power supply with settings of 90 V for 30 mins. Agarose gel will be viewed by a gel imager to observe the quantity of DNA band. Qualified DNA which shows one band without any fragment in the gel image will be stored for genotyping in a further step.

3.6.2 Working DNA Preparation

Final working DNA concentration used in PCR is 75 ng/µL. Concentration and volume of working was calculated using formula below.

$M_1V_1 = M_2V_2$

M_1 = concentration of stock DNA M_2 = concentration of working DNA

V_1 = volume of stock DNA V_2 = volume of working DNA

3.7 PCR Optimization

Gradient PCR was performed to check for the optimum annealing temperature of rs1501299 and rs1801282 tetra primers sets. The annealing range was ranged approximately ± 10 °C of the average melting temperature of all primers for each SNP.

Average melting temperature of rs1501299 primers:

$$= \frac{55.6\ °C + 57.3\ °C + 58.7\ °C + 55.0\ °C}{4}$$

= 56.65 °C

Therefore, annealing temperature ranged 45 °C – 65 °C had been set in Biorad Thermal Cycler. The optimum annealing temperature of rs1501299 primers was 60 °C.

Average temperature of rs1801282 primers:

$$= \frac{58.2\ °C + 57.8\ °C + 58.5\ °C + 55.5\ °C}{4}$$

= 57.5 °C

Meanwhile, annealing temperature ranged 57.5 °C – 67.5 °C had been set in Biorad Thermal Cycler. The optimum annealing temperature of rs1801282 primers was 62 °C.

3.7.1 Primer Design

Primer's sequence and its amplicon size used for rs1501299 was adopted from Hashemi and his co-authors in 2013. Meanwhile, the primer design used for rs1801282 including sequence and amplicon size was taken from Masud and Ye in 2013. Information of all primers used had been stated in Table 3.4. For tetra primer amplification refractory mutation system polymerase chain reaction (ARMS-PCR), two sets of inner and outer primers had been applied, each with forward and reverse form. Both outer primers are used as unspecified internal control while one mismatch in the 3'terminus of both inner primers create their specificity upon binding on targeted polymorphism (Medrano and De Oliveira, 2014) The principle of tetra-primer ARMS PCR was shown in Figure 3.3.

Primers synthesised by Integrated DNA Technologies were in the form of lyophilized material which needed to be reconstituted with 1 x TE buffer. Working primers mix had been prepared from stock primers by diluting with distilled water and mixed all primers in 0.5 mL microcentrifuge tube which stored in – 20 °C. Different annealing temperatures have been tested to obtain optimum annealing temperature.

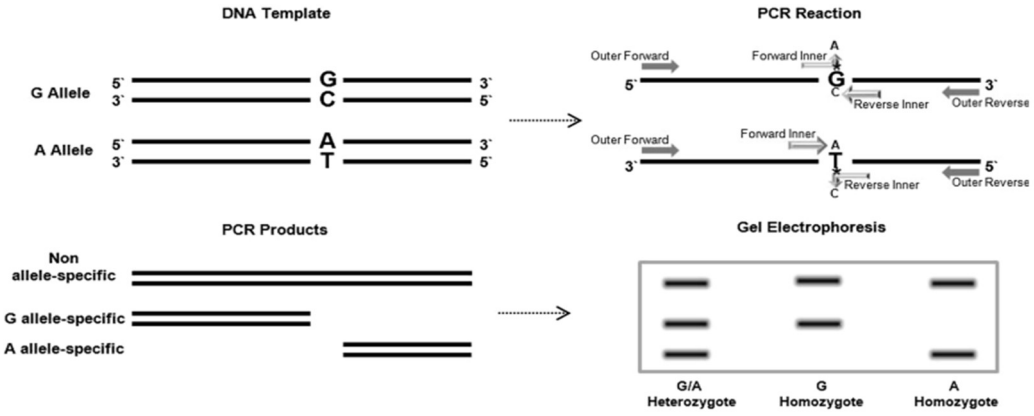


Figure 3.3: Principle of Tetra-primer ARMS PCR.

Table 3.4: Sequence, target and amplicon size of primers used.

SNP	Primer/Allele	Primer Sequence 5'- 3'	Amplification	Amplicon Size (bp)
rs1501299	Inner forward (F_I)	CCTCCTACACTGATATAAACTATATGAGGG	IF-OR	244
	Inner reverse (R_I)	TGTGTCTAGGCCTTAGTTAATAATGAACGA	OF-IR	292
	Outer forward (O_F)	GAGCTGTTCTACTGCTATTAGCTCTGC	OF-OR	476
	Outer reverse (O_R)	GAATATGAATGTACTGGGAATAGGGATG		
rs1801282	Inner forward (F_I)	GAAACTCTGGGAGATTCTCCTATTGTC	IF-OR	221
	Inner reverse (R_I)	GTATCAGTGAAGGAATCGCTTTCAGC	OF-IR	288
	Outer forward (O_F)	AACTTTTTGTCACAGCTGGCTCCTAATA	OF-OR	495
	Outer reverse (O_R)	CAACGAGCTAAGCATTAAAATACTGGA		

3.8 Genotyping

The PCR mixture used in genotyping of rs1501299 and rs1801282 was prepared in final volume of 10µL with 75 ng/µL final DNA concentration as shown in Table 3.5 (a).

Table 3.5(a): Preparation of rs1501299 and rs1801282 PCR mixture.

Reagents	Initial Concentration	Final Concentration	Volume (µL)
Master mix	2 x	1 x	5
F_i **R_i** **F_o** **R_o**	4 µM	0.4 µM	1
Working DNA	25 ng/µL	75 ng/µL	3
sdH_2O	-	-	1
	Total		10

Genotyping for both rs1501299 and rs1801282 had been conducted via tetra-primer ARMS PCR using a Biorad single block thermal cycler. The cycling condition had been listed in Table 3.5 (b). PCR for both gene variants use similar cycling conditions except for annealing temperature.

Table 3.5(b): PCR cycling condition of rs1501299 and rs1801282.

Event	Temperature (°C)	Duration	No. of cycle
Initial denaturation	94 [ab]	3 mins	1
Denaturation	94 [ab]	30 secs	30
Annealing	60[a] / 62[b]		
Extension	72 [ab]		
Final extension	72 [ab]	3 mins	1
Hold	12 [ab]	∞	

[a]: cycling condition of rs1501299; [b]: cycling condition for rs18012812.

Agarose gel was prepared by mixing and melting 1x TAE buffer with 2% agarose gel. Different volumes of 1x TAE buffer had been used for gel tray in different sizes, of which 20 ml and 30 ml was required for small and big gel, respectively. Afterwards, complete melted agarose had been added with 1 µL GelRed pre-staining dye and pre-stained agarose gel was loaded into a gel tray. Filled gel tray was stand stilled for 30 mins to allow gel solidification.

After completion of PCR, 3 µL of PCR products was mixed with 1 µL 1x DNA loading dye. After the 50 bp DNA ladder and non-template control had been loaded in the 1st and 2nd well, each PCR mixture had been loaded into every remaining well. Setting for agarose gel electrophoresis was 90 V for 33 minutes.

Thereafter, gel was viewed under UV transilluminator, and amplicon band size was interpreted by comparing the position of the DNA band with the DNA ladder. Genotype of the DNA sample was estimated via matching of sample amplicon size with different alleles together with internal control created by outer primers. Amplicon size of each genotype found in rs1501299 and rs1801282 had been listed in Table 3.6.

Table 3.6: Amplicon size for genotypes of rs1501299 and rs1821282.

	rs1501299		**rs1801282**	
	genotype	**amplicon size (bp)**	**genotype**	**Amplicon size (bp)**
Wild type	GG	244	CC	221
Mutant	TT	292	GG	288
Heterozygous	GT	244 & 292	CG	221 & 288
Internal control	-	476	-	455

3.9 DNA Sequencing

One heterozygous and one homologous sample for rs1501299 and rs1801282 was randomly selected for direct nucleotide sequencing. DNA samples were subjected to DNA purification using FavorPrep™ GEL/PCR Purification Kit. Two sets of PCR mixture had been prepared for each DNA sample with a final concentration of 75 ng/μL and final volume of 50 μL per mixture. Genotyping of DNA samples had been carried out using outer primers only and amplicon size was examined after completion of agarose gel electrophoresis.

PCR products of the same DNA sample had been mixed together and added with 450 μL of FADF buffer. After the mixture was mixed well by using a vortex, all PCR products were transferred into the FADF column which had been placed onto a collection tube. The mixture had been centrifuged at 11, 000 x g for 30 seconds and flow-through in the collection tube was discarded. Afterwards, 750 μL of wash buffer was added into the FADF column followed by centrifugation at 11, 000 x g for 30 seconds and flow-through had been discarded. After the FADF column was centrifuged at 18, 000 x g for 3 minutes, the FADF column had been replaced onto an elution tube. A volume of 30 μL elution buffer was aliquoted onto the membrane of column and column was sent to centrifuge at 18, 000 x g for 1 minutes after standing for 1 minute. Purified DNA was subjected to Nanodrop for concentration and purity identification.

All PCR products with concentration >5 ng/μL with purity between 1.8-2.0 at A260/A280 ratio had been sent for automated Sanger sequencing at Apical Scientific Sdn. Bhd. Result of DNA sequencing was interpreted by ABI PRISM 3730xl Genetic Analyzer while alignment and assembly of DNA sequence had been carried out by DNA baser sequence assembly software version 5.15.

3.10 Statistical Analysis

IBM SPSS Statistics 22 software has been applied for analysis of raw data in questionnaire, demographic distribution, FBG, WC, BP, TC as well as genotypes.

For FBG, WC, BP, and TC, normality had been carried out by the Shapiro–Wilk test and Kolmogorov–Smirnov test. Afterwards, One-way ANOVA had been applied for normally distributed MRFs measurements between CMBC types that act as independent while plasma glucose level, total cholesterol level, blood pressure as well as waist circumference had been used as dependent variables. Meanwhile, the Kruskal Wallis test had been applied for non-normally distributed MRFs measurement. Chi-square tests were carried out between CMBC types and MRFs which had been categorised based on cut-off points. The association between CMBC types and genotype of rs1801282 and rs1501299 had also been assessed via the Chi-square test.

CHAPTER 4

RESULTS

4.1 Study Subjects and Demographic Distribution

According to the calculated sample size, 81 subjects had been required to achieve 90% CI with 20% drop off rate. In this study, a total of 85 subjects who fulfilled inclusion and exclusion criteria had been recruited thus sample size required had been fulfilled. There were 33 males (38.8%) and 52 females (61.2%) participated in this study. The ethnicity were consisted of 89.4% Chinese, 8.2% Indian, 1.2% for each Bumiputera and Malay. 75.3% (n = 64) subject are 18 – 22 years old, 20.0% (n = 17) found to be 23 – 27 years old while 4.7% (n = 4) of subjects fall between 28 – 32 years old. The average age of recruited subjects is 22 years old. The demographic distribution of each subject will be showed in Appendix A.

4.2 Distribution of CMBC type Among Study Subjects

In this study, based on the CCMQ, a total of 15 CMBC types were identified. Table 4.1 tabulated the distribution of CMBC type among the 85 subjects while Figure 4.1 presents the frequency of body constitution type.

Table 4.1: Distribution of CMBC type among subjects.

Body Constitution Type	Number of Subjects (n)	Cumulative Number of Subjects (n)	Percentage (%)
Gentleness	19	19	22.3
Yang Xu	10	29	11.8
Yin Xu	16	45	18.8
Qi Xu	12	57	14.1
Tan Shi	2	59	2.4
Shi Re	2	61	2.4
Xue Yu	2	63	2.4
Te Bing	4	67	4.7
Qi Yu	10	77	11.8
Yin Xu & Qi Xu	2	79	2.4
Yang Xu & Qi Xu	1	80	1.2
Shi Re & Qi Yu	2	82	2.4
Xue Yu & TeBing	1	83	1.2
Te Bing & Qi Yu	1	84	1.2
Yin Xu & Qi Xu & Shi Re	1	85	1.2
Total	85		100

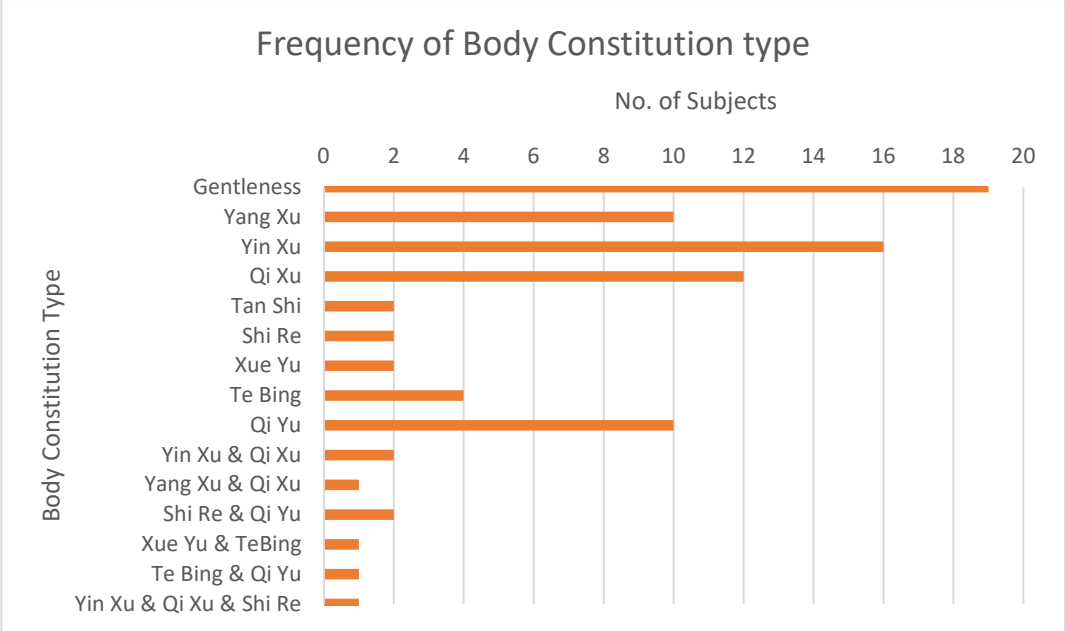


Figure 4.1: Distribution of body constitution type with its frequency.

Gentleness with balanced CMBC were found at the highest prevalence among all subjects (n = 19, 22.4%), followed by Yin Xu (n = 16, 18.8%), Qi Xu (n = 12, 14.1%), Yang Xu and Qi Yu. (n = 10, 11.8 %). Meanwhile, Tan Shi, Shi Re, and Xue Yu showed the least percentage of 2.4% (n = 2) among all CMBC. There was only 1 subject who classified as triple CMBC types. Result of CCMQ of each subject had been presented in Appendix C.

Minor single CMBC types including Tan Shi, Shi Re, Xue Yu and Te Bing were being grouped together in further analysis. All six combinational CMBC was found with only 1 or 2 study subjects and grouped together as multiple CMBC types.

4.2.1 Prevalence of CMBC Type in Relation to Genders

Table 4.2 indicates the distribution of CMBC among different genders in subject population. There was significant difference between gender and different CMBC types at p-value of 0.044 in Chi-Square test. Female was found predominantly with the body constitution of Qi deficiency, followed by Yang deficiency, Qi stagnation and Yin deficiency.

Table 4.2: Chi-Square test of frequency of CMBC types among different gender in subject population.

