GENOTYPE-BASED *MTHFR* MRNA EXPRESSION IN A COHORT OF UNDERGRADUATE STUDENTS WITH PREHYPERTENSION

By

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ABSTRACT

GENOTYPE-BASED *MTHFR* MRNA EXPRESSION IN A COHORT OF UNDERGRADUATE STUDENTS WITH PREHYPERTENSION

CATHERINE SIEW LEE YENG

Prehypertension is a complex, multifactorial disease that involves genetic and environmental factors. In recent years, *MTHFR* was identified associated with blood pressure. Epidemiological studies showed that the gene variant C677T of *MTHFR* increased the risk of hypertension. However, there was lack of similar epidemiological studies on prehypertension. Therefore, the study aims to determine the prevalence of prehypertension among a cohort of Universiti Tunku Abdul Rahman (UTAR) students, identify the association of the T allele of *MTHFR* C677T variant with prehypertension and *MTHFR* mRNA expression of prehypertension individuals with different conditions. A total of 80 students (32 males and 48 females) from UTAR were recruited. Twenty students were with prehypertension. The prevalence of prehypertension in males (70%) was higher than females (30%). DNA and RNA were extracted from buccal cells. C677T genotyping was identified using PCR-RFLP. The prevalence of CC, CT and TT genotypes were 57.5%, 32.5% and 10.0% respectively. In this study, *MTHFR* C677T gene variant showed association with prehypertension and T allele is a genetic risk factor of getting prehypertension. *MTHFR* mRNA expression levels of CC, CT and TT genotypes in four different conditions: normal with genetic condition (A), normal without genetic condition (B), prehypertension with

genetic condition (C) and prehypertension without genetic condition (D) were evaluated. The *MTHFR* gene expression of TT genotype was higher in group A, B and D but lowest in group C. *MTHFR* mRNA was expressed higher in prehypertension cases in each genotype as compared with normal blood pressure. According to the findings, the gene expression levels was significant affected by genetic risk factor and environmental factor such as diet behaviour.

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In addition, my sincere thanks also goes to my parents who have given me the strength to complete this project. Last but not least, I would like to acknowledge my gratitude to all the respondents who participated in this research.

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 my other degree at UTAR or other institutions.

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CATHERINE SIEW LEE YENG

APPROVAL SHEET

This final year project report entitled "**GENOTYPE-BASED *MTHFR* MRNA EXPRESSION IN A COHORT OF UNDERGRADUATE STUDENTS WITH PREHYPERTENSION**" was prepared by CATHERINE SIEW LEE YENG 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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(Assistant Professor Dr. Phoon Lee Quen) Date: ……

Supervisor

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Date: _____

PERMISSION SHEET

It is hereby certified that **CATHERINE SIEW LEE YENG** (ID:**15ADB07264**) has completed this final year project entitled "**GENOTYPE-BASED MTHFR MRNA EXPRESSION IN A COHORT OF UNDERGRADUATE STUDENTS WITH PREHYPERTENSION**" under the supervision of Assistant Professor Dr. Phoon Lee Quen, from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to the Universiti to upload the softcopy of my final year project 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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(CATHERINE SIEW LEE YENG)

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LIST OF ABBREVATIONS

Ala	Alanine
BMI	Body mass index
BP	Base pair
cDNA	Complementary DNA
DBP	Diastolic blood pressure
DEPC	Diethylpyrocarbonate
dH_2O	Sterile distilled water
DHA	Docosahexaenoic acid
DNA	Deoxynucleotide acid
DNase I	Deoxyribonuclease I
dNTPs	Dexoynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic Acid
Hinf	*Haemophilus influenza*
HWE	HardyWeinberg Equilibrium
mRNA	Messenger RNA
MTHFR	Methylenetetrahydrofolate reductase
NaCI	Sodium chloride
NaOH	Sodium hydroxide
PCI	Phenol-chloroform isoamyl
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid

RNase A	Ribonuclease A
RT-PCR	Real-time polymerase chain reaction
SBP	Systolic blood pressure
SDS	Sodium dodecylsulphate
TBE	Tris-boric acid-EDTA
TE	Tris-EDTA
UV	Ultraviolet
Val	Valine

CHAPTER 1

INTRODUCTION

According to the Seventh Report of the Joint National Committee (JNC 7), it has introduced a new classification which was "prehypertension". The range of systolic blood pressure (SBP) is 120-139 mmHg and diastolic blood pressure (DBP) is 80-89 mmHg. This new designation is mainly for the individual who was in prehypertension condition. They can reduce the blood pressure by adopting the healthy lifestyle (National High Blood Pressure Education Program, 2004). For the normal adult's blood pressure, the SBP and DBP was 120 mmHg and 80 mmHg respectively. When an individual diagnosed with prehypertension and still remain their unhealthy lifestyle condition, this will faster them toward the progress of the hypertension stage. According to the World Health Organization (WHO), when SBP and DBP was above 140 mmHg and 90 mmHg, that person was in hypertension stage (World Health Organization, 2013).

Hypertension is the most common disease that seen in the primary health care setting. This will lead to develop stroke, heart attack, renal failure and others disease. The hypertension recently has been identified as the risk factor for the global disease burden. As we know, the prehypertension is a major risk factor of the hypertension and also cardiovascular disease. A cohort study in Americans population have showed that the individual with prehypertension was associated with cardiovascular morbidity and mortality as compared to the normal blood pressure (Arch et al., 2004). Some studies were reported that prehypertension

was associated with the diseases such as coronary artery disease, myocardial infarction, microalbuminuria and the insulin resistance state (Qureshi et al., 2005; Cordero et al., 2006; Lee et al., 2006). The author was reported that the individual with the prehypertension have 31%, 49% and 44% in the increased risk of the coronary heart disease, stroke and total cardiovascular event when compared to the normal blood pressure individual (Wang et al., 2014).

Prevalence of the prehypertension can be varies among country, population and ethic. A cohort of study in Guangzhou have been done with the report of the prevalence of prehypertension was 35.15% (43.75% in men while 23.56% in women). They also concluded that prehypertension in men was higher than women in age 18-70 years old (Wang et al., 2014). The another study of the population in India showed that 55% of the 624 subjects which aged above 20 years old had prehypertension and higher in the men (Prasanna et al., 2015). A study among the Korean adults which was 20 years old above showed that 31.6% prevalence of the prehypertension and also higher in men (Choi et al., 2006).

In recent years, prehypertension showed an increasing trend in university students along. A total of 754 students of the University of Philippines Visayas were involved in the study and showed 13.9% of the students was prehypertension (36.1 % men and 6.8% women) (Pengpid et al., 2014). Another study was conducted among 232 medical students in university in Saudi Arabia. The result demonstrated that the prevalence of the prehypertension in males and females were 58.8% and 52.1% respectively. The study also included the relevant data analysis on the risk factor which was family history, obesity,

sedentary life and excess salt intake (Alanazi et al., 2018). In Malaysia, a study on psychological determines of prehypertension among first year undergraduate students in a public university was carried out. The data showed that the prevalence of the prehypertension among 495 students was 30.1 % (Balami et al., 2014). The prevalence of prehypertension was getting higher because university students always exposed to the unhealthy environment condition such as no enough sleeping, sedentary lifestyle and others (Gyamfi et al., 2018).

Prehypertension is a warning sign which means that individual could have a higher risk to develop hypertension. The mechanism of elevated risk for the cardiovascular diseases in prehypertension individuals was presumed to be same as the hypertension patients. Prehypertension is not a disease state. The JNC 7 suggested that the blood pressure can be reduced or back to the normal through the optimal weight control such as diet control and exercise (Izzo, 2007). Therefore, because of the determinants are modifiable, the management of the prehypertension risk factor may reduce the progression of rising blood pressure. Moreover, prevention and control high blood pressure should be addressed to students and to enhance their self-awareness of the prehypertension in young adults (Gyamfi et al., 2018).

Prehypertension and hypertension is a multifactorial disease which combination of the genetic factor and environmental factor. Mutations in methylenetetrahydrofolate reductase (*MTHFR*) gene was identified and showed associated with the risk of hypertension. From the study of Pei and Daniel (2008), the heritability of hypertension was in the range of 30% to 60%. According to

the Bianca (2012), the findings showed that individual with heterozygotes genotype (CT) have only 60% of the enzyme activity. For individual with homozygous mutant genotype (TT), it only had 30% of the enzyme activity. Most of the studies showed that the evidence of the *MTHFR* polymorphism was associated with the risk of hypertension (Alghasham et al., 2012; Nassereddine et al., 2015; Ghogomu et al., 2016). However, there are lack of studies in the association of the *MTHFR* polymorphism and prehypertension.

1.1 Objectives of the study

Prehypertension is a complex, multifactorial disease that involves genetic and environmental factors. Furthermore, the awareness of the young adults of the hypertension was low. Hypertension is not only cause by the gene variant while it may probably due to the interaction of the environmental factor (Williams et al., 2000). Therefore, in this study, the case-control association study of *MTHFR* C77T polymorphism and prehypertensive was studied. The *MTHFR* gene expression of different genotype and condition was evaluated.

The objectives of the study were as follow:

i. To study the prevalence of the prehypertension among students from Universiti Tunku Abdul Rahman (UTAR), Kampar campus, Perak.
ii. To identify the *MTHFR* C677T genotypes of the normal and prehypertension samples.
iii. To investigate if T allele of *MTHFR* C677T is a genetic risk factor for prehypertension.
iv. To evaluate *MTHFR* mRNA expression in prehypertension individuals with different genotypes compared to the normal individuals in same condition.
v. To evaluate *MTHFR* mRNA expression in prehypertension individuals and normal individuals with same genotypes in different conditions.

CHAPTER 2

LITERATURE REVIEW

2.1 Prehypertension and Hypertension

In our body circulating system, blood is carried from heart to other parts of the body in the blood vessels. Therefore, the blood pressure is created when the force of blood pushing against the arteries through heart pumping (World Health Organization, 2013). The condition which the pressure is persistently increased in the blood vessels known as hypertension. Normally, the systolic blood pressure (SBP) and diastolic blood pressure (DBP) will be measured in millimetres of mercury (mmHg). For the normal blood pressure, the SBP and DBP was 120 mmHg and 80 mmHg respectively. When the SBP and DBP was at the range of 120-139 mmHg and 80-89 mmHg, that individual was in prehypertension condition. For the hypertension patient, the SBP and DBP was greater than 140 mmHg and 90 mmHg respectively. The classification of blood pressure are demonstrated in Table 2.1. Hypertension also called as the "silent killer" because there was no any symptoms or warning signs. Therefore, many people will not go to check for it (KhanAcademy, 2018).

2.2 Classification of Hypertension

2.2.1 Essential and Secondary Hypertension

Hypertension can divide into two groups which were primary or essential hypertension and secondary hypertension. Most of the 90% to 95% of the

hypertension cases was from the essential hypertension while only 2% to 10% cases was from secondary hypertension (Alexander, 2018). The cause of the essential hypertension still unknown but there have multiple of the mechanisms that are linked with the blood pressure control. Essential hypertension not only cause by the genetic factors which based on its mode of inheritance, it also included environmental factor such as diet, sedentary lifestyle, high salt food intake, stress and overweight (Hearte, 2018).

For the secondary hypertension, it can be caused by a consequence of the medical problem which include sleep apnea, kidney failure, congenital defect in the blood vessels, certain medications or drugs, thyroid problem or alcohol (Richard, 2018).

2.2.2 Stages of the Blood Pressure

According to the Seventh Report of Joint National Committee (JNC7), the blood pressure can be classified into four categories (Table 2.1).

Table 2.1: Classification of blood pressure for adults

Blood Pressure Classification	**SBP(systolic blood pressure) (mmHg)**	**DBP(diastolic blood pressure) (mmHg)**
Normal	<120	And <80
Prehypertension	120-139	Or 80-89
Stage 1 hypertension	140-159	Or 90-99
Stage 2 hypertension	≥ 160	Or ≥ 100

(National High Blood Pressure Education Program, 2004).