Gender	Body Constitution Type n (%)							p-value
	Gentleness (n = 19)	**Yang deficiency (n = 10)**	**Yin deficiency (n = 16)**	**Qi deficiency (n = 12)**	**Single Group[a] (n = 10)**	**Qi stagnation (n = 10)**	**Multiple Group[b] (n = 8)**	
Male	13 (39.4)	2 (6.1)	6 (18.2)	2 (6.1)	4 (12.1)	2 (6.1)	4 (12.1)	0.044*
Female	6 (11.5)	8 (15.4)	10 (19.2)	10 (19.2)	6 (11.5)	8 (15.4)	4 (7.7)	

* Significant difference with $p < 0.1$.

[a]single group of CMBC type consists of Tan Shi, Shi Re, Xue Yu and Te Bing.

[b]multiple group CMBC type consists of Yin Xu & Qi Xu, Yang Xu & Qi Xu, Shi Re & Qi Yu, Xue Yu & Te Bing, Te Bing and Qi Yu, Yin Xu & Qi Xu & Shi Re.

4.3 Prevalence of Metabolic Risk Factors Among Study Subjects

Table 4.3 demonstrates the distribution of MRFs among 85 study subjects. Among the study subject, the prevalence of prediabetes was found at 8.3% (7 subjects) and central obesity at 15.3% (13 subjects). Both prevalence stated above were found to be lower than general prevalence in Malaysian population, which are 14.4% and 52.6%, respectively (Fryar et al., 2017; Akhtar et al., 2022). Calculate prevalence of hypertension is 12.9% (11 subjects) which also show a lower prevalence in compared with 35.3% among Malaysian adult as in reported by MOH in year 2015. On the other hand, hypercholesterolemia showed higher prevalence of 41.2% in this studied population than prevalence rate (38.1%) published by MOH (MOH, 2020). Data of all MRFs of each subject were tabulated in Appendix B.

Table 4.3: Distribution of MRFs among subjects.

Measurement	Metabolic Risk Factor	Range	No. of subject (n)	Percentage (%)
Fasting glucose level	Normal	⩽ 5.6 mmol/L	78	91.76
	Prediabetes	5.6 – 6.9 mmol/L	7	8.24
Systolic & Diastolic blood pressure	Normal	SBP: <120 mmHg and DBP: < 80 mmHg	56	65.88
	Elevated hypertension	SBP: 120 - 129 mmHg and DBP: < 80 mmHg	13	15.29
	Hypertension Stage 1	SBP: 130-139 mmHg or DBP: 80 - 90 mmHg	11	12.94
	Hypertension Stage 2	SBP: >140 mmHg or DBP: < 90 mmHg	5	5.88
Waist Circumference	Normal (Male)	< 90 cm	27	31.76
	Central Obesity (Male)	≥ 90 cm	6	7.06
	Normal (Female)	< 80 cm	45	52.94
	Central Obesity (Female)	≥ 80 cm	7	8.24
Total serum cholesterol level	Normal	< 200 mg/dL	50	58.82
	Borderline High	200 - 239 mg/dL	33	41.18
	High	≥ 240 mg/dL	2	

4.4 Genomic DNA Analysis

DNA was extracted using Favorgen DNA Extraction Kit from buffy coat layer isolated from whole blood sample. The purity and concentration of the extracted DNA were examined using Nanodrops and genomic gel electrophoresis. The average of extracted DNA concentration is 93.59 ng/µL while mean of DNA purity is 1.85 at A260/280 ratio. Concentration of extracted DNA and its purity had been included in Appendix D. The presentative genomic gel image was presented in Figure 4.2.

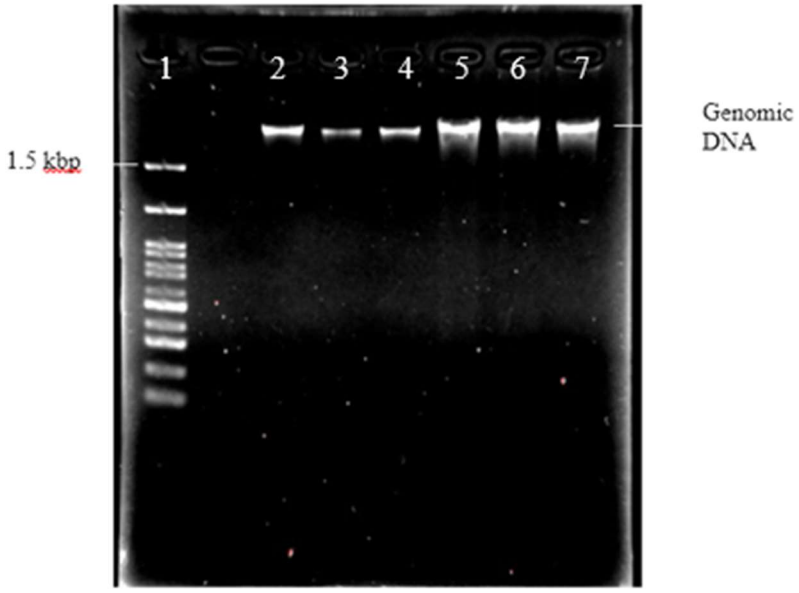


Figure 4.2: Representative genomic gel image.
Lane 1: 100 bp DNA ladder; Lane 2: sample 044; Lane 3: sample 045; Lane 4: sample 046; Lane 5: sample 047; Lane 6: sample 048; Lane 7: sample 049

4.5 Genotype of Metabolic Gene Variants (rs1501299 & rs1801282)

All the extracted DNA was diluted into the same working DNA concentration of 25 ng/μL for the genotyping purpose. Genotyping for rs1501299 and rs1801282 were performed via Tetra-primer ARMS-PCR. The PCR product was analysed by using electrophoresis with 2% (w/v) agarose gel.

Gradient PCR had been carried out to determine the optimized annealing temperature of primer sets for rs1501299 and rs1801282 and results had been listed in Figure 4.3 and Figure 4.4, respectively. The annealing temperature for rs1501299 had been set as 60 °C while 62 °C had been used for annealing temperature for rs1801282. Test run results had been presented in Figure 4.5 (rs1501299) and Figure 4.6 (rs1801282). The genotype of all subjects had been presented in appendix while gel electrophoresis result had been shown in appendix F.

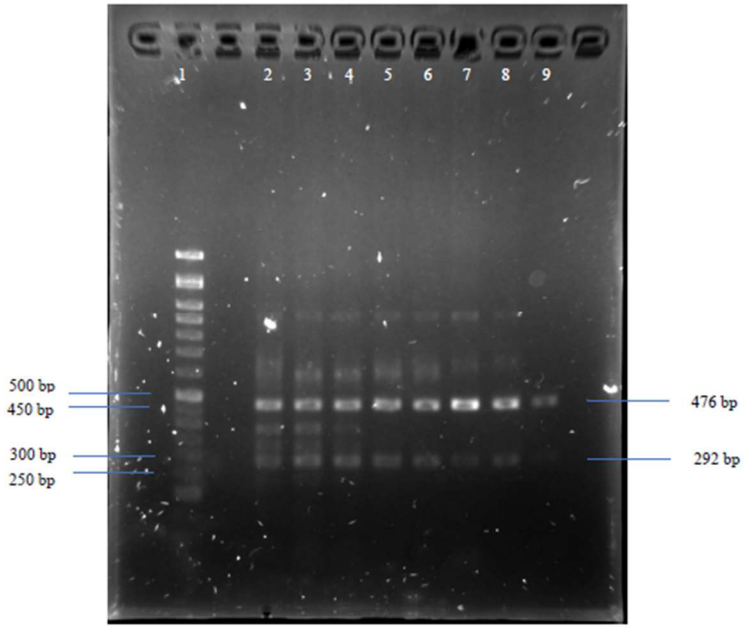


Figure 4.3: Gradient PCR result of rs1501299.
Lane 1: 50 bp Ladder; Lane 2: 45.0°C; Lane 3: 46.4°C; Lane 4: 48.8°C; Lane 5: 52.6°C; Lane 6: 57.1°C; Lane 7: 60.9°C; Lane 8: 63.4°C; Lane 9: 65.0°C

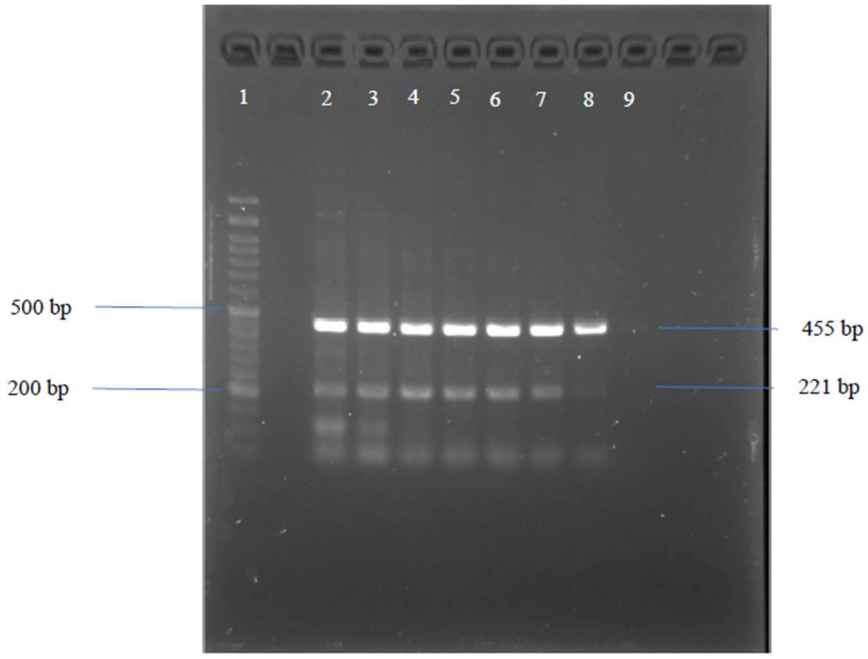


Figure 4.4: Gradient PCR result of rs1801282.
Lane 1: 50 bp Ladder; Lane 2: 47.5°C; Lane 3: 48.9°C; Lane 4: 51.4°C; Lane 5: 54.9°C; Lane 6: 59.7°C; Lane 7: 63.5°C; Lane 8: 66.0°C; Lane 9: 67.5°C

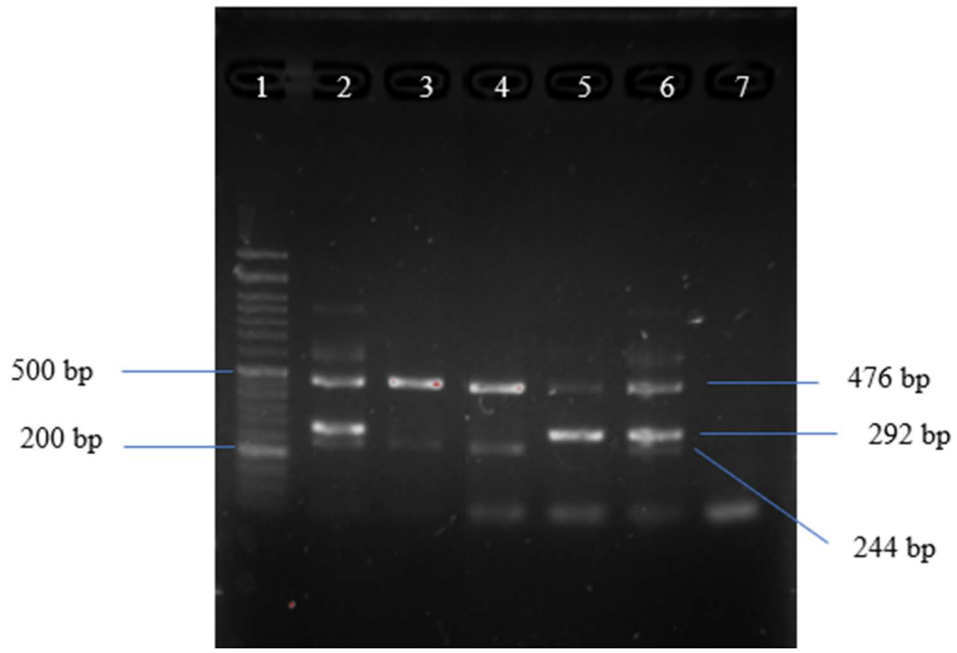


Figure 4.5: Test run result of rs1501299 with 60 °C annealing temperature. Lane 1: 50 bp Ladder; Lane 2: Test sample 001; Lane 3: Test sample 002; Lane 4: Test sample 003; Lane 5: Test sample 004; Lane 6: Test sample 005; Lane 7: Non-template control

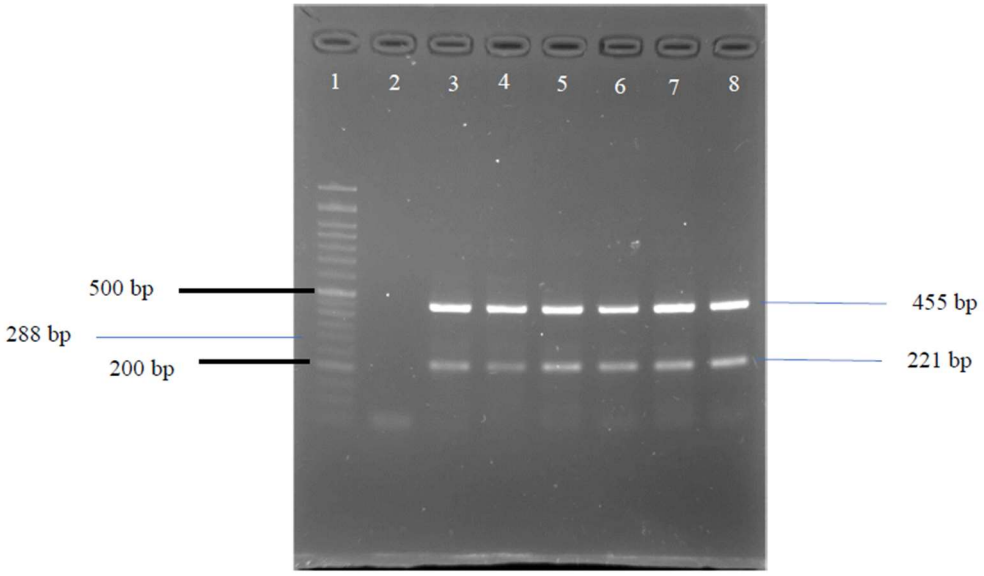


Figure 4.6: Test run result of rs1801282 with 62 °C annealing temperature. Lane 1: 50 bp Ladder; Lane 2: NTC; Lane 3: sample 067; Lane 4: sample 069; Lane 5: sample 070; Lane 6: sample 071; Lane 7: sample 072; Lane 8: sample 074

4.5.1 Genotype of apM1 Variant: rs1501299

Figure 4.7(a) shows the representative genotyping gel image for rs1501299. In analysis, wildtype G allele and mutant T allele were detected at 244 bp and 292 bp, respectively. DNA band with amplicon size of 476 bp was the internal control that amplified using the outer primers set on the target gene. As in lane 3, presence of single DNA band at 244 bp, denoted as genotype of wildtype (GG). Lane 4 to 6 detected with presence of single DNA band at 292 bp, identified as homozygous mutant with TT genotype. Meanwhile, two DNA bands at position of 292 bp and 244 bp reveal heterozygous genotype (GT) together with internal control of 476 bp. All the samples were run in parallel with non-template control to examine for any contamination throughout the PCR preparation. Lane 2 with absence of any amplicons, indicating the PCR was done without contamination.

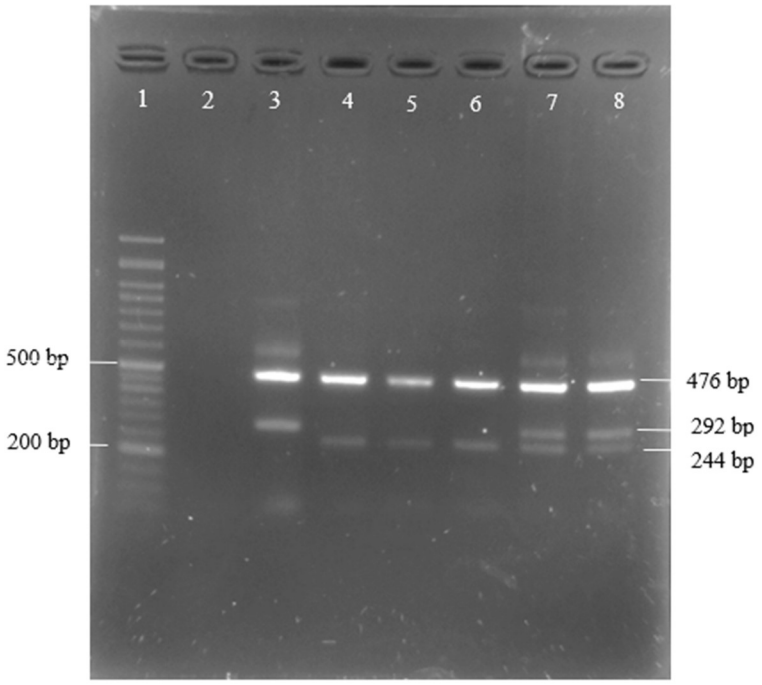


Figure 4.7(a): Representative agarose gel image of rs1501299 genotyping.
Lane 1: 50 bp Ladder; Lane 2: Non-DNA template; Lane 3: sample 005 (TT); Lane 4: sample 017 (GG); Lane 5: sample 007 (GG); Lane 6: sample 022 (GG); Lane 7: sample 001 (GT); Lane 8: sample 040 (GT)

4.5.2 Genotype of PPAR*y* Variant: rs1801282

Figure 4.7(b) demonstrates the representative genotyping gel image for detection of rs1801282. Amplicon size at 221 bp shows wildtype G allele while 288 bp indicates mutant C allele. Internal control amplicons at 455 bp should be present in all the samples, indicating successful amplification of the target gene. Presence of only 221 bp as in Lane 3 to 6, indicated as wildtype genotype (GG). Lane 7 and 8 showed all the three amplicons at 221 bp, 288 bp and 455 bp, indicating a heterozygous genotype (CG). Lane 2, NTC showed no amplicons, and no contamination was created during PCR preparation.

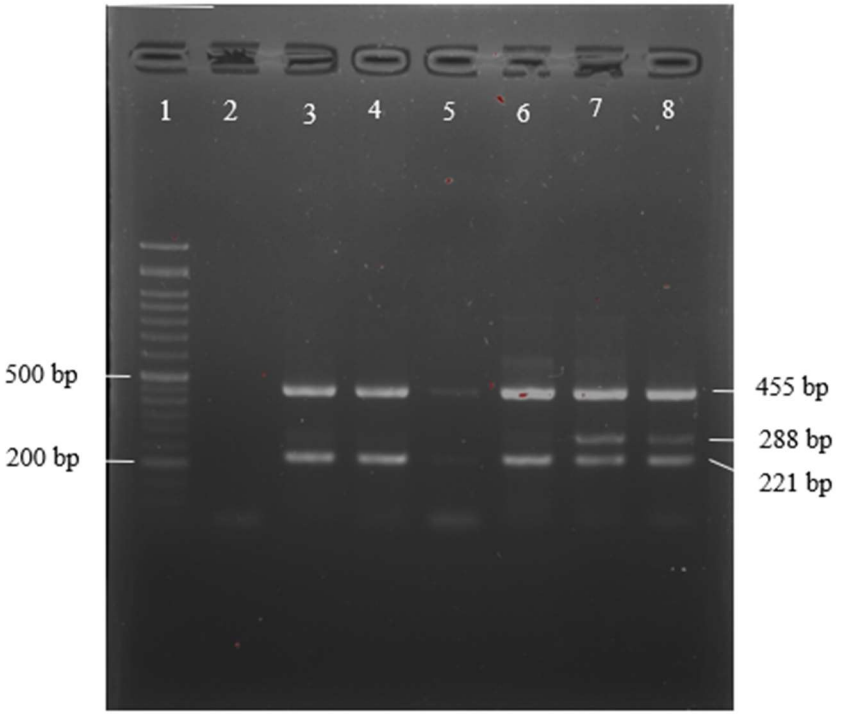


Figure 4.7(b): Representative agarose gel image of rs1801282 genotyping. Lane 1: 50 bp Ladder; Lane 2: Non-DNA template; Lane 3: sample 017(GG); Lane 4: sample 005 (GG); Lane 5: sample 010 (GG); Lane 6: sample 006 (GG); Lane 7: sample 007 (GC); Lane 8: sample 022 (GC)

4.6 DNA Sequencing

Two DNA samples had been selected randomly for sequencing analysis to validate the genotyping result for rs1501299 and rs1801282. The DNA sequencing was conducted by 1st base gene lab using ABI sequencer. Sequencing results were analyzed and aligned using DNA Baser, which was presented in Figure 4.8 (a) and Figure 4.8 (c). Besides, SNP information was also retrieved from National Library of Medicine (NCBI) SNP database to validate with the sequences of target SNP. From Figure 4.8 (a) to (d), both SNPs rs1501299 and rs1801282 were detected with the correct target site with presence of specific allele. This has further verified the genotyping result of tetra primer ARMS PCR.

(i) (ii)

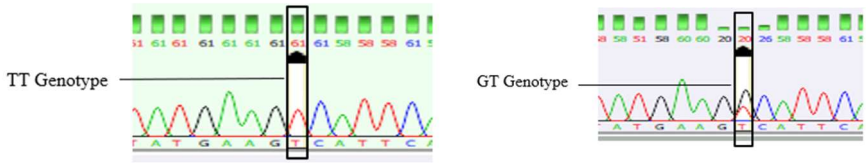


Figure 4.8 (a): Chromatogram of rs1501299 DNA sequencing.
(i) Presence of single red peak, indicating presence of T allele only, TT genotype for rs1501299. (ii) Presence of two peak, red and black peak, indicating presence of G and T alleles, detected as heterozygous with GT genotype.

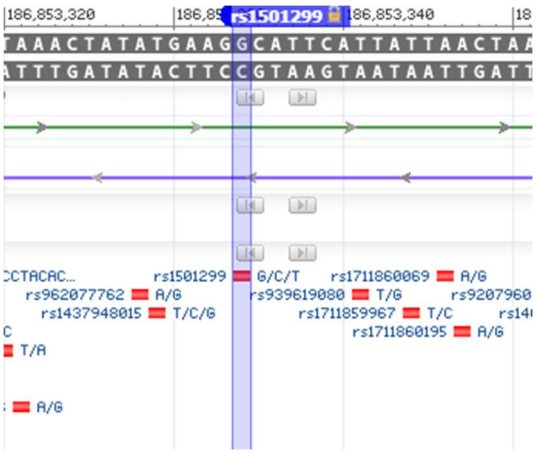


Figure 4.8 (b): Nucleotide sequence of rs1501299 (National Centre of Biotechnology Information, 2020).

(i) **(ii)**

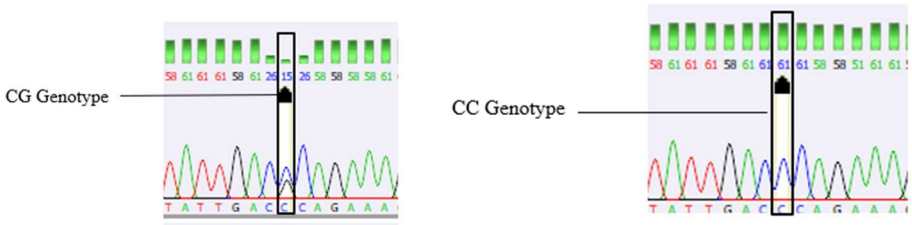


Figure 4.8 (c): Chromatogram of rs1801282 DNA sequencing
(i) Presence of two peaks, blue and black peak, indicating presence of C and G alleles, identified as CG genotype for rs1501299. (ii) Presence of a single peak, blue peak, indicating presence of C allele, detected as heterozygous with CC genotype.

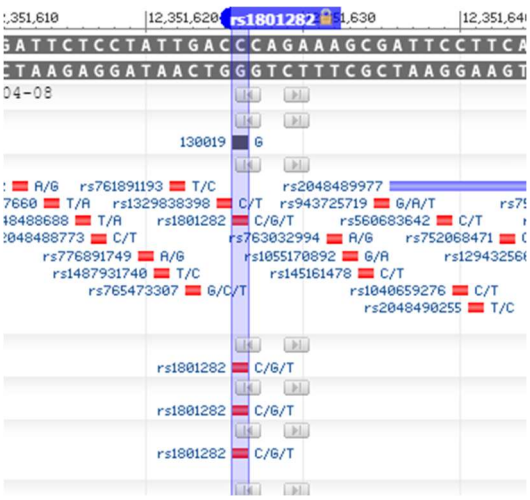


Figure 4.8 (d): Nucleotide sequence of rs1801282 (National Centre of Biotechnology Information, 2020).

4.7 Genotypic and Allelic Frequency of rs1501299 and rs1801282

Frequency of genotypic and allelic for both rs1501299 and rs1801282 is shown in Table 4.4. A total of three genotypes of rs1501299 had been found in subjects, with prevalence rate of 55.29% in wildtype homozygous (GG), 10.91% of mutant homologous (TT) as well as heterozygous (GT) with 37.65%. Minor allele frequency (MAF) was found at 0.412 (MAF >0.05), indicated as a significant SNP in this population. There were only 2 genotypes being detected for rs1801282 which are wildtype homozygous (CC) with 91.76% and heterozygous (GC) with 8.24%. Variations in rs1801282 found to be not significant in the targeted population due to presence of low MAF at 0.041 (MAF<0.05), showed that this SNP was not a significant SNP in this population.

Table 4.4: Genotype and allele frequency of rs1501299 and rs1801282.

Gene Variants	Genotypic frequency n (%)			Allelic frequency	
	GG	**TT**	**GT**	**G**	**T**
rs1501299 (G > T) n = 85	47 (55.29)	6 (10.91)	32 (37.65)	0.588	0.412
rs1801282 (G > C) n = 85	GG (91.76) 78	CC (0.00) 0	GC (8.24) 7	**G** 0.959	**C** 0.041

4.8 Normality Test for Metabolic Risk Factors

Normality tests which were Kolmogorov-Smirnov and Shapiro-Wilk tests had been executed on FBG, SBP, DBP, TC and WC for normality assessment. Result of the normality test is tabulated in Table 4.5. The data was normally distributed for SBP, DBP, TC, and log-transformed FBG, which show p-value more than 0.10 at 90% CI. However, WC found to be non-normally distributed with p-value less than 0.05 in both tests. Thereby, one-way ANOVA was carried out

for Log_{10}FBG, SBP, DBP and CHO while WC was assessed through non-parametric Kruskal Wallis test.

Table 4.5: Normality test (Kolmogorov-Smirnov and Shapiro-Wilk) on MRFs.

Metabolic Risk Factor	Kolmogorov-Smirnov		Shapiro-Wilk	
	df	Sig.	df	Sig.
Log_{10}FBG (mmol/L)	86	0.200*	86	0.377*
CHO (mg/dL)	86	0.200*	86	0.577*
SBP (mmHg)	86	0.200*	86	0.008
DBP (mmHg)	86	0.177*	86	0.001
WC (cm)	86	0.001	86	0.000

df = degree of freedom
* Significant difference with p >0.1

4.9 CMBC Types in Relation to Metabolic Risk Factors

The association between CMBC types and MRFs including prediabetes, hypertension at different stages, hypercholesterolemia and central obesity had been carried out via Chi-Square test and as presented in Table 4.6. Significant difference was interpreted when the p-value is <0.1.

In this study, there was no association between MRFs of prediabetes, hypercholesterolemia, and central obesity with body constitution (p >0.1), indicating there was no relationship between each CMBC type with these MRFs. Hypertension was found associated with the body constitution with the p-value <0.1 at 90% CI, showing there was a relationship between CMBC types and different stages of hypertension.

Table 4.6: CMBC types in relation to prevalence of metabolic risk factor.

Metabolic Risk Factor		Body Constitution Type n (%)							X^2; p-value
		Gentleness	Yang deficiency	Yin deficiency	Qi deficiency	Single Group[a]	Qi stagnation	Multiple Group[b]	
FBG	**Normal**	16 (84.2)	10 (100.0)	15 (93.8)	12 (100.0)	9 (90.0)	9 (90.0)	7 (87.5)	3.77
	Prediabetes	3 (15.8)	0 (0.0)	1 (6.3)	0 (0.0)	1 (10.0)	1 (10.0)	1 (12.5)	0.708
WC	**Normal**	17 (89.5)	8 (80.0)	11 (68.8)	11 (91.7)	10 (100.0)	9 (90)	6 (75)	6.70
	Central Obesity	2 (10.5)	2 (20.0)	5 (31.3)	1 (8.3)	0 (0)	1 (10)	2 (25)	0.349
CHO	**Normal**	12 (63.2)	5 (50.0)	6 (37.5)	9 (75.0)	6 (60.0)	5 (50.0)	7 (87.5)	7.81
	Hypercholesterolemia	7 (36.8)	5 (50.0)	10 (62.5)	3 (25.0)	4 (40.0)	5 (50.0)	1 (12.5)	0.252
BP	**Normal**	13 (58.4)	8 (80.0)	8 (50.0)	10 (83.3)	9 (90.0)	7 (70.0)	1 (12.5)	29.83
	Elevated hypertension	2 (10.5)	0 (0.0)	4 (25.0)	0 (0.0)	0 (0.0)	2 (20.0)	5 (62.5)	0.039 *
	Hypertension Stage 1	4 (21.1)	1 (10.0)	3 (18.8)	1 (8.3)	1 (10.0)	0 (0.0)	1 (12.5)	
	Hypertension Stage 2	0 (0.0)	1 (10.0)	1 (6.3)	1 (8.3)	0 (0.0)	1 (10.0)	1 (12.5)	

*Significant data with p-value <0.1

[a]single group of CMBC type consists of Tan Shi, Shi Re, Xue Yu and Te Bing

[b]multiple group CMBC type consists of Yin Xu & Qi Xu, Yang Xu & Qi Xu, Shi Re & Qi Yu, Xue Yu & Te Bing, Te Bing and Qi Yu, Yin Xu & Qi Xu & Shi Re.

One-Way ANOVA has been carried out to compare the mean of SBP, DBP, CHO and Log_{10}FBG between CMBC types. Table 4.7 depicted the metabolic assessment readings with CMBC types. There were no significant differences between SBP, DBP and CHO with different CMBC types ($p > 0.1$, 90% CI). Results shows that there was a significant difference between Log_{10}FBG with different CMBC types.

Meanwhile, Kruskal Wallis test was carried out to compare the difference between WC on different CMBC types. There was no significant difference between WC and CMBC type at p-value of 0.293 as shown in Table 4.8.

Table 4.7: CMBC types in relation to metabolic risk factor (One-Way ANOVA).

Metabolic Risk Factor	Body Constitution Type (mean ± SD)							p-value
	Gentleness (n = 19)	Yang deficiency (n = 10)	Yin deficiency (n = 16)	Qi deficiency (n = 12)	Single Group[a] (n = 10)	Qi stagnation (n = 10)	Multiple Group[b] (n = 8)	
$Log_{10}FBG$	0.70 ± 0.37	0.66 ± 0.30	0.70 ± 0.39	0.69 ± 0.34	0.68 ± 0.37	0.68 ± 0.43	0.70 ± 0.31	**0.086 ***
CHO	190.11 ± 29.27	197.40 ± 19.94	203.00 ± 26.25	191.83 ± 19.51	192.30 ± 23.22	192.90 ± 18.10	174.38 ± 22.03	0.234
SBP	114.11 ±11.96	109.50 ±19.99	115.88 ±16.06	109.42 ±17.52	104.60 ±11.03	110.20 ± 14.92	121.13 ± 14.81	0.256
DBP	72.89±7.39	74.00±14.73	75.19±8.92	70.67±8.35	66.00±7.63	72.10±9.12	77.50±6.07	0.152

*Significant data with p-value less than 0.1.