Normally, prehypertension is not a disease category but it has higher risk to develop to hypertension compared to the normal blood pressure person. The person with prehypertension stage does not need to receive medication. The prehypertension patient can prevent high blood pressure by changing the lifestyle, doing exercise and dietary behaviour. However, hypertension patients need to take the anti-hypertensive medication to control the blood pressure (National High Blood Pressure Education Program, 2004).

2.3 Prevalence of the hypertension and prehypertension

Hypertension is an important public health challenge in worldwide. The prevalence of the hypertension was different among countries or among subgroups of population in a country. A study in the United States showed that the prevalence of hypertension among adults with 18 years old and above was 29.1% (Tatiana et al., 2013). In India, the researcher found that there was 29.8% of the population diagnosed with hypertension (Raghupathy et al., 2014). World Health Organization (WHO) (2013) showed that approximately 40% of the adults aged 25 and above had hypertension. The highest prevalence of hypertension was found in the African Region (46%) while the lowest prevalence was found in Americans which was 35%. In Malaysia, the prevalence of the hypertension was 32.7% (National High Blood Pressure Education Program, 2004). Hypertensive was not gender dependent. Gender shows no significant different among hypertension patients. However, there was significant different in different ethnic group in Malaysia. In Sabah and Sarawak, the highest prevalence of hypertensive was found in Malay populations (34.0%),

followed by Chinese population (32.3%) and India population (30.6%) (Ministry of Health Malaysia, 2013).

Several studies on prevalence of prehypertension were carried out. In worldwide, the prevalence of the prehypertension was 31% (National Health and Nutrition Examination Survey (NHANES), 1999-2000). It also showed that the percentage of prehypertension in men was higher than women (Roger et al., 2011). In a cross sectional survey, prehypertension in a cohort of undergraduate students from seven ASEAN countries including Laos, Indonesia, Malaysia, Myanmar, Thailand, Vietnam and Philippines was studied. The study showed that the prevalence of prehypertension and hypertension among undergraduate students was 19.0% and 6.7% respectively (Peltzer et al., 2017). At the same time, a cross sectional study was done in a University in Ghana. Among the 540 of the students, the prevalence of prehypertension and hypertension was 26.1% and 2.2% respectively (Gyamfi et al., 2018). The prevalence of prehypertension in a university varies from one country to another country. In Malaysia, the prevalence of prehypertension among undergraduate students in University Putra Malaysia (UPM) was 30.1% (Balami et al., 2014).

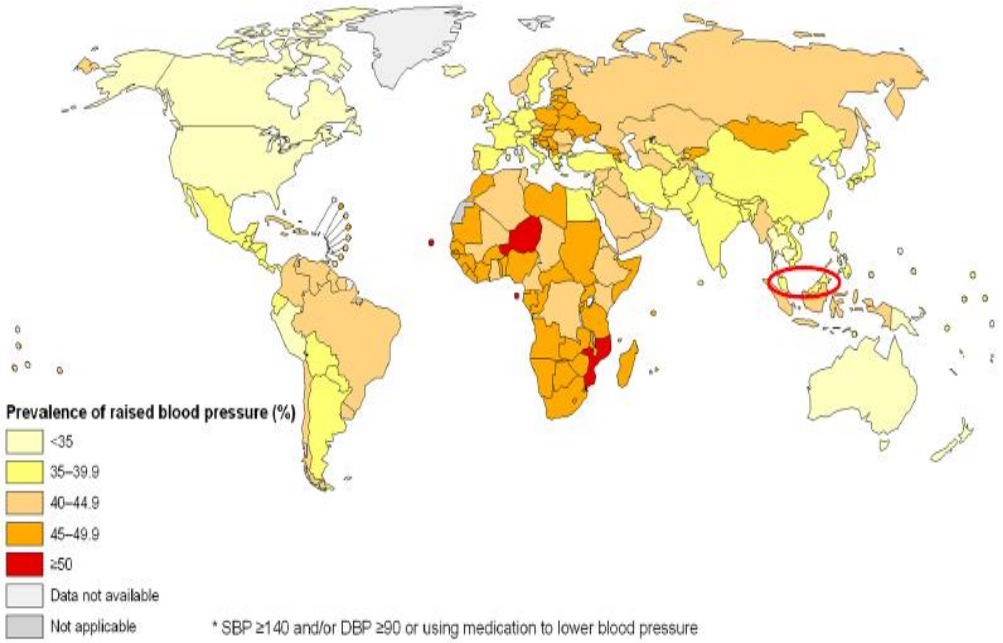

Figure 2.1: Worldwide of the prevalence of the high blood pressure in adults ages 25 and above (World Health Organization, 2013).

2.4 Risk factor of hypertension

Many factors that will lead to hypertension. Hypertension is caused by genetic factor, dietary behaviour and environmental factors. An individual who has family history of hypertension will have higher risk to get hypertension. The environmental factors include obesity, high intake of the salt foods, lack of exercise, stress, age, alcohol consumption, smoking and sleep apnea (National Health Service, 2016).

2.4.1 Obesity

Studied showed that hypertension was highly associated with obesity. Maniecka at al. (2011) found that 80% of hypertension cases in Poland was associated to overweight. In addition, a study in Framingham also reported that the prevalence

of the obesity lead to hypertension cases in men and women were 26% and 28% respectively (Wilson et al., 2002). Subhija et al. (2012) also found that higher body mass index (BMI) had higher risk of getting hypertension. The prevalence of hypertension was 51.06% for the BMI value range between 25 and 30. For BMI value between 30 to 35, 35 to 40 and above 40, the prevalence of the hypertension cases were 25%, 6.38% and 3.72% respectively.

2.4.2 High salt food intake

One of the risk factor that will altered the blood pressure is high salt intake. High salt intake will increased the sodium in body. Reduction of salt intake had a significant effect on the blood pressure (He and MacGregor, 2002). Elzbieta and Krzysztof (2018) found that 30% of the Western population was diagnosed with hypertension and prehypertension because of excessive salt consumption. Some of reported stated that the amount of daily salt intake between 3 and 6 g per day was consider high when compared to the reference range which was between 1.5 to 2.4 g per day. In conclusion, high salt intake will increased the incidence of getting hypertension (Steven, 2016).

2.4.3 Lack of physical activity

Several studies reported that exercising will reduced around 5-7 mmHg of the systolic and diastolic blood pressure of hypertension patients (Sheila and Scott, 2015). Salman and AL-Runeaan (2009) stated that those who exercise regularly had 62% of lower risk to get hypertension compared to non-exercising person.

Besides Salman and Al-Runeaan also found that sedentary behaviour will be associated with the risk of developing hypertension. Sedentary behaviour such as driving and using computer showed higher risk of getting hypertension cases among Spanish adults (Beunza et al., 2007).

2.4.4 Consumption of alcohol

Alcohol consumption is one of the risk factor of getting hypertension. Some researchers found that small amount of the alcohol which was less than half of one drink per day will have an advantage to normal blood pressure (Briasoulis et al., 2012). Skliros et al. (2012) showed that individuals with heavy drinking alcohol have higher risk of hypertension. Furthermore, Nanchahal et al. (2000) reported that when alcohol consumption above 15 times per week, it will increased the prevalence of hypertension.

2.4.5 Stress

Stress can stimulate hypertension by stimulating vasoconstricting hormones in system. In rural area of Maharashtra, the risk of hypertension increases when stress elevates. Mental stress between males and women were vary. Males showed significant associated to hypertension which were 7.27% compared to females (3.44%) (Sachin et al., 2014). In the study of Liu et al. (2016), the researchers found that chronic psychosocial stress had an association with hypertension and the raise of blood pressure may become a risk factor to develop hypertension (Kulkarni et al., 1998).

2.7 Genetic risk factor

Hypertension is a multifactorial disease which is caused by the combination of genetic background and environmental factors. Some epidemiological studies suggested that genetic variants will increased the risk of getting hypertension (Izawa et al., 2003). Pei and Daniel (2008) found that the heritability of hypertension was in the range of 30% to 60%. Hypertension is not caused by a specific gene. It is caused by interaction of multiple gene variations that are associated to hypertension (Williams et al., 2000).

2.8 Methylenetetrahydrofolate reductase gene *(MTHFR)*

2.8.1 *MTHFR* gene and its polymorphism

Methylenetetrahydrofolate reducatase gene is located at the p arm of chromosome 1 at position 36.22. It encodes an enzyme which composed of 656 amino acid (Genetic Home Reference, 2018). *MTHFR* consists of 11 exons which ranging in size from 102 base pair to 432 base pair and the complementary DNA sequence is 2.2 kilobases long (Goyette et al., 1998). *MTHFR* gene will transcribed and produced an enzyme called methyenetetrahydrofolate reductase (MTHFR). MTHFR is important in folate metabolism and DNA synthesis. The *MTHFR* mRNA was expressed in low abundance in most of the tissues which approximately 2.8 and 7.2 to 7.7 kb. While in brain, placenta and stomach, it expressed approximately 9.5 kb (Gaughan et al., 2000).

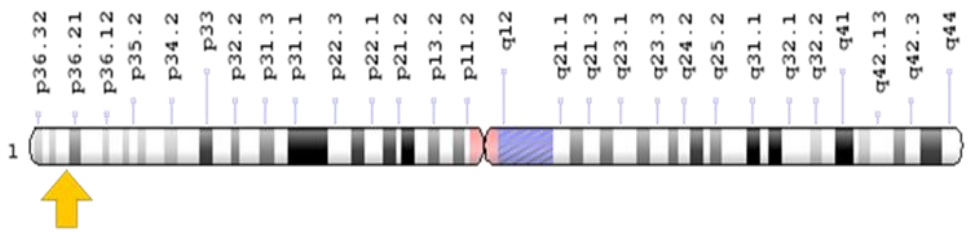

Figure 2.2: *MTHFR* gene (Genetic Home Reference, 2018)

MTHFR enzyme will convert folic acid into active form which involve the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The 5-methyltetrahydrofolate than will act as the substrate to convert the amino acid homocysteine to methionine. Methionine is important to make a protein and others compounds (Genetic Home Reference, 2018).

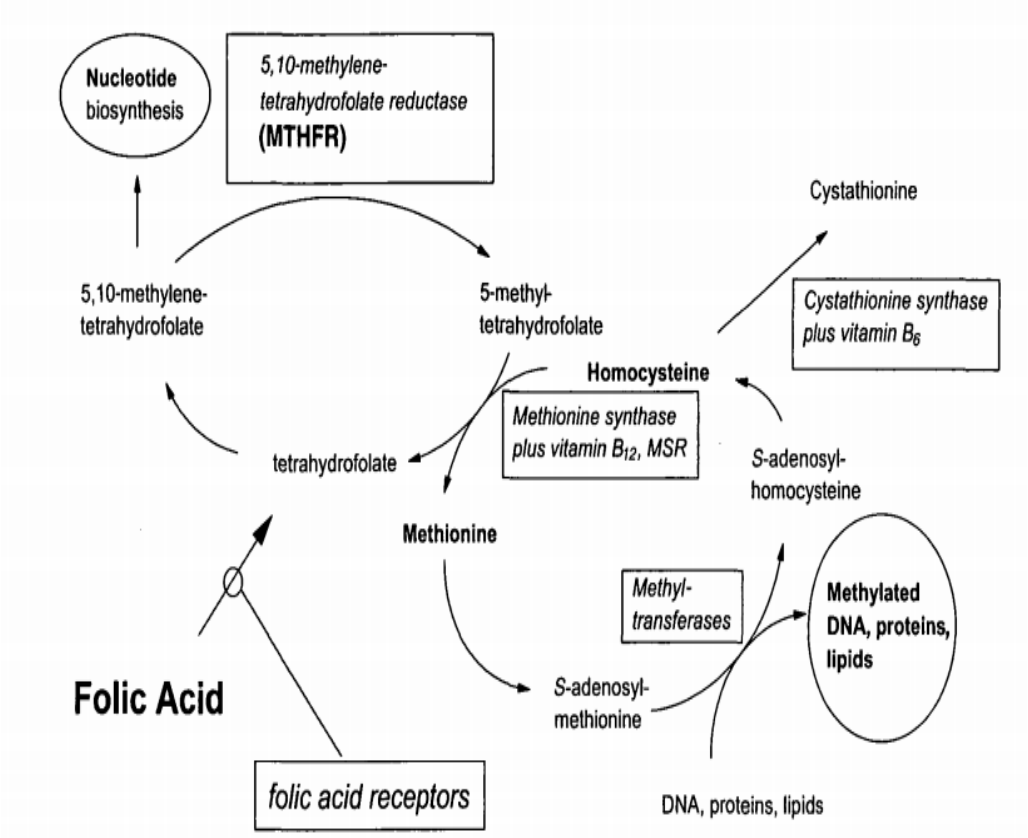

Figure 2.3: Metabolic pathways of the folic acid (Pogribna, et al., 2001)

There are two common gene variants of *MTHFR*: C677T and A1298C. In this study, the polymorphism of *MTHFR* C677T (rs1801133) was studied. The C677T is a point mutation that convert a cytosine (C) to thymine (T) at the position 677 in the exon 4 of the *MTHFR* gene. This will caused the displacement of alanine to valine (Ala222Val) at codon 222. There are three type of the genotype which was wild type (C677C), heterozygote (C677T) and homozygous mutant (T677T). The enzyme activity will be different of each type of the genotype. According to Rozen (1997) and Bianca (2012), heterozygotes had only 60% of the enzyme activity while the homozygotes mutant only expressed 30% of the enzyme activity. Homozygote mutant (TT) individual will had higher risk of accumulation of the homocysteine in the blood if they do not have enough folate intake. However, when they consumed adequate amount of folate food or supplement, this problem can be overcome (Lorenzo and Yang, 2000). In a cohort of study in Qassim region, *MTHFR* polymorphism was found associated with the reduction of the *MTHFR* enzyme activity and increased the risk of the hypertension (Alghasham et al., 2012).