[a]single group of CMBC type consists of Tan Shi, Shi Re, Xue Yu and Te Bing

[b]multiple group CMBC type consists of Yin Xu & Qi Xu, Yang Xu & Qi Xu, Shi Re & Qi Yu, Xue Yu & Te Bing, Te Bing and Qi Yu, Yin Xu & Qi Xu & Shi Re.

Table 4.8: CMBC types in relation to WC (Kruskal Wallis test).

Metabolic Risk Factor	Body Constitution Type (mean ± sd)							p-value
	Gentleness (n = 19)	Yang deficiency (n = 10)	Yin deficiency (n = 16)	Qi deficiency (n = 12)	Single Group[a] (n = 10)	Qi stagnation (n = 10)	Multiple Group[b] (n = 8)	
WC	75.99±7.66	75.20±11.31	78.83±10.99	74.44±10.17	72.59±5.61	76.15±14.45	83.03±10.46	0.293

[a]single group of CMBC type consists of Tan Shi, Shi Re, Xue Yu and Te Bing
[b]multiple group CMBC type consists of Yin Xu & Qi Xu, Yang Xu & Qi Xu, Shi Re & Qi Yu, Xue Yu & Te Bing, Te Bing and Qi Yu, Yin Xu & Qi Xu & Shi Re.

4.10 CMBC Types in Relation to Metabolic Gene Variants (rs1501299 and rs1801282)

Chi-square test had been executed to assess the relationship between different CMBC types and rs1501299 as well as rs1801282 genotypes. Table 4.9 depicted the genotypes and the CMBC types. There was no significant difference between different genotypes of rs1501299 among seven different CMBC types ($p > 0.1$, 95% CI). Similar findings were demonstrated between different CMBC types and rs1801282 as there was no significant difference between each genotype and CMBC types ($p > 0.1$; 90% CI).

Table 4.9: CMBC types in relation to metabolic gene variants.

Gene Variant	Genotype	Body Constitution Type n (%)							X^2; p-value
		Gentlenes s	Yang deficiency	Yin deficiency	Qi deficiency	Single Group[a]	Qi stagnatio n	Multiple Group[b]	
rs1501299	**GG**	10 (52.6)	8 (80.0)	9 (56.3)	7 (58.3)	6 (60.0)	5 (50.0)	2 (25.0)	10.07 0.610
	TT	2 (10.5)	1 (10.0)	1 (6.3)	1 (8.3)	1 (10.0)	0 (0.0)	0 (0.0)	
	GT	7 (36.8)	1 (10.0)	6 (37.5)	4 (33.3)	3 (30.0)	5 (50.0)	6 (75.0)	
rs1801282	**CC**	18 (94.7)	7 (70.0)	14 (87.5)	12 (100.0)	9 (90.0)	10 (100.0)	8 (100.0)	9.61 0.142
	CG	1 (5.3)	3 (30.0)	2 (12.5)	0 (0.0)	1 (10.0)	0 (0.0)	0 (0.0)	

[a]single group of CMBC type consists of Tan Shi, Shi Re, Xue Yu and Te Bing

[b]multiple of CMBC type consists of Yin Xu & Qi Xu, Yang Xu & Qi Xu, Shi Re & Qi Yu, Xue Yu & Te Bing, Te Bing and Qi Yu, Yin Xu & Qi Xu & Shi Re.

4.11 Metabolic Gene Variants (rs1501299 and rs1801282) in Relation to Metabolic Risk Factors

4.11.1 Metabolic Gene Variants (rs1501299 and rs1801282) in Relation to Prevalence of Metabolic Risk Factors

Association between each genotype of rs1501299 and metabolic risk factor had been carried out by Chi-Square test. Table 4.10 shows that there is no association between rs1501299 and all metabolic risk factor including prediabetes (X^2 (3) = 0.59, p = 0.741), central obesity (X^2 (3) = 0.31, p = 0.857), hypertension (X^2 (5) = 0.78, p = 0.448) as well as hypercholesterolemia (X^2 (3) = 3.15, p = 0.207). Chi-Square test also had been carried out between rs1801282 genotype and all metabolic risk factors including prediabetes (X^2 (3) = 0.59, p = 0.741), central obesity (X^2 (3) = 0.31, p = 0.857), hypertension (X^2 (5) = 5.78, p = 0.448) as well as hypercholesterolemia (X^2 (3) = 3.15, p = 0.207). Result had been presented in Table 4.11. The outcome also showed no association between rs1801282 genotype and all metabolic risk factors.

Table 4.10: rs1501299 in relation to prevalence of metabolic risk factors.

Metabolic Risk Factor		Genotype rs1501299			X^2; p-value
		GG	TT	GT	
FBG	Normal	43 (91.5)	6 (100.0)	29 (90.6)	0.59 0.741
	Prediabetes	4 (8.5)	0 (0.0)	3 (9.4)	
WC	Normal	39 (83.0)	5 (83.3)	28 (87.5)	0.31 0.857
	Central Obesity	8 (17.0)	1 (16.7)	4 (12.5)	
CHO	Normal	26 (55.3)	2 (33.3)	22 (68.8)	3.15 0.207
	Hypercholesterolemia	21 (44.7)	4 (66.7)	10 (31.3)	
BP	Normal	32 (68.1)	4 (66.7)	20 (62.5)	5.78 0.448
	Elevated hypertension	5 (10.6)	0 (0.0)	8 (25.0)	
	Hypertension Stage 1	7 (14.9)	1 (16.7)	3 (9.4)	
	Hypertension Stage 2	3 (6.4)	1 (16.7)	1 (3.1)	

Table 4.11: rs1801282 in relation to prevalence of metabolic risk factors.

Metabolic Risk Factor		Genotype rs1501299		X^2; p-value
		GG	GT	
FBG	Normal	71 (91.0)	7 (100.0)	0.59 0.741
	Prediabetes	7 9.0)	0 (0.0)	
WC	Normal	66 (84.6)	6 (85.7)	0.31 0.857
	Central Obesity	12 (15.4)	1 (14.3)	
CHO	Normal	47 (60.3)	3 (42.9)	3.15 0.207
	Hypercholesterolemia	31 (39.7)	4 (54.1)	
BP	Normal	50 (64.1)	6 (85.7)	5.78 0.448
	Elevated hypertension	12 (15.4)	1 (14.3)	
	Hypertension Stage 1	11 (14.1)	0 (0.0)	
	Hypertension Stage 2	5 (6.4)	0 (0.0)	

4.11.2 Metabolic Gene Variant (rs1501299 and rs1801282) in Relation to Measurements of Metabolic Risk Factors

Table 4.12 and 4.13 present the one-way ANOVA result for rs1501299 genotype and rs1801282 with log_{10}FBG level, systolic and diastolic blood pressure, respectively. Table 4.14 presents the Kruskal Wallis comparison analysis for rs1501299 and rs1801282 genotypes with waist circumference. All tests show outcomes of p-value larger than 0.1 thus no significant difference between bath metabolic gene variants and measurements of metabolic risk factors at 90% CI. Besides, Kruskal Wallis test conducted also show no significant difference in WC among each genotype of rs1501299 and rs1801282.

Table 4.12: rs1501299 in relation to measurement metabolic risk factors.

rs1501299	**Genotype**			**p-value**
	GG (n = 47)	TT (n = 6)	GT (n = 32)	
Log_{10}FBG	0.69±0.40	0.69±0.35	0.68±0.40	0.745
CHO	195.26±23.49	186.72±22.29	205.17±35.48	0.132
SBP	111.68±15.70	112.81±11.12	114.67±25.28	0.732
DBP	73.15±10.07	72.47±7.41	70.00±12.76	0.875

Table 4.13: rs1801282 in relation to measurement metabolic risk factors.

rs1801282	**Genotype**		**p-value**
	CC (n = 78)	**CG (n = 7)**	
Log_{10}FBG	0.69±0.39	0.68±0.23	0.765
CHO	192.24±24.19	198.29±26.44	0.531
SBP	112.78±15.02	107.14±11.96	0.224
DBP	73.04±9.51	68.57±4.58	0.338

Table 4.14: Metabolic gene variants in relation to measurement of WC (Kruskal Wallis test).

Metabolic gene variants				
rs1501299		**Genotype**		**p-value**
	GG	**TT**	**GT**	
WC	**(n = 47)**	**(n = 6)**	**(n = 32)**	0.813
	76.03±10.41	76.86±9.40	78.17±14.02	
rs1801282				
	CC		**CG**	
WC	**(n = 78)**		**(n = 7)**	0.388
	76.67±10.15		74.50±11.30	

CHAPTER 5

DISCUSSION

5.1 Prevalence of CMBC Types

The prevalence of CMBC types among studied subjects shows the highest prevalence in gentleness with 22.4%. This indicated that the majority of study subjects consisted of a balanced constitution with low chance of developing disease and tended to have higher quality of life regarding health (Wong et al., 2013). This result is compatible with findings in China in which gentleness was reported at the highest prevalence rate (Wang and Zhu, 2009). Some inconsistency on distribution of CMBC types was observed as the subsequent predominant CMBC types found in China population were Qi deficiency (13.42%), dampness heat (9.08%) and Yang deficiency (9.04%) whereas our study presented the second most were Yin deficiency (18.8%) and Qi deficiency (14.1%) (Wang and Zhu, 2009). This discrepancy presented that the geographical location and climate factors could contribute to different CMBC types.

Yin deficiency shows the second highest prevalence of biased CMBC in Malaysia, which can be explained by unique dietary habits among different countries. High abundance of fried food is available in Malaysia such as fried banana (pisang goreng) and crunchy fish cake (keropok) are considered as “heating food” which will produce excess internal heat within body and subsequently causing fluid loss in body as explained in TCM theory (Huang and

Wu, 2002). Overabundance of internal heat will lead to fluid (Yin) retention which leads to formation of Yin deficiency CMBC (Li et al., 2017). Meanwhile, biased CMBC type with high prevalence in China is Qi deficiency, which may be caused by seasonal change which creates a sudden change in atmosphere temperature that had not occurred in Malaysia (Paresis and Amelanotic, 2013).

Besides, another major CMBC type, Qi stagnation (11.8%) was found in this study. This could be attributed as the studied population consisted mainly of university students. In earlier study, university students had been reported with high prevalence of anxiety due to the academic and study stress (Mohammad et al., 2021). According to TCM theory, psychological stress including depression and anxiety will lead to liver dysfunction, which is the main organ accounted for by Qi circulation within the body thus leading to Qi stagnation (Li et al., 2017). A contradict result in phlegm dampness among studied population and China population had been found, which are 2.4% and 9.08%, respectively. Formation of phlegm dampness is contributed by hot and humid environments such as Guang Zhou in China which will lead to high consumption of food with high sugar and fat (Deng and Lai, 2013; Li et al., 2022). However, there is a very low prevalence of phlegm dampness in this studied Malaysian population who experience hot and humid weather. This can be explained by limited sample size and involvement of subjects in specific geographical conditions (Chan and Chien, 2008).

In comparison with another CMBC study carried out in the Malaysian population which was reported by Chong et al. in year 2018, a similar prevalence trend had been found. In that study, Qi deficiency (17.4%), Yin deficiency (14.4%), Qi stagnation (13.9%) and Yang deficiency (10.4%) show highest prevalence among all biased constituents, as consistent with the findings reported in this study. In fact, people in the same population will experience similar environments such as weather and culture, which contribute to the formation of similar CMBC types. Besides, there is a 9.4% prevalence rate of all multiple CMBC types, which only show 1 or 2 subjects in each multiple CMBC type. In fact, one person may possess single or multiple CMBC types at the same time due to their imbalanced lifestyle and eating preference (Lai et al., 2021).

5.1.1 Prevalence of CMBC Types Among Genders

In this study, the higher prevalence of all biased CMBC types in female could be die to slightly higher in number of subjects belongs to female (61.2%, n = 52), which indicate a smaller male population (38.8%, n = 33) had been recruited. A small sample size might not be able to estimate an actual population parameter among a specific population.

CMBC types which show largest deviation in prevalence among different gender is Qi deficiency, followed by Qi stagnation and Yang deficiency. All three BC types found to be associated with dysmenorrhea. (Chong et al., 2018; Gao et al., 2018). All three CMBC types had been associated with abnormal Qi

and Xue circulation which had been related to dysmenorrhea that may be caused by impedance in blood supply toward uterine (Oyelowo, 2007; Sun, 2010).

5.2 Prevalence of Individual Metabolic Risk Factor

The metabolic risk factors investigated in this study were FBG, BP, TC and WC. By referring to cut-off point for FBG, BP, TC and CHO as defined by AHA, 8.3% of subjects were found to be prediabetes, which show a considerably lower rate compared to the general prevalence of prediabetes (14.39%) in Malaysian population (Akhtar et al., 2022). This discrepancy could be due to the specific age range of subjects involved in this study, which have an average of 22 years old. Young adults would show a lower prevalence rate of prediabetes compared to elderly (Akhtar et al., 2022). In fact, aging is found to be a crucial factor in development of chronic metabolic disorder such as T2DM, due to impairment of reduced cell mass and physical inactivation (Suastika et al., 2012). Besides, this deviation may also be due to the major ethnicity in this study as Chinese. Until now, Chinese had been presented with the lowest prevalence of diabetes mellitus compared with Indians and Malay (Akhtar et al., 2022).

Prevalence of central obesity reported in this study was 15.3% in all subjects, which is much lower as compared with the general prevalence (52.6%) of central obesity as reported by MOH in the year 2019. In fact, there is a large deviation in prevalence of central obesity in different age groups (Pell et al., 2016). Prevalence of central obesity (11.9) among people in 21-25 years old which reported by Pell et al. in 2016 could be a more precisely referred prevalence for

this project as 81.17% (n = 68) of subjects recruited in this study had been classified in 21-25 years old.

Based on the pressure range as defined by AHA at 130/80 mmHg, the prevalence of hypertension in this study was 12.9%. This prevalence was found much lower when compared with the prevalence of hypertension among Malaysian population as reported in 2015 was at 35.3% (Ministry of Health, 2018). Again, this deviation can be corrected by adjusting the prevalence of HTN with a more specific population. Prevalence of hypertension of university students in Malaysia was found to be 10.0%, which shows a higher compatibility with prevalence of hypertension found in this project which involved University students in majority (Soo et al., 2020).

The prevalence of hypercholesterolemia reported in this study was found consistent with the official prevalence of hypercholesterolemia in Malaysia, which are 41.2% and 38.1%, respectively (Ministry of Health, 2019). Higher prevalence of hypercholesterolemia found to be associated with high fat food intake among university students in Malaysia (Cheng and Kamil, 2020).

5.3 Genotype of Metabolic Gene Variants (rs1501299 & rs1801282)

Genotyping of rs1501299 and rs1801282 had been conducted through Tetra primer ARMS PCR as this method is time saving and cost effective (Lajin et al., 2013). A total of four primers had been used which consisted of two outer primer and two inner primer sets, each set consists of forward and reverse primer. The outer primer sets allow amplification of non-specific DNA sequence which

target SNP located among this sequence. This amplified non-specific DNA sequence will serve as template for inner primer and act as internal control in genotyping (Zhang et al., 2013).

Afterwards, inner primers or allele specific had been used to differentiate different SNPs found in amplified fragments. In more details, a mismatch had been designed on the 3' terminus of each forward and reverse inner primer (Medrano and De Oliveira, 2014). For rs1501299, inner forward primer used to target wildtype G allele while DNA strand with mutant T allele will be replicated by reverse inner primer (Hashemi et al., 2013). Meanwhile, forward, and reverse inner primer had been used to allow replication of C and G allele, respectively (Masud and Ye, 2013). As a result, SNP can be differentiated as two different sizes of fragment which produced by outer forward and inner reverse primer set as well as forward inner and reverse inner primers (Medrano and De Oliveira, 2014).

5.4 Genotypic and allelic frequency of rs1501299 and rs1801282

Allelic frequency of rs1501299 in this study is 0.588 and 0.411 for wildtype G allele and mutant T allele. This was found inconsistent with the allelic frequency published by NCBI with 0.729 and 0.271 for major and minor allele frequency among Asian population. This discrepancy could be due to differences in the studied ethnicity in Asian population in which this study consisted of 90% of Chinese subjects. The allele frequency of rs1501299 in this study was found more consistent with the Han Chinese population with wild type and mutant allele at 0.691 and 0.309, indicating Chinese in the study population could be

from the same originates among Han Chinese (Tsai et al., 2014). The MAF of rs1501299 was at 0.411, revealing that rs1501299 was a significant variant among the Malaysian population with a cut-off threshold of 0.05.

For rs1801282, the major or wildtype C allele show allelic frequency of 0.959 while minor or mutant G allele show 0.041 allele frequency. This result was found compatible with allelic frequency in Asian population as published in NCBI (C allele = 0.96; G allele = 0.04). Minor allele frequency of rs1801282 is less than 0.05 which indicates rs1801282 is an insignificant variant among the Malaysian population. This is found consistent with Asian population as reported in NCBI SNP.

5.5 Normality Test for Metabolic Risk Factors

Kolmogorov-Smirnov together with Shapiro-Wilk tests had been carried out for normality tests on measurement of metabolic risk factors. Shapiro-Wilk tests proved to be higher-power in normality tests while Kolmogorov-Smirnov is more suitable for sample size larger than 50 (Razali and Wah, 2011; Mishra et al., 2019). CHO, SBP, DBP showed normal distribution while FBG showed normal distribution after log transformation which aimed to reduce data skewness (Changyong et al., 2014). In other words, WC found to be non-normally distributed which may be resulted by relatively small sample size in this project. According to the central limit theorem, sample size is directly proportional to the tendency of having a normal distribution (Arya and Kumar, 2012).

5.6 CMBC Types in Relation to Metabolic Risk Factors

5.6.1 CMBC Types in Relation to Prevalence of Metabolic Risk Factor

According to the Chi-square test, p-value <0.1 indicates a significant association between the prevalence of MRFs with different CMBC types (Chan, 2003). Hypertension is the only metabolic risk factor found associated significantly with CMBC types with p-value of 0.038 (p-value < 0.1). Yin deficiency is found to be positively correlated with hypertension which shows compatible findings in Taiwan Chinese population (L et al., 2021). This could be attributed as Yin deficiency individuals were impaired in body fluid circulation, inefficient body fluid replenishment would lead to accumulation of internal heat within the body. Build-up of heat or Yang hyperactivity said to be contributed to hypertension (Liao et al., 2021).

Meanwhile, hypercholesterolaemia and central obesity found no association with CMBC types with p-value of 0.708 and 0.349, respectively. This result is controversial with previous studies, in which phlegm dampness and Qi deficiency were found to be a significant risk factor in hyperlipidaemia (Li et al., 2019). Phlegm dampness will result from inner stagnation of phlegm within the body. This will contribute to irregularity in transportation and transformation of Qi in spleen and liver (Li et al., 2019). Impaired Qi circulation in spleen and liver will lead to inappropriate kidney function in transpiration as well as gasification (Li et al., 2019). Defects in liver and spleen function which are responsible for Qi production will lead to formation of Qi deficiency CMBC which in turn impedes spleen function and creates a vicious cycle (Li et al., 2019).

5.6.2 CMBC types in Relation to Measurement of Metabolic Risk Factors

Among all the metabolic risk factors (Log_{10}FBG, CHO, WC, SBP and DBP) that were analysed in this study, only log-transformed FBG found to be significant difference among CMBC groups, with p-value of 0.086. Yang deficiency was found to have the lowest FBG level among all CMBC types, which indicates there is no association of this CMBC type with DM. The result is contradictory to previous findings which show a positive correlation between Yang deficiency and DM as reported by Lee et al. in 2015. In TCM theory, people with Yang deficiency showed a diminished energy in conducting physiological activity and affecting functioning of spleen and liver, which is the main organ in regulation of glucose metabolism (Xu et al., 2017). As a result, abnormal energy metabolism will lead to DM. This conflicting result can be explained by findings that different CMBC types had been related with the same disease among different populations. For instance, Yin deficiency is found to be positively associated with DM in the Han Chinese population while phlegm dampness shows identical correlation among the Uighur population (Dang et al., 2018). Thus, this may conclude that CMBC types would present the predisposition to certain disease.

5.7 CMBC Types in Relation to Metabolic Gene Variants (rs1501299 and rs1801282)

Chi square test was carried out and no significant difference between CMBC types with rs1501299 and rs1801282 was shown, with p-value >0.1. This was found to contradict with the findings by Wu and co-author in 2010, which is the only study which investigates association between CMBC types and APM1 and

PPARy variants among 4 CMBC types (Gentleness, Phlegm dampness, Yang deficiency and Yin deficiency). In this study, mutant G allele of rs1801282 found to be negatively associated with Yin deficiency constitution. People with mutant G allele show a repressed lipolysis in adipose tissue which in turn increases insulin sensitivity in adipose tissue (Stumvol et al., 2001). This action is counteracting the predisposition of T2DM development in Yin deficiency, which impaired fluid circulation found to be associated with disorder in hypothalamic pituitary-adrenal axis function (Bruehl et al., 2007; hu et al., 2017).

Nevertheless, the association between CMBC types and PPARy Pro12Ala showed no significant difference in this study. Incompatibility of results with previous findings may be caused by different population studies, which consists of different critical gene variants in metabolic disorder development (Sirugo, 2019). Meanwhile, rs1501299 also has no association with all CMBC types, revealing a similar result in the study stated above (Wu et al., 2010). This project also shows no correlation between rs1501299 with the five other CMBC types among the Malaysian population.

5.8 Metabolic Gene Variants (rs1501299 and rs1801282) in Relation to Metabolic Risk Factors

Chi-square analysis showed no significant association between metabolic risk factor among different genotypes of rs1501299, with p-value >0.1. Consistent findings were seen with a meta-analysis conducted by Han and co-author who reported no association had been found between rs1501299 and prevalence of T2DM. However, a mutant allele of rs1501299 had been found to be positively

associated with abdominal obesity in the Russian population (Shramko et al., 2021).

Similar to rs1501299, genotypes of rs1801282 show no significant difference in all metabolic risk factors measurement in this project. Similar results had been suggested by Radha et al. in 2006 that there was no significant difference in genotype of rs1801282 among diabetic patients and healthy patients in the South Asian population. However, the C mutant allele of rs1801282 had been associated with lower risk of T2DM development in the Japanese population, (Mori et al, 2001).

These contradictory results can be explained by different gene polymorphisms may be found in different populations (National Library of Medicine, 2022). This opposite result may be caused by small sample size carried out in this project which could not provide a definitive conclusion on the association between rs1501299 and rs1801282 with MRFs. Moreover, development of metabolic disorder is multifactorial.

5.9 Limitations

This study consists of a relatively smaller sample size which will cause underestimation of validity in a study and might not be able to show a true finding or show similar results with previous studies that were carried out on a large scale. Besides, only 3 out of 5 metabolic risk factors which had been defined as metabolic syndrome were measured in this study. Metabolic risk factors included in diagnostic criteria of metabolic syndrome show a higher

accuracy in estimation of metabolic disorder development based on CVD mortality and total mortality (Samson and Garber, 2014).

5.10 Future Recommendations

A larger sample size was suggested to achieve 95% CI which can provide a higher reliability in estimation of ant parameters within a population (Hazra, 2017). Moreover, lipid profiles with higher specificity for example measurement of triglyceride level and high-lipoprotein cholesterol level which had been proved as better indicators for atherogenic dyslipidaemia can be measured as metabolic risk factors (Zhu et al., 2018).

CHAPTER 6

CONCLUSION

Overall, Gentleness or balanced CMBC type showed the highest prevalence. The predominant biased CMBC types in the Malaysian population were Yin deficiency, Qi deficiency, Yang deficiency and Qi stagnation. The prevalence of CMBC was found to be various among different populations but similarity within the same population. Females showed a higher prevalence in all single biased BC types when compared with male. Prevalence of metabolic risk factors of FAG, WC and BP was found to be lower in compared with prevalence published by MOH, which may be due to younger age group and biased ethnicity in population studied. Among all MRFs measured, BP and FBG found significant differences among BC types, in which Yin deficiency showed higher prevalence in subjects with hypertension while Yang deficiency showed a lower FBG among subjects. For metabolic gene variants tested, rs1501299 was found to be a significant variant among Malaysian population while rs1801282 considered as insignificant variant, which both results were consistent with significance of variant in Asia population. Both metabolic gene variants showed no association with CMBC types at 90% confidence interval. There was also no significant difference in genotype of metabolic gene variants with metabolic risk factors among the population studied. However, limited sample size in this study may not provide a conclusive finding for association of rs1501299 and rs1801282 among different BC types and metabolic risk factors in Malaysia population.

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APPENDIX A

Subject ID	Age	Gender	Ethnicity
001	21	MALE	Chinese
002	21	MALE	Chinese
003	27	MALE	Chinese
004	22	FEMALE	Chinese
005	21	FEMALE	Chinese
006	22	FEMALE	Chinese
007	22	MALE	Chinese
008	22	FEMALE	Chinese
009	23	FEMALE	Chinese
010	23	MALE	Indian
011	22	MALE	Chinese
012	21	MALE	Chinese
013	21	FEMALE	Chinese
014	21	FEMALE	Chinese
015	22	FEMALE	Indian
016	21	FEMALE	Chinese
017	18	FEMALE	Chinese
018	22	MALE	Indian
019	21	FEMALE	Chinese
020	21	MALE	Chinese
021	21	MALE	Chinese
022	21	FEMALE	Chinese
023	22	MALE	Chinese
024	23	FEMALE	Chinese
025	23	FEMALE	Chinese
026	23	FEMALE	Chinese
027	28	MALE	Chinese
028	20	FEMALE	Chinese
029	21	MALE	Chinese
030	21	MALE	Chinese
031	21	FEMALE	Chinese
032	21	FEMALE	Chinese
033	21	MALE	Chinese
034	21	FEMALE	Chinese
035	21	MALE	Chinese
036	23	MALE	Chinese
037	23	MALE	Chinese
038	22	FEMALE	Chinese
039	23	FEMALE	Indian
040	20	MALE	Chinese
041	21	FEMALE	Chinese
042	21	MALE	Chinese
043	21	FEMALE	Chinese
044	24	FEMALE	Indian
045	20	FEMALE	Chinese
046	21	FEMALE	Chinese

047	21	FEMALE	Chinese
048	21	FEMALE	Chinese
049	28	MALE	Chinese
050	21	FEMALE	Chinese
051	21	FEMALE	Chinese
052	21	FEMALE	Chinese
053	21	FEMALE	Chinese
054	21	FEMALE	Chinese
055	20	FEMALE	Chinese
056	21	FEMALE	Chinese
057	24	FEMALE	Bumiputera
058	22	FEMALE	Indian
059	21	FEMALE	Chinese
060	23	FEMALE	Chinese
061	22	MALE	Chinese
062	18	MALE	Chinese
063	18	FEMALE	Chinese
064	23	FEMALE	Indian
065	21	MALE	Chinese
066	25	MALE	Chinese
067	32	MALE	Malaly
068	21	FEMALE	Chinese
069	21	FEMALE	Chinese
070	24	FEMALE	Chinese
071	21	MALE	Chinese
072	22	FEMALE	Chinese
073	22	MALE	Chinese
074	24	MALE	Chinese
075	21	MALE	Chinese
076	31	MALE	Chinese
077	21	FEMALE	Chinese
078	26	FEMALE	Chinese
079	21	FEMALE	Chinese
080	21	MALE	Chinese
081	20	MALE	Chinese
082	19	FEMALE	Chinese
083	22	FEMALE	Chinese
084	22	FEMALE	Chinese
085	19	FEMALE	Chinese

APPENDIX B

Subject ID	FBG (mmol/L)	TC (mg/dL)	SBP (mmHg)	DBP (mmHg)	WC (cm)
001	5.2	164	122	80	70.2
002	5.7	188	117	72	75.0
003	4.6	184	125	74	88.0
004	4.4	193	99	69	69.5
005	4.4	228	95	66	70.5
006	4.6	215	104	79	69.5
007	4.7	184	125	76	63.5
008	4.6	217	103	67	78.0
009	5.2	201	98	73	79.5
010	5.6	126	122	80	78.0
011	5.3	219	124	76	80.0
012	4.9	200	112	66	72.0
013	4.3	190	116	81	69.0
014	4.4	158	108	77	72.0
015	5.3	131	120	79	79.0
016	5.1	209	116	70	76.0
017	4.6	204	103	55	72.0
018	5.4	144	116	77	83.5
019	4.4	180	108	68	64.0
020	5.3	203	136	76	70.0
021	5.3	185	156	90	101.0
022	4.7	239	100	65	66.0
023	5.1	228	150	97	97.0
024	5.3	206	112	72	72.0
025	4.2	224	102	67	62.0
026	4.7	171	94	65	71.0
027	5.4	170	102	73	91.0
028	5.0	202	127	74	92.0
029	4.8	167	139	85	75.5
030	5.1	186	111	73	75.5
031	4.9	199	93	68	64.5
032	4.1	162	99	67	73.0
033	4.7	178	104	66	81.5
034	4.6	195	106	73	65.5
035	4.9	201	129	88	82.0
036	4.3	201	114	66	79.0
037	5.2	211	138	90	112.0
038	4.7	223	87	69	73.0
039	5.6	187	94	60	64.0
040	4.7	172	129	75	89.0
041	5.1	187	117	70	79.5
042	4.9	146	136	80	88.0
043	4.9	194	95	64	71.5
044	6.4	226	121	83	86.4
045	5.0	214	103	68	72.5

046	4.9	226	85	60	63.0
047	4.8	203	111	72	72.0
048	4.9	192	127	77	87.2
049	5.9	177	122	78	81.0
050	4.0	190	105	76	64.5
051	4.6	193	95	58	75.5
052	4.3	187	111	61	67.0
053	5.7	184	121	79	86.5
054	4.3	195	99	64	77.0
055	4.7	204	108	69	65.0
056	4.2	191	108	77	63.0
057	5.1	167	127	78	72.5
058	4.8	207	115	72	74.5
059	4.8	203	95	63	68.5
060	4.6	202	105	69	70.0
061	4.7	166	119	70	83.0
062	5.1	165	124	71	77.5
063	4.8	192	117	90	102
064	4.7	206	106	66	97.5
065	5.0	154	123	79	78.5
066	4.7	218	124	85	90.5
067	4.3	215	159	111	92.5
068	4.9	195	102	74	74.0
069	5.1	191	96	66	71.0
070	4.4	187	103	65	69.0
071	4.6	201	111	69	76.0
072	4.8	243	113	71	68.5
073	5.5	176	111	76	79.6
074	4.7	183	124	89	74.0
075	4.5	161	123	65	87.5
076	4.9	163	102	60	72.7
077	5.1	211	108	71	64.2
078	4.8	191	107	68	70.5
079	5.1	198	117	82	68.3
080	4.6	158	112	63	72.8
081	5.7	170	111	73	71.2
082	4.7	204	92	60	65.9
083	5.1	220	95	63	96.0
084	5.2	244	96	62	75.5
085	5.2	238	91	66	73.0