2.8.2 *MTHFR* polymorphism associated with hypertension

Many studies reported that *MTHFR* polymorphism C677T was associated to hypertension. A total of 114 studies among East Asians and Cucasians was done. In that study, *MTHFR* C677T was found strongly associated with hypertension (Boyi et al., 2014).

In a cohort of study in Isreal, *MTHFR* polymorphism was found associated with the diastolic blood pressure (DBP). There was significant different of DBP with different genotypes. Wildtype (CC) genotype showed lower mean of DBP (71.4 mmHg). The mean of DBP for heterozygous (CT) genotype and homozygous mutant (TT) genotype was 74.3 mmHg and78.9 mmHg respectively (Eliyahu and Ruth, 2015). In Japan, there was significant associated of *MTHFR* gene and blood pressure (Takeuchi et al., 2010). Suchita et al. (2007) also found that homocysteine level was significant higher in heterozygous CT and homozygous mutant TT genotype when compared to the wildtype CC genotype. Therefore, C677T and T677T have higher risk of getting hypertension. Besides, in a cohort of Taiwanese population study, *MTHFR* polymorphism with low plasma folate level was found associated with the risk of getting hypertension ($p < 0.001$) (Lin et al., 2013).

Furthermore, several studies showed that homozygote mutant TT genotype had higher risk of getting hypertension when compare to the individual with the heterozygote CT genotypes (Heux et al., 2004; Qian et al., 2007; Iihan et al., 2008). In addition, Juan et al. (2015) found that individual with *MTHFR* C677T had lower risk of getting hypertension by alternating the living behaviour and environmental factor. Many case-control association studies were carried out. Yet, there is very limited work was done in prehypertension.

2.8.3 Metabolic pathway and pathogenesis of *MTHFR* polymorphism

T allele of *MTHFR* gene will reduced the enzyme activity and reduced the conversion of folate into 5-methyltetrahydrofolate. As a result, the concentration of folate in serum will decreased and the concentration of the homocysteine in the plasma will be increased (Brustolin et al., 2009). A study demonstrated that the effect of homocysteine metabolism gene polymorphism such as *MTHFR* gene and folate deficiency will increased homocysteine level (Wen et al., 2017).

Homocysteine is a sulphur-containing amino acid that was involved in the metabolism of methionine. Hyperhomocysteinemia was associated with many disorder included autoimmune disorder, diabetes, birth defects, vascular and neurodegerative disorder, renal disease and also neuropsychiatric disorder. Chronic hyperhomocysteinemia was an important factor that cause in the development of the arterial hypertension. Several studies showed that plasma homocysteine levels was associated to blood pressure. Hyperhomocysteine may cause high blood pressure in some older adults. High homocysteine will damage the vascular smooth muscle and endothelial cells and causes nitric oxide production inhibition. Thus, it leads to the loss of arterial vasodilation and vascular integrity. This will cause the blood pressure increases and atherosclerosis may be developed (Jamario and Donald, 2017). Besides, homocysteine act as an indicator of oxidative stress status.

A clinical study showed that homocysteine was a determinant of the thickness of vascular and compliance in hypertensive patients (Wang et al., 2014). In addition, Kumar et al. (2016) found that *MTHFR* C677T gene polymorphism

was associated with the small vessels disease in North Indian population. Again, another study also showed that *MTHFR* C677T polymorphism was significant associated with hyperhomocystinuria and cardiovascular disease in Jammu region (Raina et al., 2016). In Hordaland Homocysteine Study (HHS), *MTHFR* polymorphism was found associated with hyperhomocysteniemia (Refsum et al., 2006). According to the study of Stehouwer et al. (2003), when there was increase 5 micromol/l of the homocysteine in plasma level, the systolic blood pressure will increase 0.7 mmHg and 102 mmHg in men and women respectively. Besides, the plasma homocycteine will decrease when treated with folic acid. The reducing of the homocysteine level by high intake of the folic acid can be supported by another study (Boushey et al., 1995). In Yang et al. (2017) study, the initial observation showed significant associated between hyperhomocysteine and hypertension.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Biological sample

This study had been approved by UTAR Scientific and Ethnical Review Committee (SERC) with ethnical approval code number (U/SERC/96/2018). The ethnical approval letter and personal data protection statement were attached in Appendix A and Appendix B respectively. The study was carried out among a cohort of undergraduate students in Universiti Tunku Abdul Rahman (UTAR), Perak campus. The study duration was from January 2018 to June 2018. The total samples in this study was 80 students which were 32 males (40%) and 48 females (60%). Majority of the sample population was Chinese ethnic group which consists of 71 students (88.7%), two students from Malay ethnic group (2.5%) while seven students were from India ethnic group (8.8%). Buccal cells from mouth wash were collected. DNA and RNA were extracted from buccal cells.

The required sample size was calculated as below:

$$n = [t^2 \text{ x } p(1-p)]/m^2$$

$$n = [1.96^2 \text{ x } 0.06(1-0.94)]/0.05^2$$

$$= 87$$

n= required sample size

T= confidence level at 95% (standard value of 1.96）

P= estimated prevalence of T677T genotype was 6%

M=margin of error at 5%

*A total of 10 respondents will be recruited in order to allow 10% drop out

3.1.2 Chemicals materials

The list of the chemical materials and instruments that were used in the study was compiled. Chemicals that were used in DNA extraction, polymerase Chain Reaction (PCR), fragment Length Polymorphism (RFLP), RNA extraction and real-time Polymerase Chain Reaction (RT-PCR) are demonstrated in Table 3.1 and Table 3.2 respectively.

Table 3.1: List of the chemical used

Chemicals	**Manufacturer, Country**
Tris	1st BASE , Singapure
Ethylenediaminetetraacetic Acid (EDTA)	R&M Chemistry, Malaysia
Sodium dodecylsulphate (SDS)	Bendosen, Malaysia
Proteinase K	Novagen, Germany
Phenol-chloroform isoamyl alcohol	EMD Milipore Corporation, USA
Cold absolute ethanol	Nacalai Tesque, Japan
RNase A	PureLink, Malaysia
Chloroform	MERCK, New Jersey
Saline solution (0.9% NaCI)	Excelvisions Medicals, Malaysia
Boric acid	MERCK, Germany
Agarose Powder	1st BASE, Malaysia
Pair wise primers	Integrated DNA Technilogies, Malaysia
2x Power Taq PCR master mix	Bioteke Corporation, Malaysia
IO SYBR Green Supermix	BioRad, United States
Hinf I buffer	Vivantis, Malaysia
Hinf I restriction enzyme	Vivantis, Malaysia
6x loading dye	Norgen, Canada
Lambda DNA/Hind III ladder	Vivantis, Malaysia
50 bp DNA ladder	Norgen, Canada
100 bp DNA ladder	PKT, Korea
1 kb DNA ladder	Gene ruler, United States
Ethidium bromide	Bio Basic, Canada
Diethylpyrocarbonate (DEPC)	Bio Basic, Canada
DNase I	Thermo Scientific, Malaysia
2- mercaptoethanol	Bio-Rad, United States
Viva cDNA Synthesis Kit - M-MuLV Reverse Transcriptase - 10X Buffer M-MuLV - 10mM dNTPs mix - Random hexamer (50 ng/uL) - Nuclease-free water	Vivantis, Malaysia
Sodium Hydroxide	MERCK, USA
Favorprep Tissue total RNA mini kit - FARB uffer - Wash Buffer 1 - Wash Buffer 2 (concentrate) - RNase-free water	Favorgen, Taiwan

Table 3.2: List of consumables and instruments

Consumables/ Instruments	Manufacturer, Country
Conical flask (50 mL)	Favorit, Malaysia
Measuring cyclinder (25mL, 50 mL, 100mL)	Favorit, Malaysia
Schott bottle (500mL, 1000mL)	Duran, Germany
Centrifuge tube	Axygen Scientific, USA
1.5 mL microcentrifuge tube	Greiner Bio-one, Germany
PCR tubes	Axygen Scientific, USA
PCR strips tubes	Axygen Scientific, USA
Pipettes	Witeg, Germany
Pipette tips	Quality Scientific Plastic, China
Water bath	Memmert, Germany
Weighing machine	Adventurer, New Jersey, USA
Electrophoresis Sets	Major Science, Taiwan
Vortex mixer	Gemmy Industrial Corporation, Taiwan
Microwave	Sharp, Malaysia
4˚ and 20˚ freezer	Pansonic, Malaysia
Fume hood	Esco, USA
Centrifuge machine (15 mL and 50 mL)	Dynamica, India
Centrifuge machine (1.5 mL)	Thermo Scientific, US
Nanophotometer	Implen GmbH, Germany
PCR machine	Eppendorf, Germany
Real time PCR machine	Bio-Rad, United State
Favorprep Tissue total RNA mini kit - Filter column - FARB Mini Column - Collection Tube - Micropestle - Elution tube	Favorgen, Taiwan
Mutlioc-It, bench-top UV-Transilluminator	Syngene, UK

3.2 Overview of the methodology

The overview of the methodology that involved in DNA and RNA extraction, genotyping identification, PCR and real-time PCR in this study are demonstrated in Figure 3.1 and Figure 3.2 respectively.