Appendix C

ID	Yang Xu	Yin Xu	Qi Xu	Tan Shi	Shi Re	Xue Yu	Te Bing	Qi Yu	Ping He	CMBC
001	54	72	72	34	21	29	21	50	31	**Yin Xu & Qi Xu**
002	79	25	53	53	21	61	39	68	31	**Yang Xu**
003	7	25	19	9	21	4	21	14	91	**Gentleness**
004	21	31	19	22	21	39	39	18	56	**Xue Yu & Te Bing**
005	14	41	25	9	21	18	32	39	53	**Yin Xu**
006	39	9	22	3	17	7	7	4	66	**Gentleness**
007	11	13	13	6	0	14	11	7	69	**Gentleness**
008	14	0	41	13	0	14	11	61	41	**Qi Yu**
009	29	56	38	38	42	43	50	29	75	**Yin Xu**
010	11	13	25	6	8	7	14	14	78	**Gentleness**
011	14	25	47	31	17	21	32	50	47	**Qi Yu**
012	4	34	22	22	29	25	54	21	72	**Te Bing**
013	18	38	44	47	46	32	14	21	41	**Tan Shi**
014	50	22	50	34	25	7	18	29	63	**Yang Xu & Qi Xu**
015	7	16	13	16	13	11	14	7	75	**Gentleness**
016	4	13	19	19	25	11	29	39	69	**Gentleness**
017	61	84	59	63	83	75	50	64	44	**Yin Xu**
018	0	41	9	0	4	18	7	7	69	**Yin Xu**
019	7	41	56	16	17	54	32	36	53	**Qi Xu**
020	7	13	9	6	29	11	14	11	84	**Gentleness**
021	36	66	34	41	29	11	11	50	56	**Yin Xu**
022	82	44	44	38	46	29	32	32	59	**Yang Xu**
023	61	41	47	56	54	36	54	50	50	**Yang Xu**
024	21	66	41	53	54	54	61	57	69	**Yin Xu**
025	57	25	41	19	38	32	7	68	47	**Qi Yu**
026	36	50	31	3	46	14	0	43	44	**Yin Xu**
027	61	31	47	19	21	54	39	32	47	**Yang Xu**
028	21	34	34	19	63	29	0	4	59	**Shi Re**
029	0	13	19	13	25	4	0	0	94	**Gentleness**
030	0	6	19	0	8	4	7	0	84	**Gentleness**
031	79	59	50	50	58	46	46	68	47	**Yang Xu**
032	32	28	41	22	25	25	39	29	44	**Qi Xu**
033	50	44	34	0	8	29	25	43	75	**Yang Xu**
034	21	38	41	31	42	43	71	39	50	**Te Bing**
035	54	63	63	22	25	46	43	36	44	**Yin Xu & Qi Xu**
036	21	34	22	19	21	29	14	64	38	**Qi Yu**
037	21	22	31	6	17	21	21	29	75	**Gentleness**
038	39	34	47	16	33	36	18	25	66	**Qi Xu**
039	11	44	31	3	13	39	32	29	72	**Yin Xu**
040	54	50	59	25	21	39	36	57	34	**Qi Xu**
041	36	69	44	44	21	64	50	46	63	**Yin Xu**

042	21	50	50	34	50	36	43	32	66	**Yin Xu & Qi Xu & Shi Re**
043	32	72	38	9	54	36	14	29	47	**Yin Xu**
044	29	38	34	13	33	21	32	18	59	**Yin Xu**
045	54	63	47	56	58	36	61	54	44	**Yin Xu**
046	39	28	41	16	17	18	14	32	41	**Qi Xu**
047	39	25	41	19	8	21	14	21	41	**Qi Xu**
048	18	34	41	9	29	32	29	46	47	**Qi Yu**
049	43	38	53	50	54	36	43	54	38	**Shi Re & Qi Yu**
050	54	50	69	41	50	43	54	54	50	**Qi Xu**
051	50	44	53	38	46	50	50	46	53	**Qi Xu**
052	7	13	31	9	17	29	4	29	59	**Qi Xu**
053	32	31	47	56	54	46	46	21	63	**Tan Shi**
054	14	13	9	22	13	18	7	18	72	**Gentleness**
055	57	38	50	34	50	54	57	75	31	**Qi Yu**
056	50	56	41	44	42	46	32	71	38	**Qi Yu**
057	36	22	28	0	4	43	39	50	56	**Qi Yu**
058	0	9	3	3	8	11	0	0	69	**Gentleness**
059	11	34	41	19	50	18	54	32	75	**Te Bing**
060	36	9	38	22	46	43	21	46	47	**Shi Re & Qi Yu**
061	32	63	31	31	25	18	21	46	66	**Yin Xu**
062	7	34	50	44	38	21	43	46	59	**Qi Xu**
063	43	34	50	31	42	39	32	57	44	**Qi Yu**
064	29	47	50	31	38	57	14	64	25	**Qi Yu**
065	0	22	19	0	8	11	4	4	72	**Gentleness**
066	57	16	50	19	29	29	46	39	47	**Yang Xu**
067	39	50	53	38	50	29	57	57	53	**Te Bing & Qi Yu**
068	11	9	19	13	13	21	25	29	75	**Gentleness**
069	68	47	28	38	42	36	32	43	63	**Yang Xu**
070	14	3	19	0	29	7	11	11	75	**Gentleness**
071	36	31	44	28	46	29	43	29	63	**Shi Re**
072	25	72	41	25	25	21	32	43	44	**Yin Xu**
073	7	19	19	6	17	14	11	4	88	**Gentleness**
074	89	78	84	78	75	75	79	79	28	**Yang Xu**
075	43	56	41	53	38	32	39	39	75	**Yin Xu**
076	18	25	16	19	25	14	18	14	75	**Gentleness**
077	29	50	38	13	13	14	14	7	69	**Yin Xu**
078	61	44	31	47	38	39	39	50	34	**Yang Xu**
079	21	19	47	13	21	25	11	32	59	**Qi Xu**
080	29	34	34	22	25	50	21	32	44	**Xue Yu**
081	25	31	28	25	29	25	25	25	63	**Gentleness**
082	18	31	28	13	29	46	25	14	69	**Xue Yu**
083	36	63	34	22	58	46	93	25	63	**Te Bing**
084	4	6	6	6	8	11	29	4	88	**Gentleness**
085	57	38	41	34	21	57	64	4	53	**Te Bing**

Appendix D

Subject ID	DNA concentration (ng/µL)	A260/A280 ratio
001	30.2	2.58
002	20.8	1.63
003	78.5	1.76
004	67.5	1.89
005	69.8	1.88
006	31.9	1.91
007	30.6	1.9
008	25.8	1.68
009	27.7	1.7
010	89.3	1.06
011	13.2	1.74
012	111.9	1.95
013	48	2.19
014	69.3	1.87
015	85.9	1.72
016	38.1	1.79
017	17.7	1.87
018	22.8	1.75
019	90.7	1.67
020	171.2	1.56
021	161.7	1.9
022	19.8	1.9
023	67.3	2.05
024	44.2	1.88
025	68.2	2.27
026	645.4	1.88
027	43.1	1.65
028	30.7	1.71
029	80.7	1.8
030	6.9	1.67
031	44.5	1.84
032	24.4	1.75
033	34.9	1.82
034	57.9	1.71
035	11.3	1.9
036	72.9	1.92
037	127.8	1.83
038	250.7	1.99
039	21.5	1.81
040	46.8	1.79
041	24.6	1.76
042	13.5	1.74
043	30	1.84
044	169.9	1.89
045	103.9	1.88

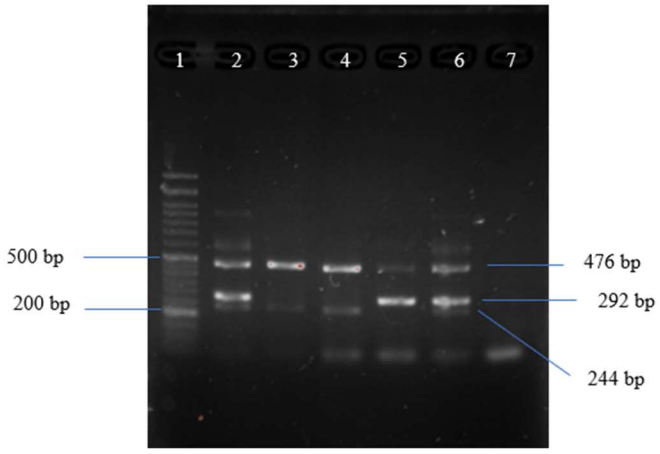
046	89.7	1.96
047	547	1.85
048	133.7	1.89
049	80.8	1.88
050	81.9	1.85
051	130.6	1.86
052	41.7	1.83
053	62.3	1.84
054	183.5	1.89
055	177.6	1.97
056	104	1.88
057	32.3	1.82
058	56.1	1.86
059	120.1	1.86
060	49.1	1.82
061	19	1.74
062	24.2	2.15
063	111.9	1.88
064	110.3	1.86
065	72	1.87
066	71.1	1.86
067	152.3	1.87
068	93.8	1.85
069	62	1.84
070	72.2	1.82
071	213.2	1.87
072	86.4	1.88
073	257.3	2.02
074	30.4	1.84
075	14.7	1.81
076	197	1.87
077	91.3	1.87
078	120.3	1.9
079	229.6	1.9
080	81.6	1.86
081	147.1	1.89
082	161.2	1.87
083	133	1.85
084	134.5	1.89
085	34.8	1.88

Appendix E

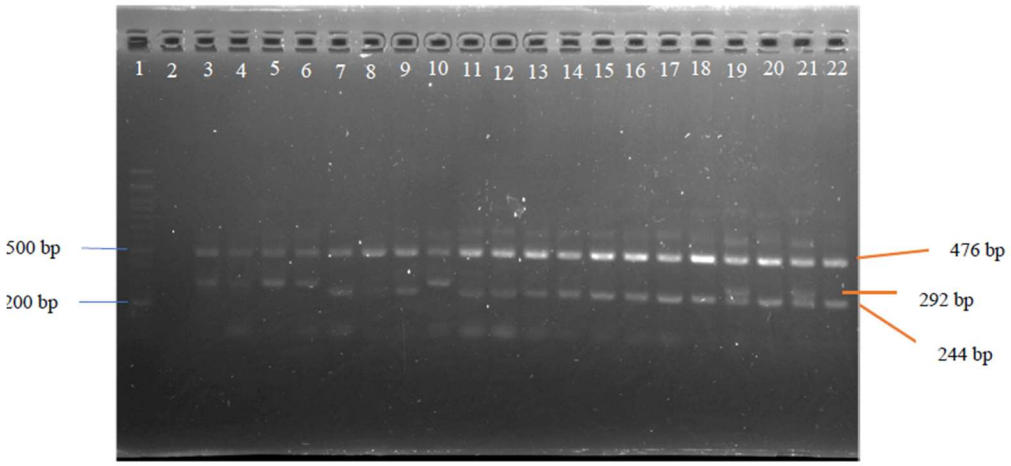
Subject ID	rs1501299 (G > T)	rs1801282 (G > C)
001	GT	GG
002	GG	GG
003	GG	GG
004	GG	GG
005	TT	GG
006	GT	GG
007	GG	GC
008	GT	GG
009	GG	GG
010	GG	GG
011	GT	GG
012	GT	GG
013	GG	GG
014	GT	GG
015	GT	GG
016	GT	GG
017	TT	GG
018	GT	GG
019	GG	GG
020	GG	GG
021	TT	GG
022	GG	GC
023	GG	GG
024	GG	GC
025	TT	GG
026	GG	GC
027	GG	GG
028	GG	GG
029	GT	GG
030	GG	GG
031	GT	GG
032	GG	GG
033	GT	GG
034	GG	GG
035	GG	GG
036	GT	GG
037	GG	GG
038	GG	GG
039	GT	GG
040	GT	GG
041	GG	GG
042	TT	GG
043	GG	GG
044	GG	GG
045	GT	GG
046	GG	GG

047	GT	GG
048	GT	GG
049	GT	GG
050	GG	GG
051	GG	GG
052	GG	GG
053	GT	GG
054	GT	GG
055	GG	GG
056	GT	GG
057	GT	GG
058	GG	GG
059	GT	GG
060	GG	GC
061	GT	GC
062	GG	GG
063	GT	GG
064	GT	GG
065	GG	GG
066	GG	GG
067	GG	GG
068	GG	GG
069	GG	GG
070	GG	GG
071	GG	GG
072	GG	GG
073	GG	GG
074	GG	GG
075	GT	GG
076	GT	GG
077	GT	GG
078	GT	GG
079	GT	GG
080	GG	GG
081	GG	GG
082	GG	GG
083	GG	GC
084	TT	GG
085	GG	GG

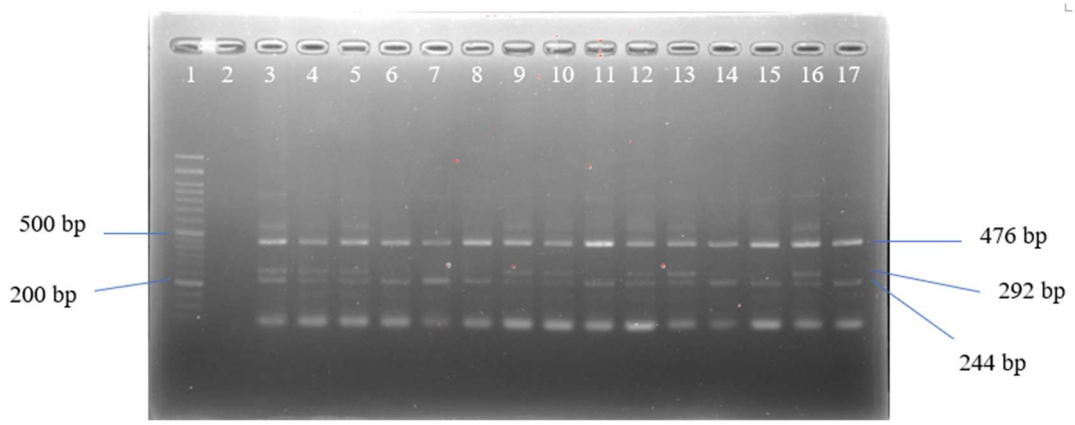
Appendix F



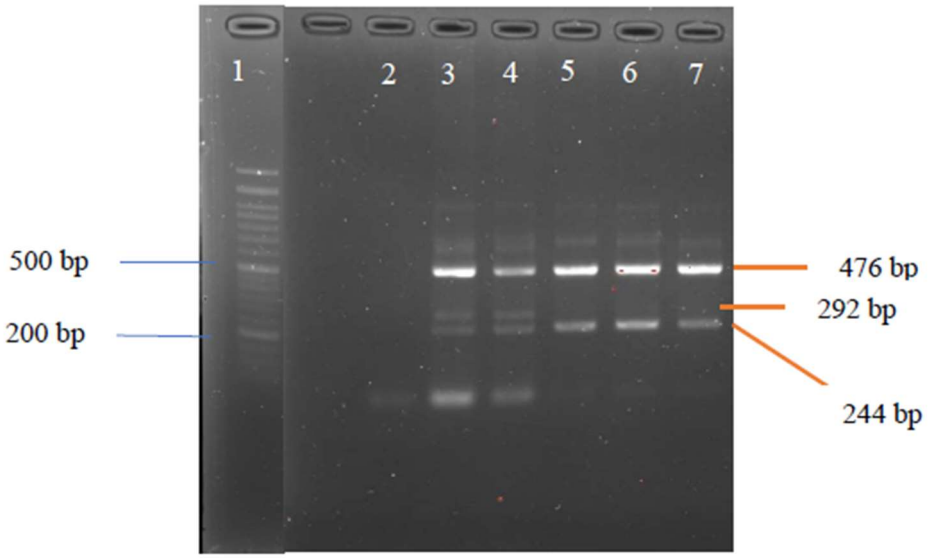
Lane 1: 50 bp Ladder; Lane 2: NTC; Lane 3: sample 001; Lane 4: sample 002; Lane 5: sample 004; Lane 6: sample 005; Lane 7: sample 006