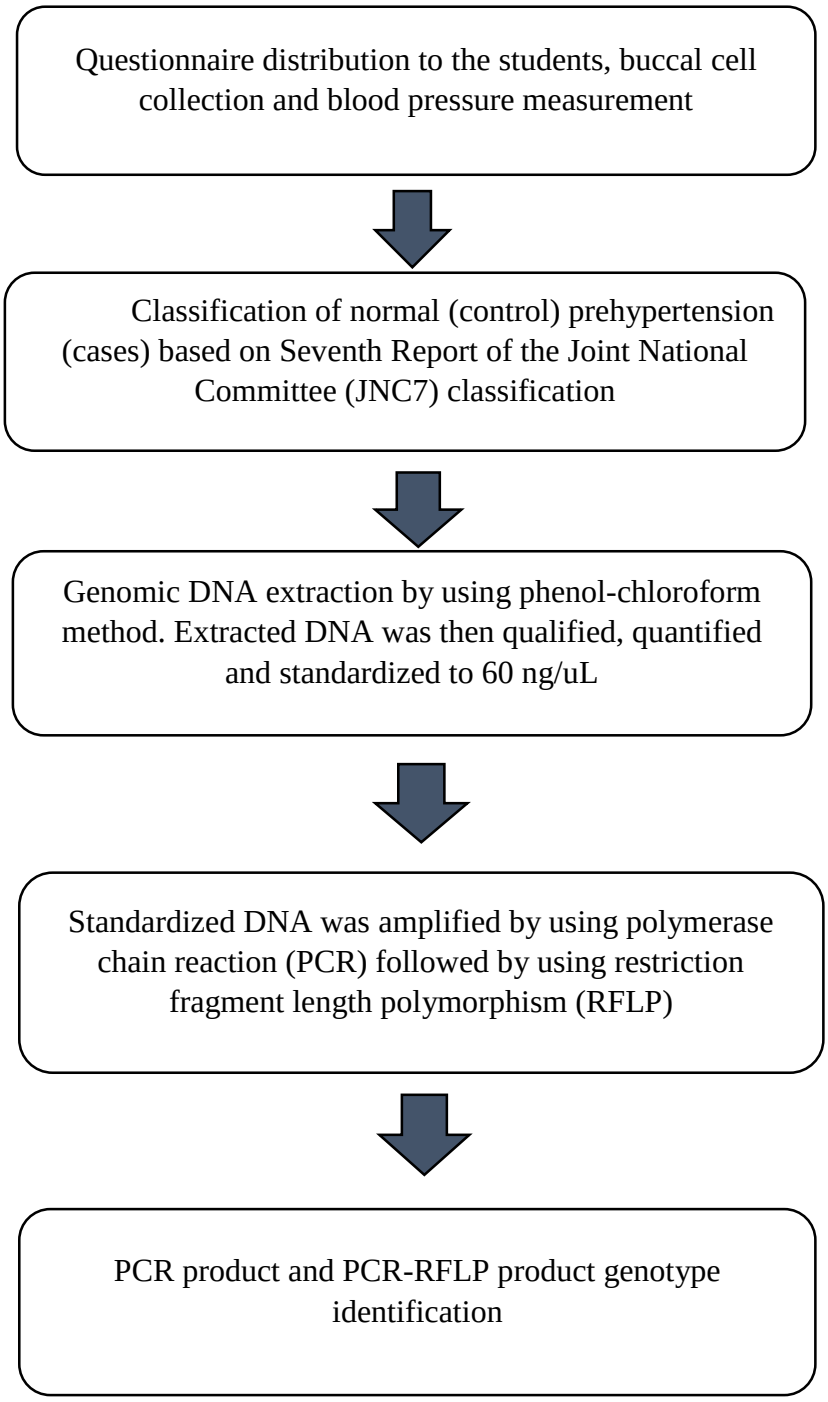

Figure 3.1: Overview methodology for Genomic DNA extraction and genotyping identification

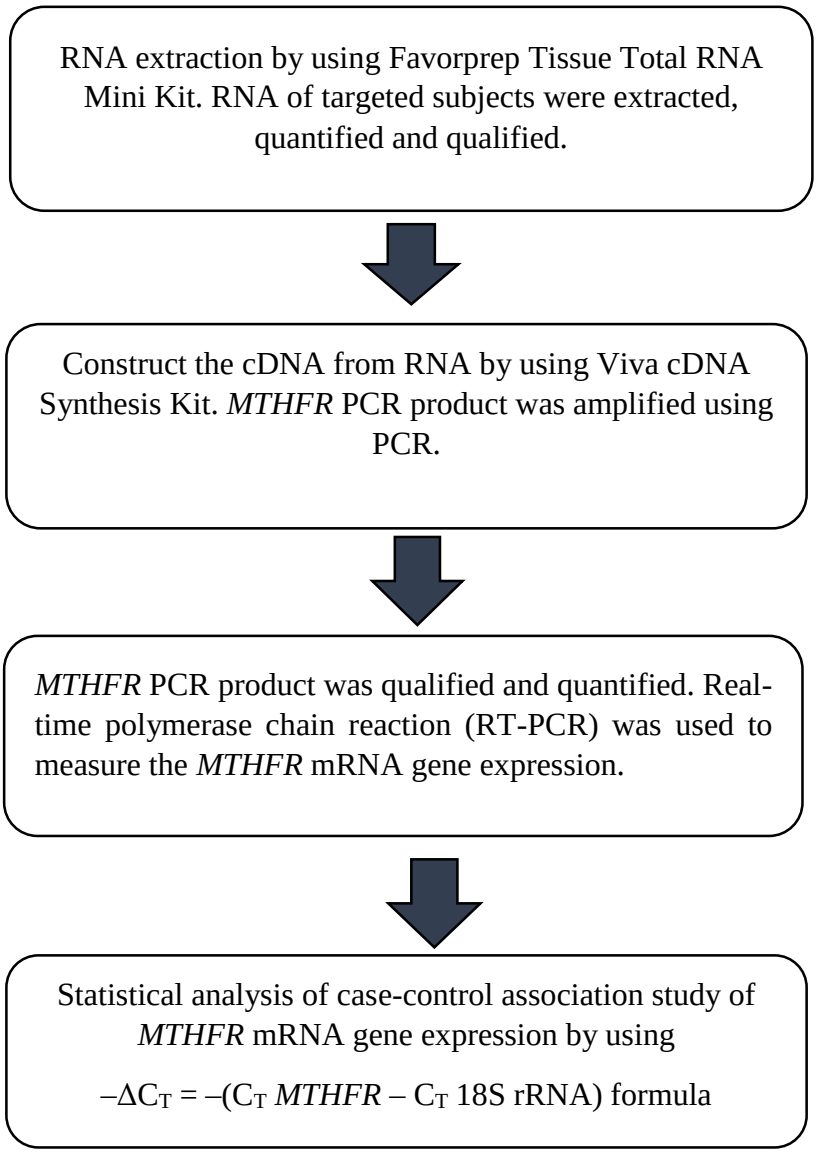

Figure 3.2: Overview methodology of RNA extraction and gene expression of mRNA

3.3 Genomic DNA Extraction

3.3.1 Consent Form and Questionnaire survey

At the initial stage, the student volunteers were given a consent form. The consent form consisted the details of title, the procedure, the risk and the confidentiality of the study. Upon reaching an agreement, a signature was obtained from the subject to represent that they are agree and aware of the risks during collecting their buccal cells for the genomic DNA and RNA extraction.

The consent form was attached together with the questionnaire (Appendix C). Subjects were required to answer the questionnaire which consisted four parts. First part is demographic data like age, gender, race, height, weight and family health history. The second part is dietary behaviour. The third session is investigate the physical activity level and the fourth part is depression, anxiety and stress scales.

3.3.2 Blood pressure measurement

Blood pressure reading which include systolic blood pressure (SBP) and diastolic blood pressure (DBP) will be measured by using an automated blood pressure monitor. The blood pressure was measured on the left arm after the subjects had rested for 5 minutes in a seated position. All the measurement were taken three times and the average value was calculated. Blood pressure was categorized based on JNC7 blood pressure classification which shown in the Table 2.1.

3.3.3 Buccal cell collection

The reason that use buccal cell collection because it poses minimal risk and discomfort to donor as it is a non-invasive method. Donors are advised not to consume any food or drink 30 minutes prior to mouth wash collection. Each donor needs to clean and rinse their mouse by mineral water to remove the food particles. After that, a 15 mL of tube that containing sterile saline (0.9% NaCI) was given to the donor and donor was requested to gargle for 2 minutes to collect buccal cells. The saline that containing buccal cells were dispensed back into the Styrofoam cup. Then, it was transferred to a sterile 15 mL of centrifuge tube. The buccal cells collection was then used for genomic DNA and RNA extraction.

3.3.4 Genomic Deoxyribonucleic Acid (DNA) Extraction

Genomic DNA was extracted from the buccal cells by using the phenol-chloroform method. The saline buffer containing buccal cells was centrifuged for 20 minutes at 10,000 rpm. The supernatant was discarded and the pellet was resuspended in 460 µL of Tris-EDTA (TE) buffer, 10 µL of Proteinase K (4 mg/mL) and 30 µL of 10% SDS. The mixture was then mixed well and vortexed before transferring into a 1.5 mL of microcentrifuge tube. The sample was incubated for 1 hour at 37˚ C. A total volume of 500 µL of Phenol-chloroform-isoamyl alcohol (PCI) with the ratios of 25:24:1 was added to the tube after incubation. The tube was inverted several times to mix well. Then the tube was centrifuged at 14,000 rpm for 10 minutes and the supernatant was carefully removed to a new 1.5 mL microcentrifuge tube without disturbing the tissue debris at the interface. Subsequently, 20 µL of RNase A (4 mg/mL) was added

into the tube for the removal of RNA. The sample was incubated at 37˚C for 5 minutes. After that, the tube was centrifuged at 13,000 rpm for 10 minutes. The supernatant was then transferred to a new 1.5 mL microcentrifuge tube. Next, a volume of 500 µL of chloroform was added into the tube and centrifuged at 14,000 rpm for 10 minutes. The top layer form was transferred carefully into a new 1.5 mL microcentrifuge tube and followed by adding 2X volume of cold absolute ethanol. After that, 40 µL of the NaCI (5M) was added to the tube and inverted for several times. The tube was then centrifuged at 14,000 rpm for 15 minutes. The supernatant was discarded and the pellet was washed with 500 µL of 70% ethanol for 2 times. The tube was centrifuged at 13,000 rpm for 5 minutes. The ethanol then discard and the pellet was then air-dried at room temperature for approximately 20 minutes. Lastly, the DNA pellet was dissolved in 50 µL of TE buffer and was stored at -20˚ C refrigerator.

3.3.5 Quantification of Genomic DNA

Genomic DNAs were quantified using NanoPhotometer (Implen). The concentration and the purity of DNA were evaluated by measuring the absorbance of genomic DNA sample at the wavelength of 260 nm and 280 nm respectively. After the NanoPhotometer was switch on and calibrated, a Submicroliter Cell was then inserted into the holder of the cell with the facing of the cell windows to the direction of light beam passage. Before start measuring of concentration DNA samples, the lid and the measurement window were cleaned with Kimwipe paper and the machine was blanked with 2 µL of the sterile distilled water. Next, a volume of 2 µL genomic DNA sample was

pipetted and transferred to the measurement window carefully. The window was then covered with a 1 mm path length lid prior to initiating the measurement. The absorbance readings at OD260 nm, OD280 nm, the ratio and also the genomic DNA concentration was recorded. Then, the measurement window and the lid were cleaned with a Kimwipe paper before measuring next genomic DNA sample. The formula of calculating the genomic DNA is shown as below:

Genomic DNA concentration = OD260 nm x 50 x 10 x Dilution factor

OD260 nm : Absorbance reading at 260 nm

50 & 10 : Substance specific factor for double stranded DNA; Lid factor

Dilution factor : Set at 1.0 for genomic DNA

This formula was also used for the calculation of the DNA concentration at the path length at 280 nm. Besides, the ratio of the absorbance at 260 nm and 280 nm ($A_{260/280}$) was used to assess the purity of genomic DNA. $A_{260/280}$ ratio of ~ 1.8 is accepted as pure DNA.

3.3.6 Qualification of genomic DNA by Gel Electrophoresis

The qualification of genomic DNA was assessed via gel electrophoresis. For gel electrophoresis, 1X Tris-Boric acid-EDTA (TBE) buffer and 1% agarose gel were prepared. The 1X TBE buffer was prepared by mixing of 10 mL of 10X TBE buffer with 90 mL of sterile distilled water in a conical flask. A total amount of 0.2 g of agarose powder was weighed and added into the conical flask that was containing 20 mL of 1X TBE buffer. The mixture was fully dissolved by using microwave. The agarose mixture was then cooled down to approximately 50˚ - 60˚ before being poured into the gel casting tray. A 0.75 mm comb was

inserted immediately into the casting tray and the agarose gel was allowed to solidify at room temperature for approximately 30 minutes. Once the gel was completely solidified, the comb was carefully removed and the agarose gel together with the casting tray was placed into a gel electrophoresis tank. The tank was then filled up with 1X TBE buffer until around 5 mm above the agarose gel was covered.

For each gel, the first lane was loaded with the 2 µL of the Lambda DNA *Hind* III ladder. Prior to loading 5 µL of the genomic DNA samples into the wells, they were mixed with 1 µL of 6x loading dye on a piece of a clean parafilm. Once everything was loaded into the wells, the gel electrophoresis was run at 90V for 30 minutes. Then, the agarose gel was stained with Ethidium Bromide for 10 minutes and destained with the water for 5 minutes. Ultimately, the gel image was viewed and captured under a UV-Transiiluminator.

3.3.7 Standardization of template DNA

All DNA samples were standardized to a final concentration of 60 ng/uL. The standardized genomic DNA was then used as a template in PCR amplification. The qualification of 60 ng/uL of genomic DNA was assessed via gel electrophoresis.

3.4 *MTHFR* C677T genotyping identification

3.4.1 PCR amplification

The *MTHFR* C677T forward primer sequence: 5' -TGA AGG AGA AGG TGT CTG CGG GA-3' and *MTHFR* C677T reverse primer sequence: 5' –AGG ACG GTG CGG TGA GAG TG-3' were used to amplify 198 bp of PCR product. Each of the PCR tube in a reaction contained 0.4 µM of forward primer, 0.4 µM of reverse primer, 1 unit of *Taq* Master Mix ,60 ng/µL DNA template and sterile distilled water. The amount volume of each components are demonstrated in Table 3.3 and Table 3.4.

The extracted genomic DNA was amplify by using polymerase chain reaction (PCR). The conditions of PCR amplification are summarized in Table 3.5. The PCR amplification began with an initial pre-denaturation step at 92˚C for 2 minutes followed by 35 cycles of denaturation, annealing and elongation at 92 ˚C for 1 minutes, 64 ˚C for 1 minutes and 72 ˚C for 30 seconds respectively. The final extension step was carried out at 72 ˚C for 7 minutes. The PCR products were then loaded on a 2% agarose gel and electrophoresis was performed at 90V for approximately 35 minutes to confirm the presence of the desired amplicon size (198 bp). The gel image was viewed and captured under UV-Transilluminator after ethidium bromide staining.

Table 3.3: Forward and reverse primers for the *MTHFR* C677T

Pair wise primer	Primer (5' to 3')	PCR amplicon (bp)
Forward	5' -TGA AGG AGA AGG TGT CTG CGG GA-3'	198
Reverse	5' -AGG ACG GTG CGG TGA GAG TG-3'	

Table 3.4: Volume and concentration of the PCR components used in PCR amplification

PCR components	Stock concentration	Final concentration	Volume (µL)
2x *Taq* Master Mix	2x	1x	9.00
Forward primer	10 µM	0.4 µM	0.72
Reverse primer	10 µM	0.4 µM	0.72
DNA template	-	60 ng/uL	1.00
Sterile distilled water	-	-	6.56
Total			18.00

Table 3.5: Conditions for PCR amplification of *MTHFR* C677T

Stage	Temperature (˚C)	Duration	Cycle
Pre-denaturation	92	2 min	1
Denaturation	92	1 min	35
Annealing	64	1 min	
Elongation	72	30 sec	
Final extension	72	7 min	1
Hold	10	∞	

3.4.2 *MTHFR* C677T genotyping using PCR-RFLP method

Once the presence of amplicon at desired size was confirmed, the PCR product was digested with *Hinf*I (*Haemophillus influenza*) restriction enzyme. The C is changed to T at the nucleotide in the exon produce a recognition site for *Hinf*I restriction enzyme. The recognition sequences and cutting sites of *Hinf*I at G^ANTC sites for 5' to 3' and CTNA^G sites for 3' to 5' have been illustrated in Figure 3.3. Besides, the components of restriction enzyme reaction are stated in Table 3.6. The mixtures were incubated in a 37˚C water bath for 2 hours. Then, the restricted PCR products were loaded into 4% of agarose gel and electrophoresed at 90V for 35 minutes. The 50 bp ladder was used. The

homozygous wild type (CC) genotype showed only one band at 198bp. Heterozygotes (CT) produces three bands: 198bp, 175bp and 23bp. While homozygous mutant (TT) showed two bands at 175bp and 23bp.

Figure 3.3: *MTHFR* gene fragment with the primer sequence and the recognition site of the *HinfI* restriction enzyme

Table 3.6: Components of restriction enzyme reaction

Gene variant	Components	Volume (µL)
MTHFR C677T	Sterile distilled water (dH_2O)	8.2
	10x HinfI buffer	1.5
	PCR product	5.0
	*Hinf*I enzyme	0.3
	Total	15.0

3.5 RNA extraction and quantification mRNA expression of *MTHFR* gene

3.5.1 Preparation and RNA Extraction

Prior to the RNA extraction, all equipment and consumables were treated with Diethylpyrocarbonate (DEPC) water to make sure it is RNase free. Firstly, the non-disposable plastic ware such as tip box was treated with 0.1M NaOH, 1 mM EDTA and DEPC treat water. The tips were soaked in 0.1% of DEPC solution for overnight and then were autoclaved before used. Furthermore,

electrophoresis tank also was treated with 0.5% SDS and DEPC treat water to removed and prevent RNase contamination.

Buccal cells were used for RNA extraction. The procedure of buccal cell collection was discussed at section 3.3.3. The RNA was extracted by using FavorPrep Tissue Total RNA Mini Kit from Favorgen manufacturer. At initial step, the 15 mL of centrifuge tube that consisting buccal cells was centrifuged at 10,000 rpm for 20 minutes at 4˚C. The supernatant was discarded and the pellet was dissolved with 350 µL of lysis buffer (FARB Buffer) and 3.5 µL of β-mercaptoethanol. The tube was then vortexed vigorously for 1 minutes to resuspend the cells completely. A filter column was placed to a collection tube and the sample mixture was transferred to the filter column. The sample was centrifuged at 18,000 xg for 2 minutes. The supernatant was transferred from the collection tube to a new 1.5 mL microcentrifuge tube and the volume of the supernatant was measured. A 1x volume of 70% RNase-free ethanol was added and mixed well by vortexing. A FARB Mini Column was placed to a collection tube and followed by the sample mixture transferred to the FARB mini column. The sample was then centrifuged again at 18,000 xg for 1 minutes. The flow-through was discarded and returned the FARB mini column back to the collection tube. A total volume of 250 µL of wash buffer 1 was added to the FARB mini column and centrifuged at 18,000 xg for 1 minutes. A volume of 60 µL of RNase-free DNase I was added to the membrane center of the FARB mini column and placed on the benchtop for 15 minutes. Next, a 250 µL of wash buffer 1 was added again to the FARB mini column and centrifuged at 18,000 xg for 1 minutes, followed by the flow-through was discard. A volume of 750

µL of wash buffer 2 was added to the FARB mini column and centrifuged at 18,000 xg for 1 minutes and washing step was repeated. After that, the FARB mini column was centrifuged at 18,000 xg for 3 minutes to spin dry the FARB mini column. The FARB mini column then placed to an elution tube and 50 µL of the RNase-free distilled water was added to the membrane center of the FARB mini column. The FARB mini column was standed for 10 minutes. Finally, the FARB mini column was centrifuged at 18,000 xg for 1 minutes to elute the RNA. RNA was stored at -80˚C.

3.5.2 Qualification and quantification RNA using NanoPhotometer

The quantification of the RNA was measured with the NanoPhotometer (Implen). The concentration and the purity of RNA were evaluated by measuring the absorbance of RNA sample at the wavelength of 260 nm and 280 nm respectively. The step of using NanoPhotometer is same as section 3.3.5. $A_{260/280}$ ratio of ~ 2.0 is accepted as pure RNA.

3.5.3 Qualification of RNA on Gel Electrophoresis

The qualification of RNA was assessed using gel electrophoresis. The agarose gel was prepared as mention in section 3.3.6. The first lane of gel was loaded with 2 µL of 1 kb ladder. The 5 µL of RNA samples were mixed with 1 µL of 6x loading dye before loading on the wells. The gel electrophoresis was run at 100V for 30 minutes. Next, the agarose gel was stained with Ethidium Bromide

for 10 minutes and destained with water for 5 minutes. Ultimately, the gel image was viewed and captured under a UV-Transiiluminator.

3.5.4 cDNA synthesis

The RNA sample was reversed transcripted to cDNA by using Viva cDNA synthesis Kit from Vivantis manufacturer. First, the RNA-primer mixture was prepared with including 15 µL of RNA sample and 1 µL of 10 mM dNTPs mix and 1 µL of random hexamer. The mixture was then incubated at 65˚C for 5 minutes and chilled on ice for 2 minutes. The mixture was gently spin down. After that, 1 µL of M-MuLv Reverse transcriptase and 2 µL of 10x Buffer M-MuLV was added to the sample mixture. The sample was then mixed gently and spin down. The mixture was incubated for 1 hour at 42˚C. Then, the mixture was incubated at 85˚C for 5 minutes to terminate the reaction. The tubes was then chilled on the ice for 2 minutes and the mixture was spin down. The cDNA was synthesis and stored at -20˚C. The concentration and purification of the cDNA can be measured by Nanophotometer.

3.5.5 PCR cDNA amplification

Two types of primers were used in the PCR amplification which was 18S rRNA as the housekeeping gene and *MTHFR* primers. The forward primer sequence of 18S rRNA was 5’ –AAC TTT CGA TGG TAG TCG CCG-3’ while the reverse primer sequence of 18s rRNA was 5’ –CCT TGG ATG TGG TAG CCG TTT-3’. The forward primer sequence of *MTHFR* was 5’ –GAA GTA CGA GCT

CCG GGT TA-3' while the reverse primer sequence of *MTHFR* was 5'-AAG ATG CCC CAA GTG ACA G-3'. Each PCR master mix contained 1 unit of *Taq* Master Mix, 0.4 µM of forward primer, 0.4 µM of reverse primer, 100 ng/µL cDNA template and sterile distilled water. The amount volume of each components are listed in Table 3.7 and Table 3.8.

PCR amplified 104 bp fragment by 18S rRNA primers and 98 bp of *MTHF* gene using *MTHFR* primers. The conditions used for PCR amplification is demonstrated in Table 3.9. The PCR amplification began with an initial pre-denaturation step at 94˚C for 5 minutes followed by 35 cycles of denaturation, annealing and elongation at 94 ˚C for 30 seconds, 60 ˚C for 30 seconds and 72 ˚C for 10 seconds respectively. The final extension step was carried out at 72 ˚C for 5 minutes. The PCR products were then loaded on a 2% agarose gel and electrophoresis was performed at 90V for approximately 35 minutes to confirm the presence of the desired amplicon size. The gel image was viewed and captured under UV-Transilluminator after ethidium bromide staining.

Table 3.7: Forward and reverse primers for the *MTHFR* mRNA

Pair wise primer		**Primer (5' to 3')**	**PCR amplicon (bp)**
Housekeeping 18S rRNA (internal control)	Forward	AAC TTT CGA TGG TAG TCG CCG	104
	Reverse	CCT TGG ATG TGG TAG CCG TTT	
MTHFR	Forward	GAA GTA CGA GCT CCG GGT TA	98
	Reverse	AAG ATG CCC CAA GTG ACA G	

Table 3.8: Concentration and volume of the PCR components used for PCR amplification

PCR components	Stock concentration	Final concentration	Volume (µL)
2x *Taq* Master Mix	2x	1x	9.00
Forward primer	10 µM	0.4 µM	0.72
Reverse primer	10 µM	0.4 µM	0.72
cDNA template	-	100 ng/µL	1.00
Sterile distilled water	-	-	6.56
Total			18.00

Table 3.9: Conditions for PCR amplification of cDNA

Stage	Temperature (˚C)	Duration	Cycle
Pre-denaturation	94	5 minutes	1
Denaturation	94	30 sec	35
Annealing	60	30 sec	
Elongation	72	10 sec	
Final extension	72	5 minutes	1
Hold	10	∞	

3.5.6 *MTHFR* mRNA gene expression level

The *MTHFR* gene expression was qualified using real time PCR. Real-time PCR is the gold standard for quantifying gene expression and it is a rapid, accurate, sensitive, cost-effective, reliable and reproducible (Yilmaz et al., 2012). Relative quantification was used to determine the changes in mRNA levels in the samples and expresses it relative to level of an internal control RNA. Relative quantifications will determined the fold changes in expression between two samples. The relative quantification calibration curve result for the gene of interest was normalized to the housekeeping gene in the same samples and

followed by the normalized numbers was compared between samples to obtain the fold changes (Qiagen, 2018).