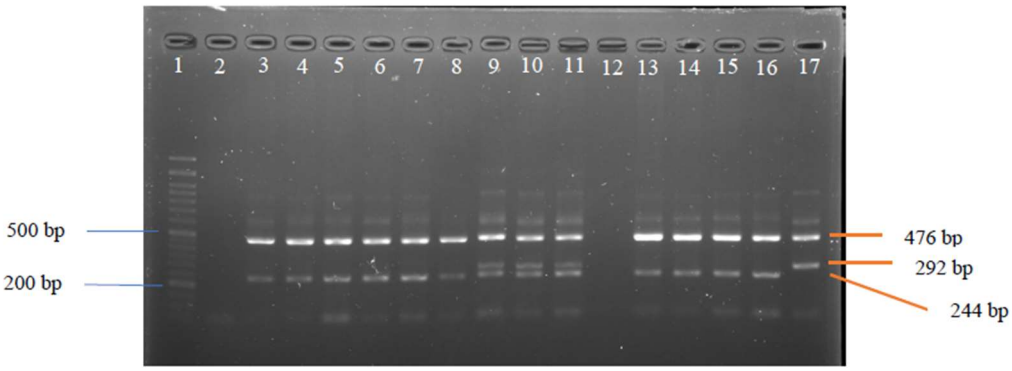
Lane 1: 50 bp Ladder; Lane 2: NTC; Lane 3: sample 015; Lane 4: sample 016; Lane 5: sample 017; Lane 6: sample 021; Lane 7: sample 020; Lane 8: sample 003; Lane 9: sample 024; Lane 10: sample 025; Lane 11: sample 026; Lane 12: sample 028; Lane 13: sample 034; Lane 14: sample 032; Lane 15: sample 030; Lane 16: sample 035; Lane 17: sample 037; Lane 17: sample 038; Lane 17: sample 039 Lane 17: sample 041; Lane 17: sample 045; Lane 17: sample 046



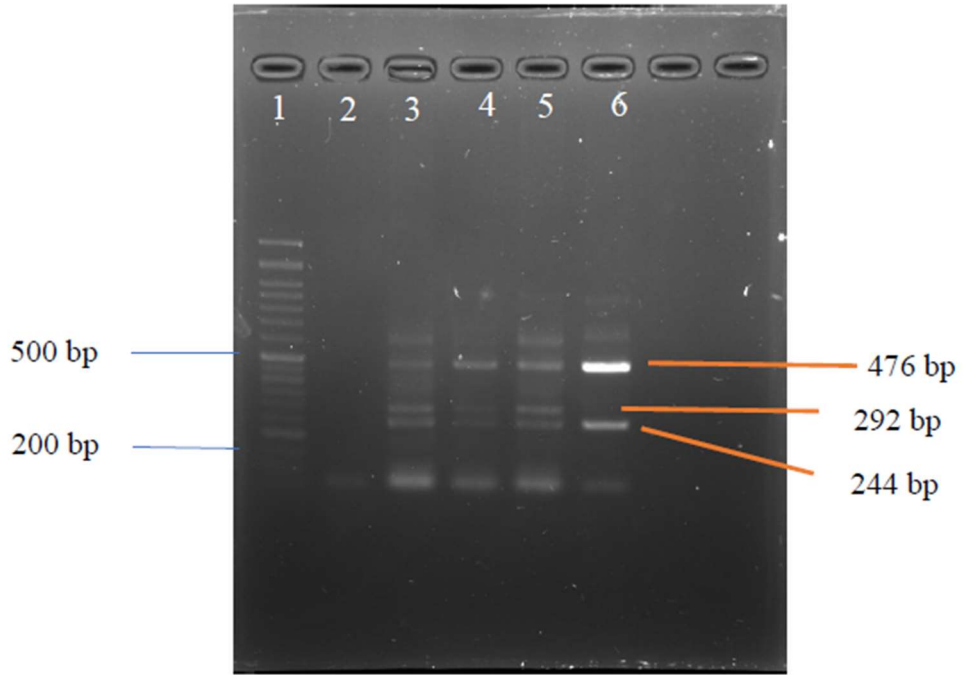
Lane 1: 50 bp Ladder; Lane 2: NTC; Lane 3: sample 047; Lane 4: sample 048; Lane 5: sample 049 Lane 6: sample 050; Lane 7: sample 051; Lane 8: sample 052; Lane 9: sample 053; Lane 10: sample 054; Lane 11: sample 055; Lane 12: sample 056; Lane 13: sample 057; Lane 14: sample 058; Lane 15: sample 027; Lane 16: sample 059; Lane 17: sample 060



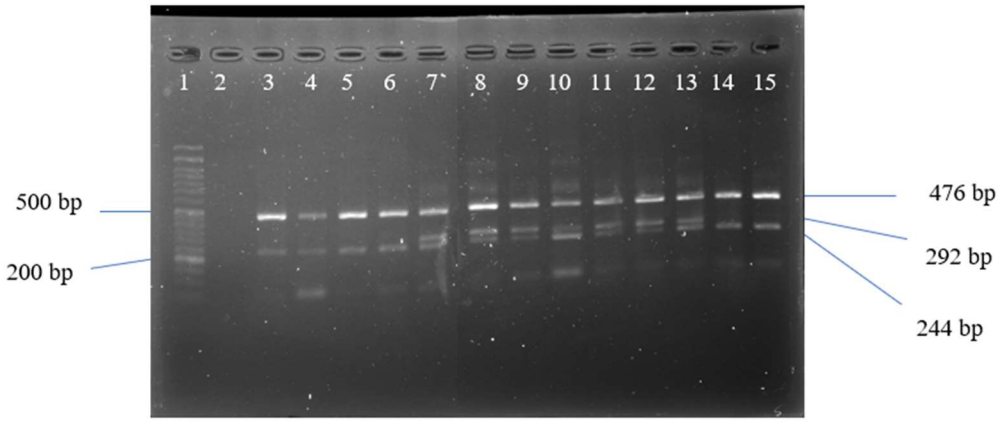
Lane 1: 50 bp Ladder; Lane 2: NTC; Lane 3: sample 063; Lane 4: sample 064; Lane 5: sample 065; Lane 6: sample 066; Lane 7: sample 067



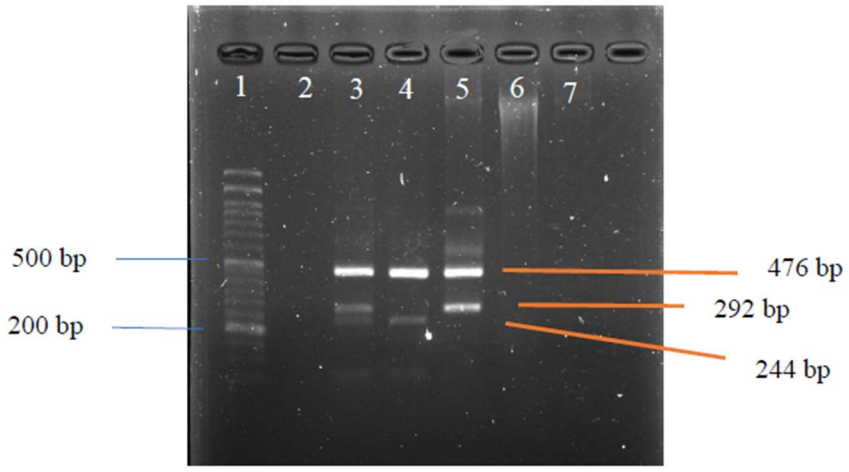
Lane 1: 50 bp Ladder; Lane 2: NTC; Lane 3: sample 068; Lane 4: sample 069; Lane 5: sample 070; Lane 6: sample 071; Lane 7: sample 072; Lane 8: sample 073; Lane 9: sample 076; Lane 10: sample 077; Lane 11: sample 078; Lane 12: sample 079; Lane 13: sample 080; Lane 14: sample 083; Lane 15: sample 081; Lane 16: sample 082; Lane 17: sample 084



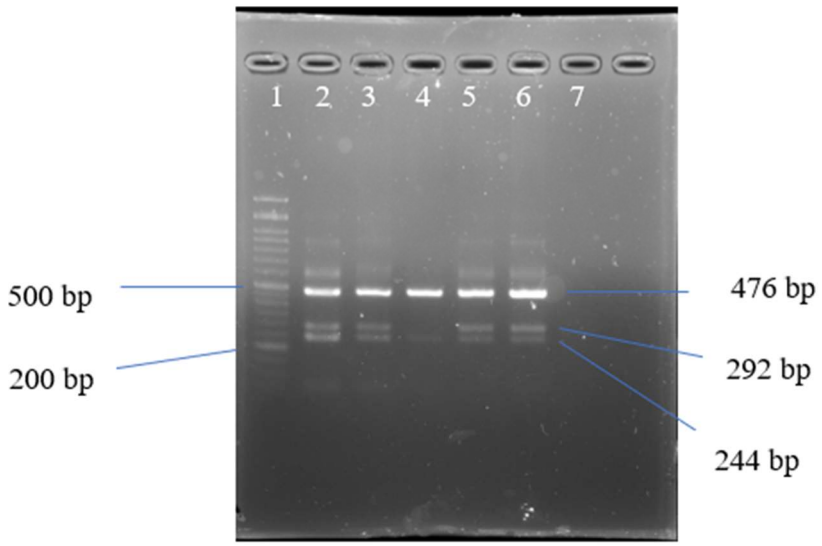
Lane 1: 50 bp Ladder; Lane 2: NTC; Lane 3: sample 008; Lane 4: sample 012; Lane 5: sample 011; Lane 6: sample 019



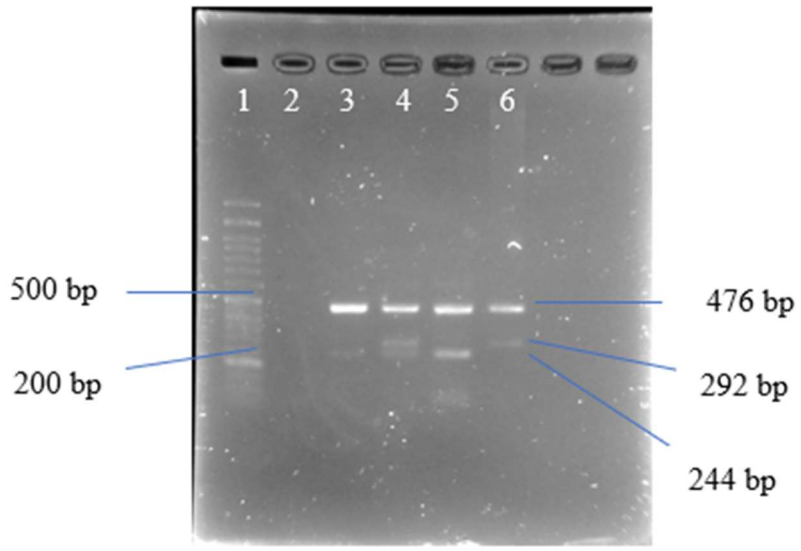
Lane 1: 50 bp Ladder; Lane 2: NTC; Lane 3: sample 007; Lane 4: sample 010; Lane 5: sample 022; Lane 6: sample 023; Lane 7: sample 014; Lane 8: sample 029; Lane 9: sample 031; Lane 10: sample 033; Lane 11: sample 036; Lane 12: sample 040; Lane 13: sample 042; Lane 14: sample 043; Lane 15: sample 044



Lane 1: 50 bp Ladder; Lane 2: NTC; Lane 3: sample 061; Lane 4: sample 062; Lane 5: sample 084; Lane 6: sample 085



Lane 1: 50 bp Ladder; Lane 2: sample 079; Lane 3: sample 013; Lane 4: sample 009; Lane 5: sample 018; Lane 6: sample ; Lane 7: NTC



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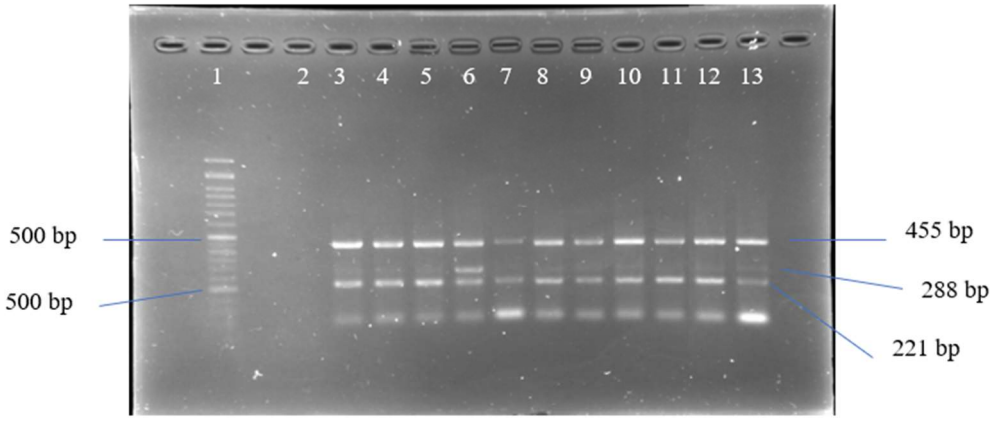
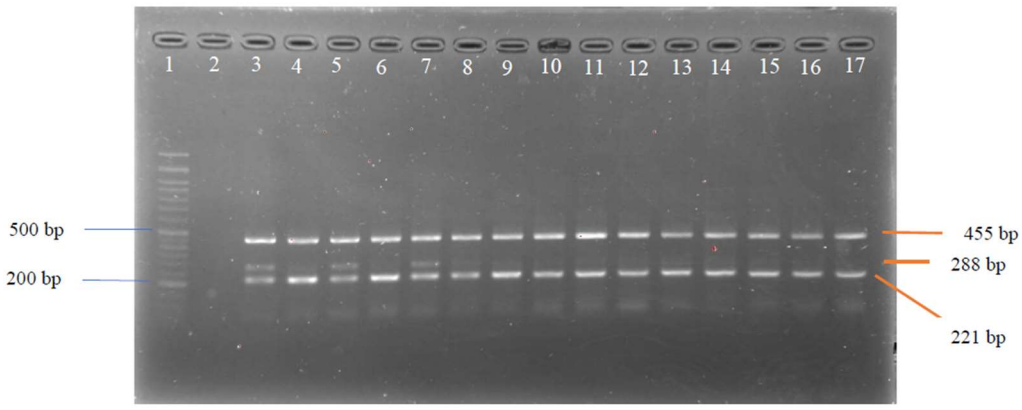
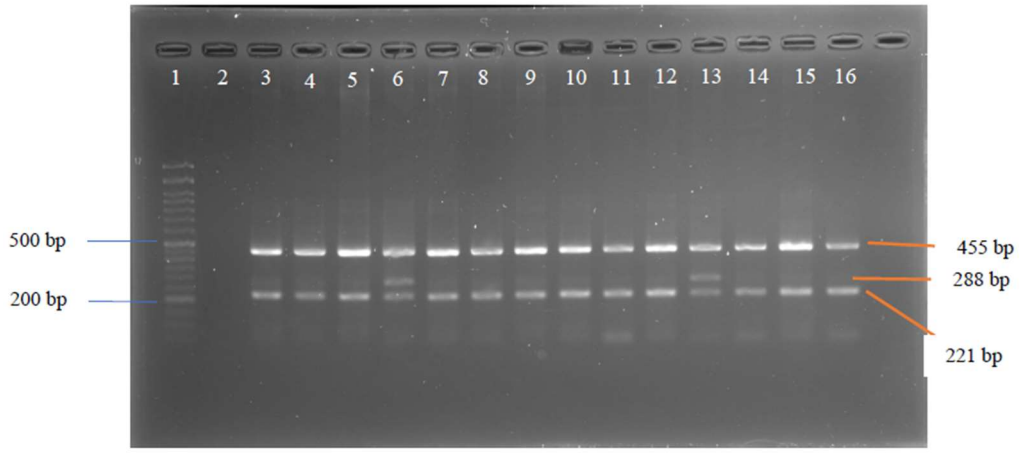