First, the iQ SYBR Green supermix was thawed and placed on the ice protected from light. The master mix assay for all reactions was prepared and the components are listed in Table 3.10. After put all the reaction materials in qPCR tubes, the tubes was then sealed with the flat caps and spin down. After that, the samples were inserted to the heat block into the real time PCR machine for amplification. The condition of the real-time PCR was shown in Table 3.11.

Table 3.10: Concentration and volume of the qPCR components used for real-time PCR amplification

qPCR components	**Stock concentration**	**Final concentration**	**Volume (µL)**
2x Iq™ SYBR Green Supermix	2x	1x	5.00
Forward primer	10 µM	0.4 µM	0.40
Reverse primer	10 µM	0.4 µM	0.40
cDNA template	-	100 ng/µL	1.00
Sterile distilled water	-	-	3.20
Total			10.00

Table 3.11: Thermal cycling for the real-time PCR amplification

Stage (Amplification)	**Temperature (˚C)**	**Duration**	**Cycle**
Pre-denaturation	94	5 min	1
Denaturation	94	30 sec	35
Annealing	60	30 sec	
Elongation	72	10 sec	
Melt Curve Analysis	65 – 95, increment 1.0 for 0.05 sec		

3.5.7 Statistical Significances of *MTHFR* mRNA expression levels

The questionnaire was evaluated. The genotype identification of *MTHFR* C677T was evaluated by using PCR-RFLP method. The distribution of allele and genotype frequency of the control and prehypertension cases of the students were evaluated by using Chi-square test. The hypothesis that genetic variant of *MTHFR* C677T gene is associated with prehypertension was tested by employing the Hardy-Weinberg Equilibrium. The *MTHFR* mRNA expression levels according to the studied polymorphism were calculated by using $2^{-(\Delta\Delta Ct)}$ method to calculate the fold change gene expression of mRNA level between samples. The *MTHFR* mRNA expression level was compared among four different groups: normal without genetic condition (A), normal with genetic condition (B), prehypertension cases with genetic condition (C) and prehypertension cases without genetic condition (D).

CHAPTER 4

RESULTS

4.1 Epidemiology of prehypertension among UTAR Kampar Students

In this study, a total of 80 study subjects were collected. There were 32 of males (40%) and 48 females (60%). Of these, the population was predominantly Chinese ethnic group which accounts for 71 (88.7%) and Indians ethnic have seven students (8.8%) and only two students from Malay ethnic group (2.5%). The overwhelming number of Chinese participants due to the majority of the students in the UTAR Kampar campus are Chinese.

4.1.1 Prevalence of prehypertension among UTAR Kampar Students and between Gender

Among the total 80 participants were involved in this study, 60 (75%) students have the normal blood pressure and 19 (23.75%) students were prehypertension and only one (1.25%) students were in hypertension stage. Due to only a number of respondents belong to hypertension category, therefore the prehypertension group and hypertension group were classified into “prehypertension” group and were integrated during statistical analysis. Overall, the prevalence of the prehypertension cases group in this study was 25% in UTAR students population. The prevalence of normal and prehypertension cases responds presented in the Figure 4.1. When comes to the gender distributions in the prehypertension

responses, males have higher prevalence to get prehypertension compared to female. There were 70% were male and 30% were females (Figure 4.2).

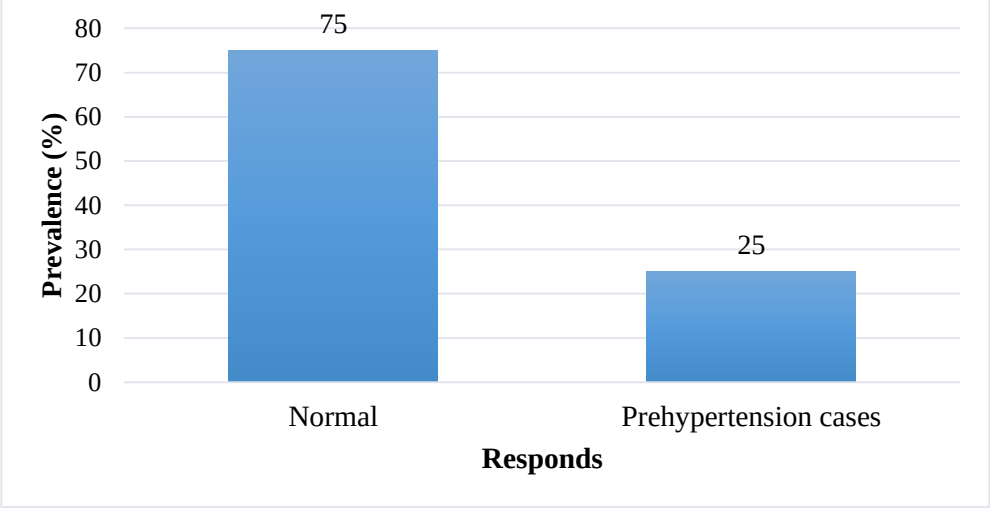

Figure 4.1: Prevalence of the normal and prehypertension cases responds of blood pressure

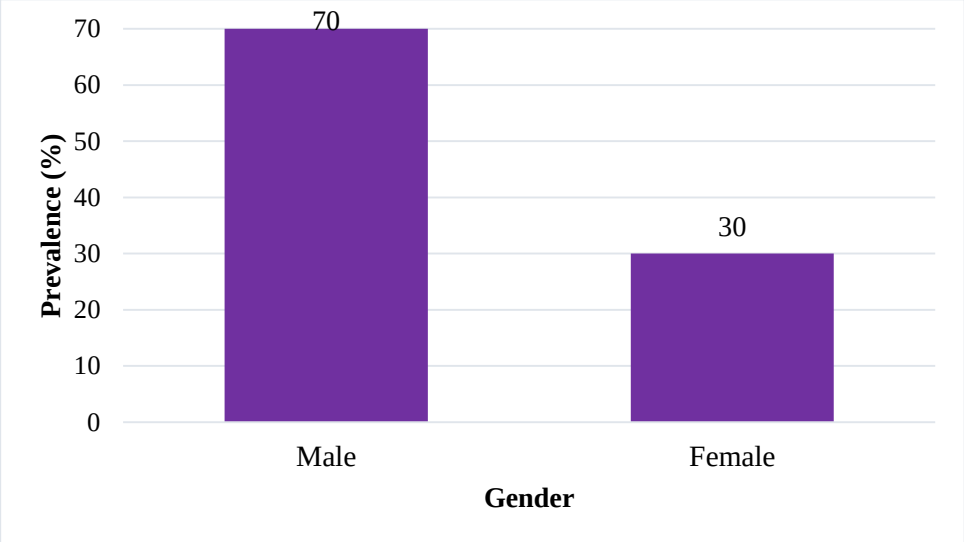

Figure 4.2: Prevalence of prehypertension between male and females

4.2 *MTHFR* C677T (Ala222Val) Polymorphism in Prehypertension

4.2.1 Evaluating Genomic DNA

The Genomic DNA was extracted from buccal cells. The genomic of DNA was qualified before proceed to PCR amplification and restriction enzyme digestion. The genomic DNA was electrophoresed on a 1% of agarose gel for approximately 30 minutes at 90V (Figure 4.3). The presence of the bands on the gel indicates that the successful extraction of genomic DNA. The concentration and purity of the genomic DNA samples are listed in Appendix E. The average of the concentration and purity of genomic DNA samples were 327 ng/µL and 1.72 respectively. The DNA samples were then standardized to 60 ng/µL and electrophoresed on 1% agarose gel. The standardized genomic DNA showed the band uniformly distributed in all the wells (Figure 4.4).

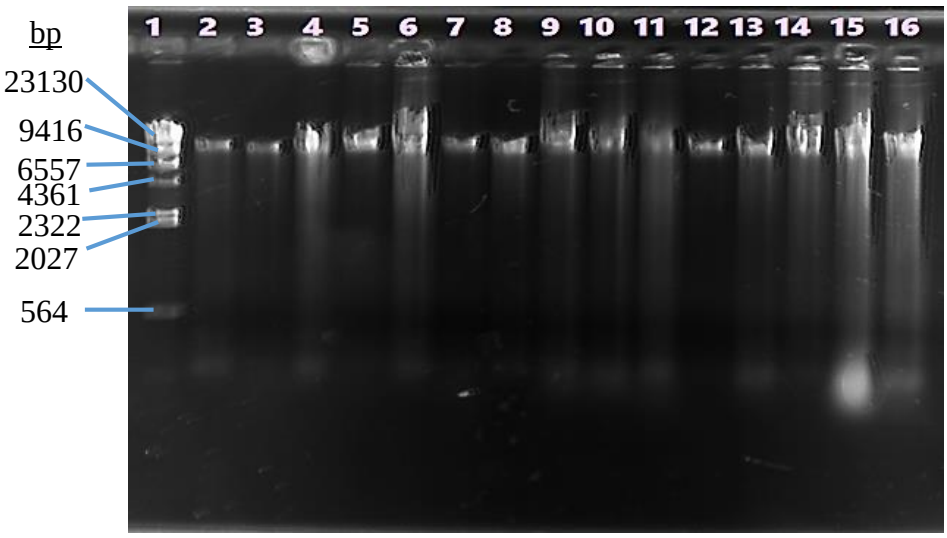

Figure 4.3 : Gel image of unstandardized genomic DNA extracted from buccal cells (1% agarose gel). Lane 1 is the Lambda DNA/*Hind*III ladder. Lane 2 to land 16 are the genomic DNA samples of different donors.

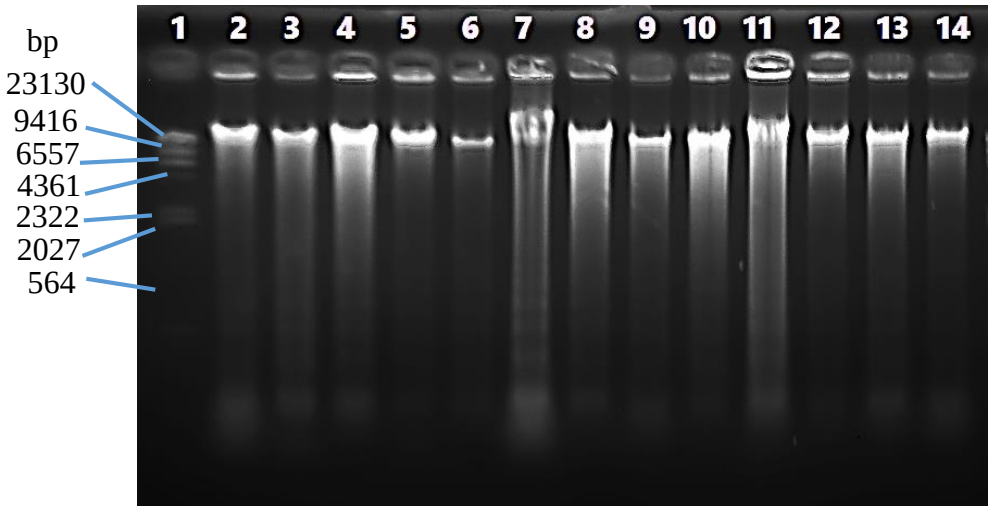

Figure 4.4: Gel image of 60 ng/µL standardized genomic DNA (1% agarose gel). Lane 1 is Lambda DNA/*Hind*III ladder. The lane 2 to lane 14 are the standardized of genomic DNA samples of different donors.

4.2.2 *MTHFR* Ala222Val (rs1801133) Genotyping

In this study, *MTHFR* C677T genotyping was determined using PCR-RFLP technique. The amplicon was located at the 3' untranslated region of exon 4 of the *MTHFR* gene with a total length of 198 bp. The PCR amplicon was then electrophoresed on a 2% of agarose gel at 90V for 35 minutes (Figure 4.5).

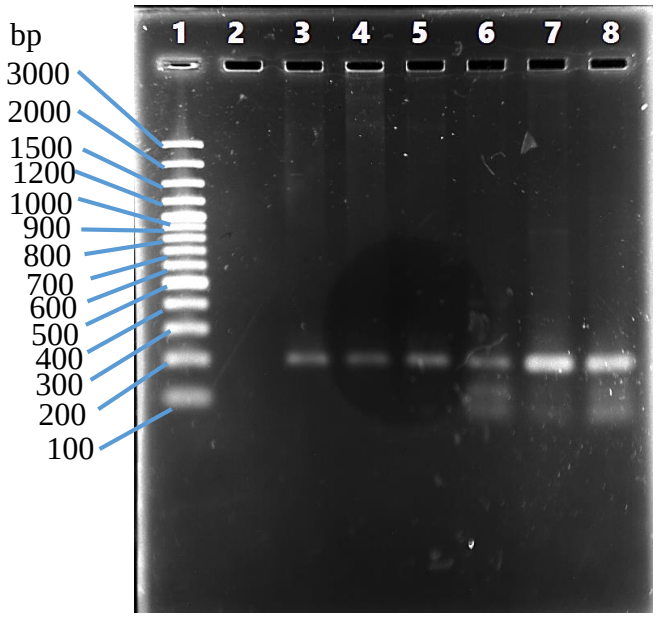

Figure 4.5: The PCR amplicons of targeted *MTHFR* gene fragment run on 2% agarose gel. Lane 1 is 100 bp DNA ladder. Lane 2 is negative control. Lane 3 to lane 8 are PCR products and the amplicon size is 198 bp.

To confirm the genotype of the desired amplicon, RFLP was performed. The digested fragments were loaded on 4% agarose gel and electrophoresed to separate the fragments corresponding to their respective size. The restriction enzyme that used to digest the *MTHFR* C677T gene variants was *Hinf* I. All the bands were well seperated and the genotypes could be clearly distinguished (Figure 4.6). For the homozygous wild type (CC) genotype, only one band at 198 bp was observed while for the heterozygous (CT) genotype showed three bands at 198 bp, 175 bp and 23 bp. For the homozygous mutant (TT) genotype, two bands were observed at 175 bp and 23 bp.

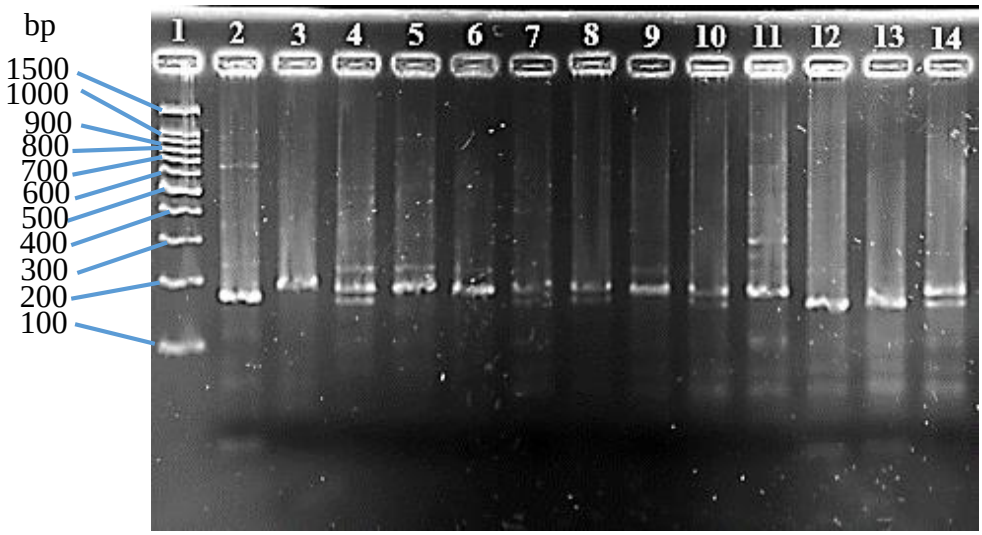

Figure 4.6: *MTHFR* C677T genotyping on a 4% agarose gel. Lane 1 is 100 bp DNA ladder. Lane 2 to lane are PCR-RFLP samples. Lanes 3, 5, 6, 9 and 11: homozygous wildtype; Lanes 4, 7, 8, 10 and 14: heterozygous; Lanes 2, 12 and 13: homozygous mutant

4.2.3 *MTHFR* C677T genotypic and allelic frequencies of the studied population

After the process of PCR-RFLP, the *MTHFR* C677T (rs1801133) genotypes were identified. A total of 80 of UTAR students participated in this study. From the volunteers, 46 students (57.5%) were homozygous wildtype CC genotype, 26 students (32.5%) were heterozygote CT genotype and eight students (10.0%) were homozygote mutant TT genotype (Table 4.1). There were 60 controls (normal) and 20 cases were prehypertension students. The genotype frequency of the normal and cases are demonstrated in Table 4.2.

The frequencies of the allele and genotype of *MTHFR* C677T in controls and cases were calculated based on the Hardy-Weinberg equilibrium. In general, the allele frequency was caculated. It is 0.75 for C allele and 0.25 for T allele (Table 4.3).

Table 4.1: Genotypic frequency of *MTHFR* gene in the studied population

Genotype	Total (n =80)
CC	46 (57.5%)
CT	26 (32.5%)
TT	8 (10.0%)

Table 4.2: Genotypic frequency of *MTHFR* in control and cases group

Genotype	Control group	Cases group
CC	35 (58.3%)	11 (55.0%)
CT	20 (33.3%)	6 (30.0%)
TT	5 (8.33%)	3 (15.0%)
Total	60 (100.0%)	20 (100.0%)

Table 4.3: Allele frequency of *MTHFR* in control group and cases group

Allele	Frequency (n= 80)	
	Control group (Normal)	Cases group (Prehypertension)
C	0.76	0.74
T	0.24	0.26
Total	1.00	1.00

4.2.4 T allele of *MTHFR* C677T is a genetic risk factor of prehypertension

Based on Hardy-Weinberg Equilibrium, it was found that *MTHFR* C677T was associated with prehypertension. In this study, H_0 and H_1 hypotheses were set and evaluated.

H_0 : The date conform Hardy-Weinberg Equilibrium. T allele is not a genetic risk factor for prehypertension.

H_1 : The date do not conform Hardy-Weinberg Equilibrium. T allele is a genetic risk factor for prehypertension.

The chi square test was in the calculation. For the control group, since the calculated χ^2 < tabulated χ^2 (3.84), so H_0 is accepted. However, for the cases group (prehypertension and hypertension), the calculated χ^2 > tabulated χ^2 (3.84),

therefore, H_0 is rejected. In conclusion, the *MTHFR* C677T gene variant was associated with prehypertension in this study. The chi square test are shown in Table 4.4 and Table 4.5.

Table 4.4: *MTHFR* C677T genotypes distribution in normal control group

Genotype	Observed (O)	Expected (E)	(O-E)	$(O-E)^2$	$(O-E)^2$ /E
C/C	35	35	0	0	0
C/T	20	22	-2	4	0.18
T/T	5	3	2	4	1.33
Total	60				$\chi^2 = 1.51$ (p =0.05)

Degree of freedom = (3-2) =1
Tabulated χ^2 =3.84

Table 4.5: *MTHFR* C677T genotypes distribution in prehypertension cases group

Genotype	Observed (O)	Expected (E)	(O-E)	$(O-E)^2$	$(O-E)^2$ /E
C/C	11	11	-1	1	0.08
C/T	6	8	-2	4	0.50
T/T	3	1	2	4	4.00
Total	20				$\chi^2 = 4.58$

Degree of freedom = (3-2) =1
Tabulated χ^2 =3.84

4.3 *MTHFR* gene mRNA expression of cases and control

4.3.1 Evaluating RNA

Total RNA was extracted from buccal cells. Among the 80 students, 22 students were selected for the further study in the mRNA expression of the *MTHFR* gene. The samples were chosen based on their genotypes. The RNA samples were

electrophoresed on 1% agarose gel for approximately 30 minutes at 100V. The gel image is demonstrated in Figure 4.5. Degradation of RNA was obtained. Optimisation of RNA extraction method should be done in future study. The concentration and purity of the RNA samples are shown in Appendix F. The average of the concentration and purity of RNA samples were 30 ng/µL and 1.75 respectively.

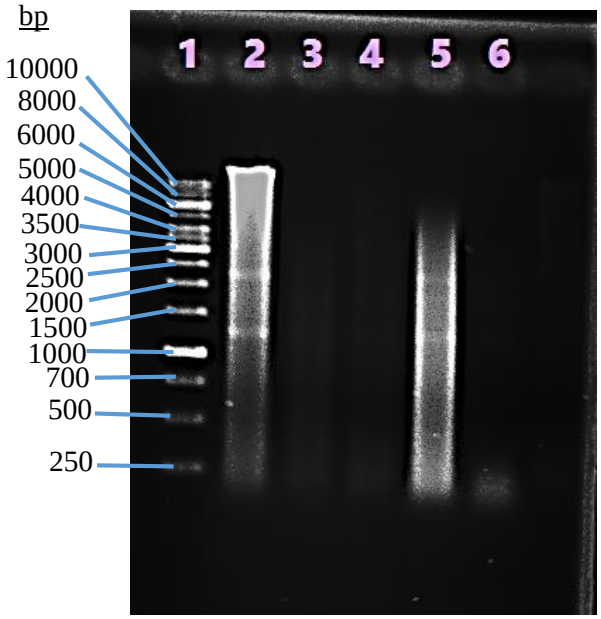

Figure 4.7: Total RNA samples (1% agarose gel). Lane 1 is 1kb DNA ladder. Lane 2 to lane 6 are the extracted RNA from five different samples. No bands were detected in Lane 3, 4 and 6 because the concentration of the total RNAs are too low.

4.4 Gene expression of *MTHFR* mRNA

4.4.1 PCR amplification

Before proceed to the real-time PCR amplification, the cDNA was standardized to 100 ng/µL. After that, the standardized of cDNA proceed to PCR amplification. Two primers had been use for the PCR amplification which were 18s rRNA primers as a housekeeping gene and *MTHFR* primers. The PCR

cDNA amplicon for 18s rRNA gene was 104 bp while for *MTHFR* gene was 98 bp (Figure 4.8).

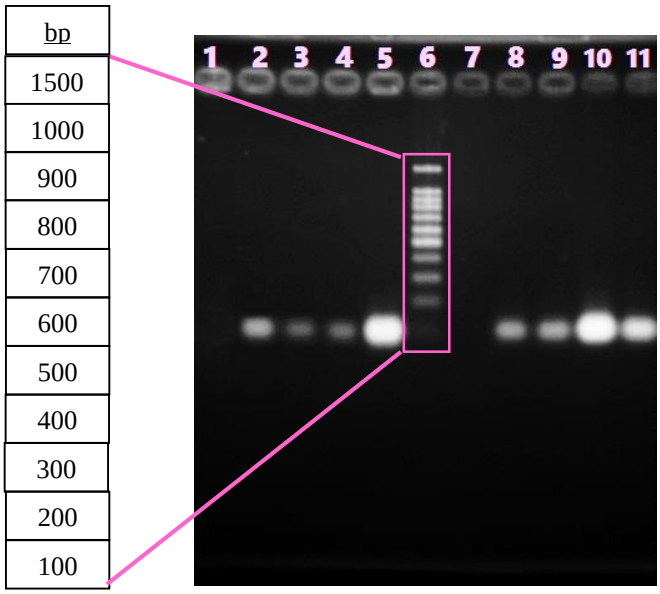

Figure 4.8: PCR products of *MTHFR* gene and 18s rRNA gene (2% agarose gel). Lane 6 is 1kb DNA ladder. Lane 1 and Lane 7 are negative controls. Lane 2 to lane 5 are PCR products with 18s rRNA primers and lane 8 to lane 11 are PCR products of *MTHFR* primers.