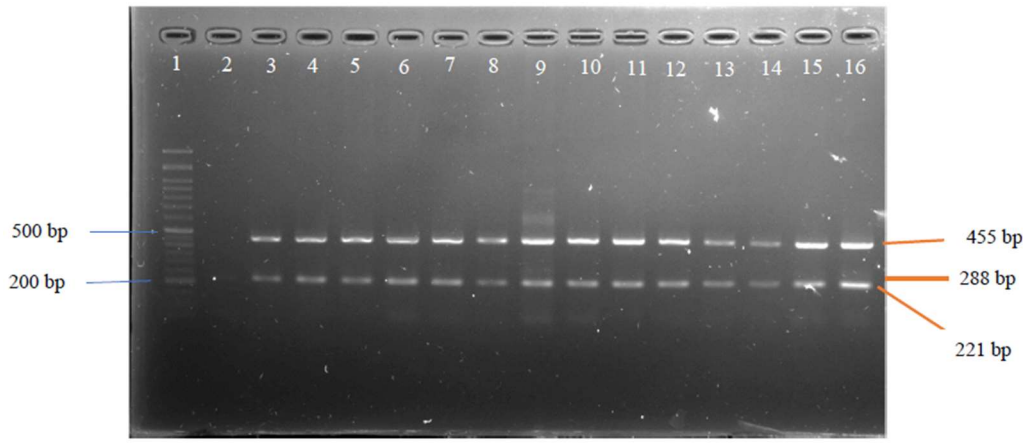
Lane 1: 50 bp Ladder; Lane 2: NTC; Lane 3: sample 004; Lane 4: sample 005; Lane 5: sample 006; Lane 6: sample 007; Lane 7: sample 010; Lane 8: sample 013; Lane 9: sample 014; Lane 10: sample 015; Lane 11: sample 016; Lane 12: sample 020; Lane 13: sample 021



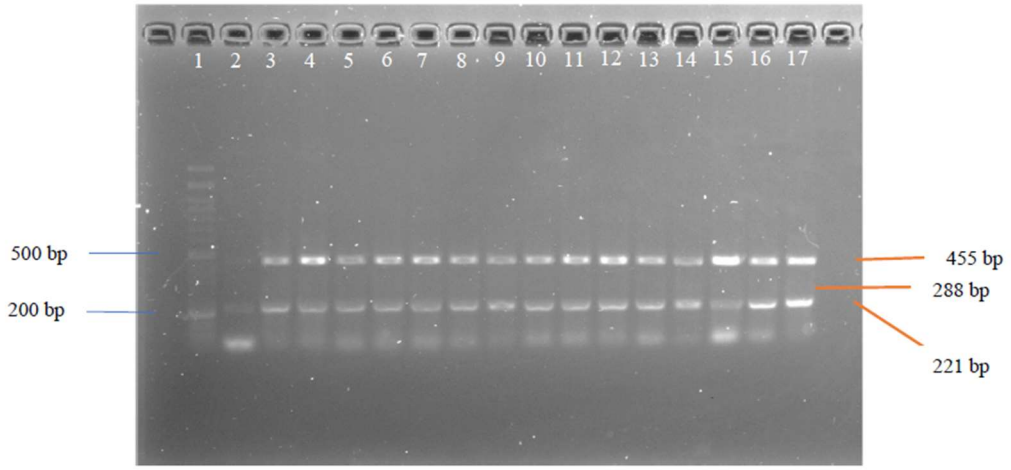
Lane 1: 50 bp Ladder; Lane 2: NTC; Lane 3: sample 022; Lane 4: sample 003; Lane 5: sample 024; Lane 6: sample 025; Lane 7: sample 026; Lane 8: sample 028; Lane 9: sample 034; Lane 10: sample 032; Lane 11: sample 030; Lane 12: sample 035; Lane 13: sample 037; Lane 14: sample 038; Lane 15: sample 039; Lane 16: sample 041; Lane 17: sample 045



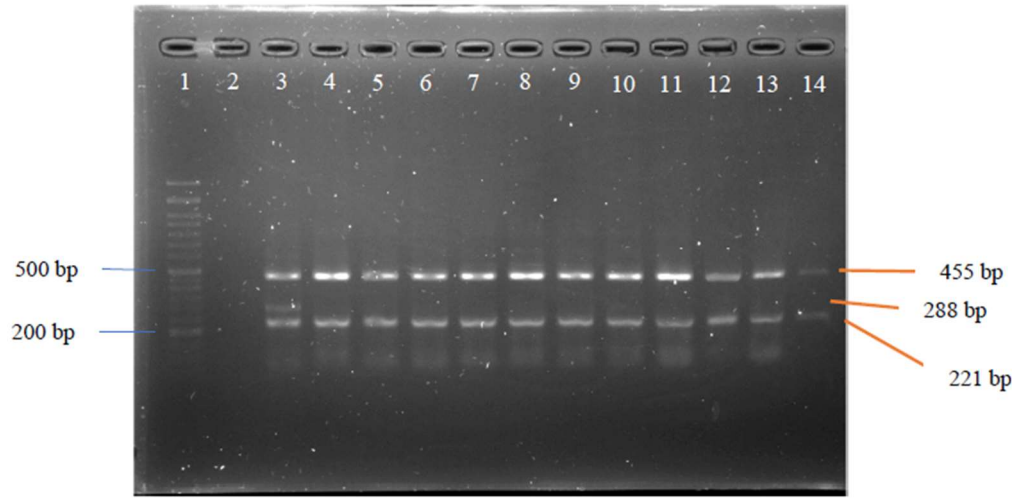
Lane 1: 50 bp Ladder; Lane 2: NTC; Lane 3: sample 078; Lane 4: sample 079; Lane 5: sample 080; Lane 6: sample 083; Lane 7: sample 056; Lane 8: sample 081; Lane 9: sample 082; Lane 10: sample 084; Lane 11: sample 008; Lane 12: sample 012; Lane 13: sample 011; Lane 14: sample 019; Lane 15: sample 023; Lane 16: sample 029; Lane 17: sample 031



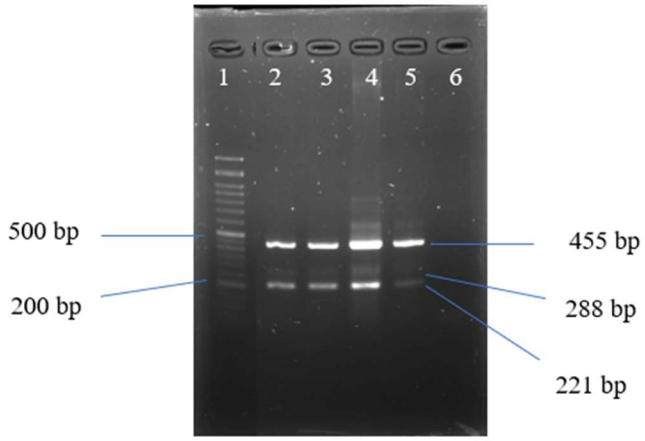
Lane 1: 50 bp Ladder; Lane 2: NTC; Lane 3: sample 040; Lane 4: sample 044; Lane 5: sample 001; Lane 6: sample 002; Lane 7: sample 009; Lane 8: sample 018; Lane 9: sample 033; Lane 10: sample 036; Lane 11: sample 042; Lane 12: sample 043; Lane 13: sample 061; Lane 14: sample 074; Lane 15: sample 075; Lane 16: sample 017



Lane 1: 50 bp Ladder; Lane 2: NTC; Lane 3: sample 046; Lane 4: sample 47; Lane 5: sample 048; Lane 6: sample 049; Lane 7: sample 050; Lane 8: sample 051; Lane 9: sample 052; Lane 10: sample 53; Lane 11: sample 054; Lane 12: sample 055; Lane 13: sample 056; Lane 14: sample 057; Lane 15: sample 058; Lane 16: sample 027; Lane 17: sample 059



Lane 1: 50 bp Ladder; Lane 2: NTC; Lane 3: sample 060; Lane 4: sample 063; Lane 5: sample 064; Lane 6: sample 065; Lane 7: sample 066; Lane 8: sample 068; Lane 9: sample 069; Lane 10: sample 070; Lane 11: sample 071; Lane 12: sample 067; Lane 13: sample 072; Lane 14: sample 073



Lane 1: 50 bp Ladder; Lane 2: sample 062; Lane 3: sample 076; Lane 4: sample 077; Lane 5: sample 085; Lane 6: NTC

APPENDIX G

UNIVERSITI TUNKU ABDUL RAHMAN
Wholly Owned by UTAR Education Foundation (Company No. 578227-M)

Re: U/SERC/240/2021

18 October 2021

Dr Tan Gim Cheong
Head, Department of Allied Health Sciences
Faculty of Science
Universiti Tunku Abdul Rahman
Jalan Universiti, Bandar Baru Barat
31900 Kampar, Perak.

Dear Dr Tan,

Ethical Approval For Research Project/Protocol

We refer to the application for ethical approval for your student's research project from Bachelor of Science (Hons) Biomedical Science programme enrolled in course UDDD3108. We are pleased to inform you that the application has been approved under Expedited Review.

The details of the research projects are as follows:

No	Research Title	Student's Name	Supervisor's Name	Approval Validity
1.	Association of Traditional Chinese Medicine (TCM) Body Constitution with Metabolic Syndromes	Fong Yu Qi	Dr Teh Lai Kuan	18 October 2021 – 17 October 2022

The conduct of this research is subject to the following:

(1) The participants' informed consent be obtained prior to the commencement of the research;

(2) Confidentiality of participants' personal data must be maintained; and

(3) Compliance with procedures set out in related policies of UTAR such as the UTAR Research Ethics and Code of Conduct, Code of Practice for Research Involving Humans and other related policies/guidelines.

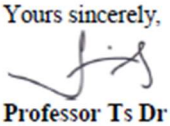
(4) Written consent be obtained from the institution(s)/company(ies) in which the physical or/and online survey will be carried out, prior to the commencement of the research.

Kampar Campus : Jalan Universiti, Bandar Barat, 31900 Kampar, Perak Darul Ridzuan, Malaysia
Tel: (605) 468 8888 **Fax**: (605) 466 1313
Sungai Long Campus : Jalan Sungai Long, Bandar Sungai Long, Cheras, 43000 Kajang, Selangor Darul Ehsan, Malaysia
Tel: (603) 9086 0288 **Fax**: (603) 9019 8868
Website: www.utar.edu.my

Should the students collect personal data of participants in their studies, please have the participants sign the attached Personal Data Protection Statement for records.

Thank you.

Yours sincerely,



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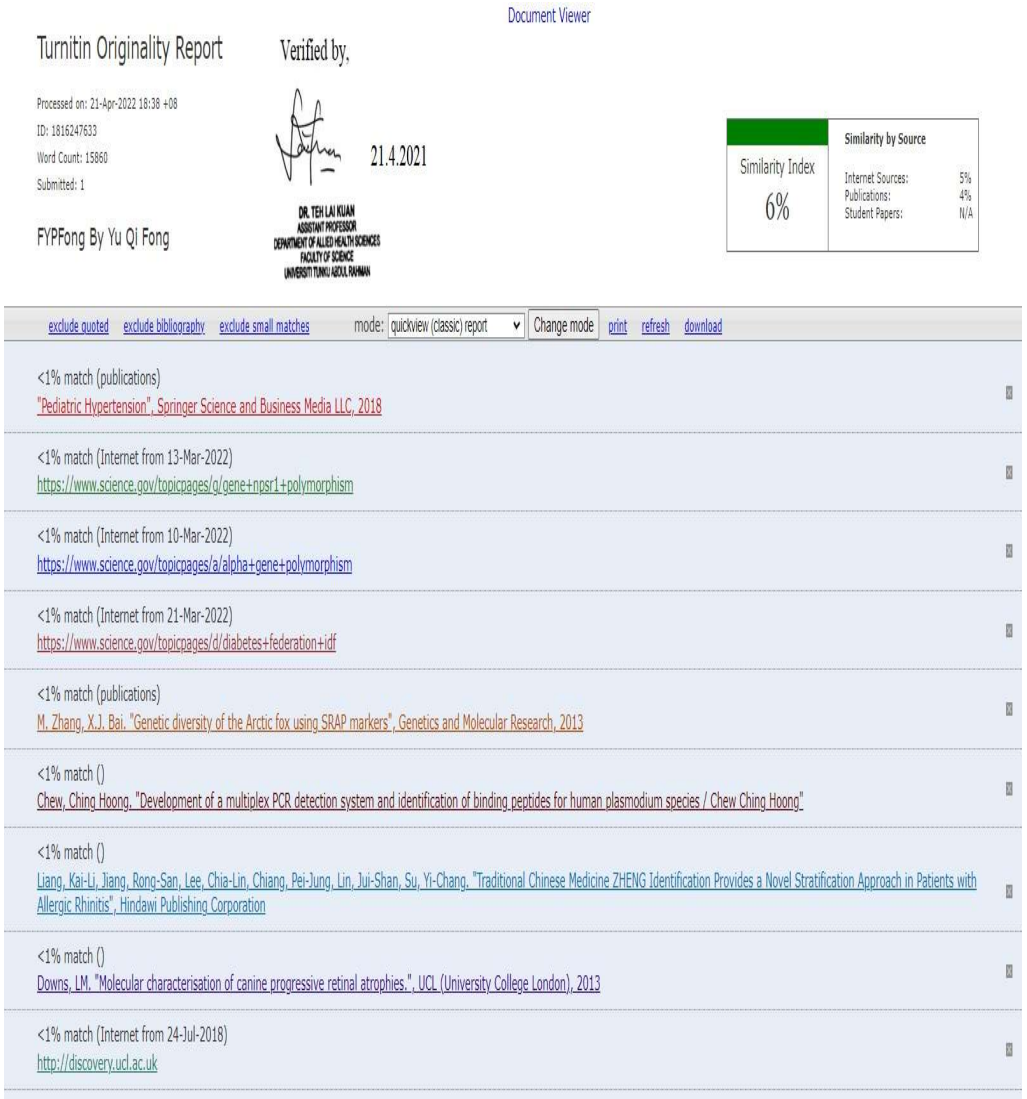
APPENDIX F



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ASSISTANT PROFESSOR
DEPARTMENT OF ALLIED HEALTH SCIENCES
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Programme / Course	Bachelor of Science (HONS) Biomedical Science
Title of Final Year Project	Association Between Chinese Medicine Body Constitution and Variants in Metabolic Gene (rs1501299 & rs1801282)

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Date: 21.4.2021

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Name: _____
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