4.4.2 Real-time PCR amplification

The gene expression of *MTHFR* gene was measured using real-time PCR. The method that was used to analyse data form real time PCR was relative quantification method. The calculation formula for relative quantification are $2^{-\Delta\Delta Ct}$ and involved the parameter of the differences (Δ) between the threshold cycle (Ct). The steps for calculation as followed:

$$\Delta Ct = Ct \text{ (target gene)} - Ct \text{ (Housekeeping gene)}$$

$$\Delta\Delta Ct = \Delta Ct \text{ (average of sample)} - \Delta Ct \text{ (average of control)}$$

$$\text{Fold gene expression} = 2^{-(\Delta\Delta Ct)}$$

In this study, *MTHFR* gene expression level of 22 students out of 80 participants were evaluated based on their genotypes in different conditions. *MTHFR* mRNA expression with different genotype between normal (control) and prehypetension (cases) samples in same groups were compared. Besides, *MTHFR* mRNA expression in normal (control) and prehypertension (cases) with same genotype but in different groups were evaluated. Therefore, 22 of students were divided into four groups according to the questionnaire. The four groups include normal without genetic condition (A), normal with genetic condition (B), prehypertension with genetic condition (C) and prehypertension without genetic condition (D). The Ct value and melting curve if each sample with different genotype are demonstrated in Figure 4.9 and Figure 4.10.

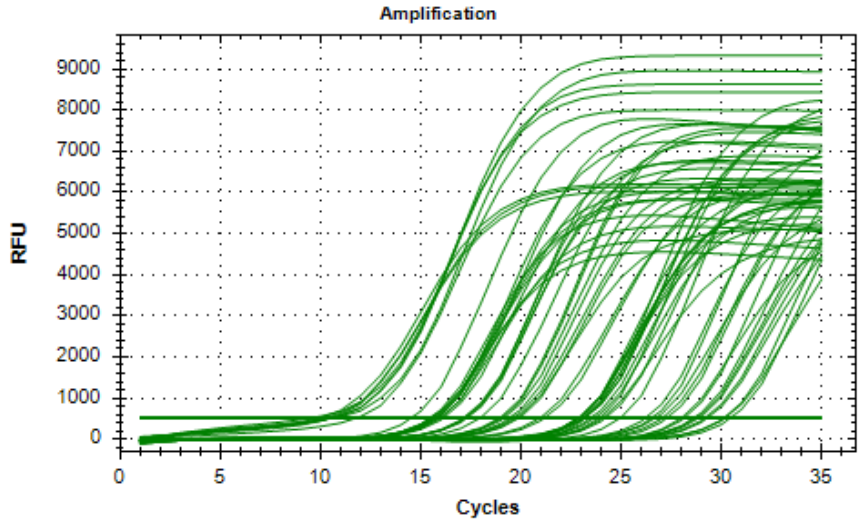

Figure 4.9: The Ct value of *MTHFR* gene of different samples and 18s rRNA gene (control)

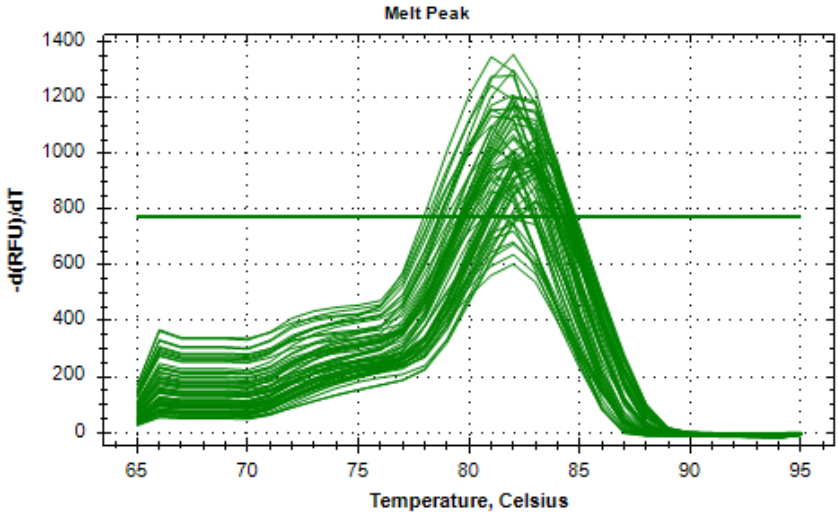

Figure 4.10: The melting curve of each sample. The melting point for each samples are 81˚C ± 1˚C.

4.5 *MTHFR* gene expression in prehypertension cases with different genotype compared to normal group in same condition

In this study, the *MTHFR* gene expression of three genotypes (homozygous wildtype genotype (CC), heterozygote genotype (CT) and homozygous mutant (CC)) were evaluated.

4.5.1 Comparison of *MTHFR* gene expression among CC, CT and TT in normal blood pressure group with genetic condition

Table 4.6 and Figure 4.11 show the gene expression of *MTHFR* genotype in a normal group with genetic condition. The homozygote wildtype CC genotype used as the control and compared to the gene expression for the heterozygote CT and homozygous mutant TT genotypes. Therefore, the fold change of gene expression for CC genotype is 1.0. Although the CT genotype expressed lower than the CC genotype, but it almost similar to the TT genotype. The CT and TT

genotype only expressed at a 0.02 fold change expression compared to control gene.

Table 4.6: *MTHFR* gene expression CC, CT and TT in normal group with genetic background

	Genotype		
	CC	**CT**	**TT**
Houskeeping gene (18s rRNA) (Ct)			
1	-	11.51	14.87
2	20.50	11.40	15.82
Average	20.50 ±0.00	11.46±0.08	15.32±0.67
Target gene (*MTHFR*) (Ct)			
1	23.80*	25.68	26.42
2	28.43	24.30	31.06
Average	28.43±0.00	25.00±0.98	28.74±3.28
Gene expression calculation			
ΔCt = Ct (*MTHFR*-18s rRNA)	7.93	13.54	13.39
ΔΔCt = ΔCt average(sample-control)	0.00	5.61	5.11
$2^{-(\Delta\Delta Ct)}$	1.00	0.02	0.02

Remark: * The data was eliminated because it was suspected as inaccurate reading.

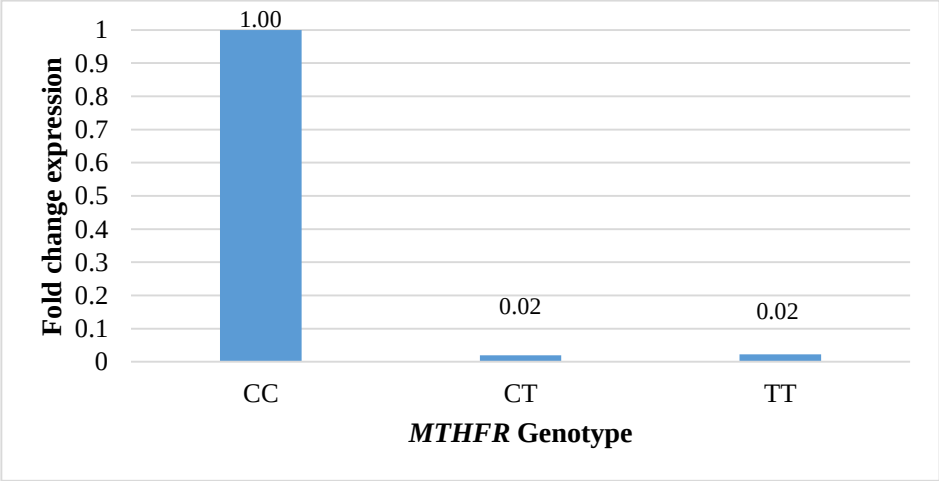

Figure 4.11: Comparison of *MTHFR* gene expressions among CC, CT and TT in normal group with genetic condition

4.5.2 Comparison of *MTHFR* gene expression among CC, CT and TT in normal blood pressure group without genetic condition

Next, Table 4.7 and Figure 4.12 show the *MTHFR* gene expressions of different genotype in normal group without genetic condition. The homozygote wildtype CC genotype was compared to the gene expression of heterozygote CT and homozygous mutant TT genotype. The fold change of gene expression for CC genotype was 1.0. For the heterozygote CT genotype, the fold change expression was 0.22x or almost 80% lower than CC genotype. However, the gene expression of TT genotype was 1.53x higher than CC genotype.

Table 4.7: *MTHFR* gene expression of CC, CT and TT in normal group without genetic condition

	Genotype		
	CC	**CT**	**TT**
Houskeeping gene (18s rRNA) (Ct)			
1	28.92*	10.30	13.60
2	16.90	10.82	23.03
Average	16.90±0.00	10.56±0.38	18.32±6.67
Target gene (*MTHFR*) (Ct)			
1	-	23.10	24.54
2	27.21	23.02	31.49
Average	27.21±0.00	23.06±0.06	28.02±4.91
Gene expression calculation			
ΔCt = Ct (*MTHFR*-18s rRNA)	10.31	12.50	9.70
ΔΔCt = ΔCt(sample-control)	0.00	2.19	-0.16
$2^{-(\Delta\Delta Ct)}$	1.00	0.22	1.53

Remark: * The data was eliminated because it was suspected as inaccurate reading.

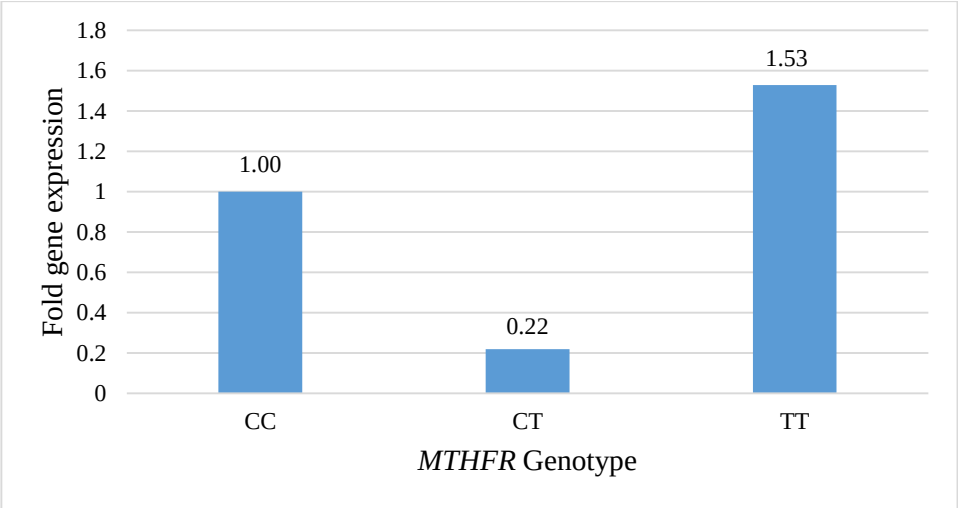

Figure 4.12: Comparison of *MTHFR* gene expressions among CC, CT and CT in normal group without genetic condition

4.5.3 Comparison of *MTHFR* gene expression among CC, CT and TT in prehypertension cases with genetic condition

Next, the *MTHFR* gene expression of different genotypes in prehypertension cases with genetic condition was showed in Table 4.8 and Figure 4.13. The *MTHFR* gene expression of homozygote wildtype CC, heterozygote CT and homozygous mutant TT genotypes was evaluated. The fold gene expression of CC genotype was the highest (1.0x) and followed by CT genotype (0.27x). TT genotype had the lowest of gene expression (0.14x).

Table 4.8: *MTHFR* gene expression of CC, CT and TT in prehypertension cases with genetic condition

	Genotype		
	CC	**CT**	**TT**
Housekeeping gene (18s rRNA) (Ct)			
1	16.24	17.86	19.89
2	18.99	10.11	15.70
Average	17.16±1.94	13.99±5.48	17.80±2.96
Target gene (*MTHFR*) (Ct)			
1	23.92	24.24	27.47
2	26.60	22.81	29.06
Average	25.26±1.89	23.53±1.01	28.27±1.12
Gene expression calculation			
ΔCt = Ct (*MTHFR*-18s rRNA)	7.65	9.54	10.47
ΔΔCt = ΔCt(sample-control)	0.00	1.89	2.82
$2^{-(\Delta\Delta Ct)}$	1.00	0.27	0.14

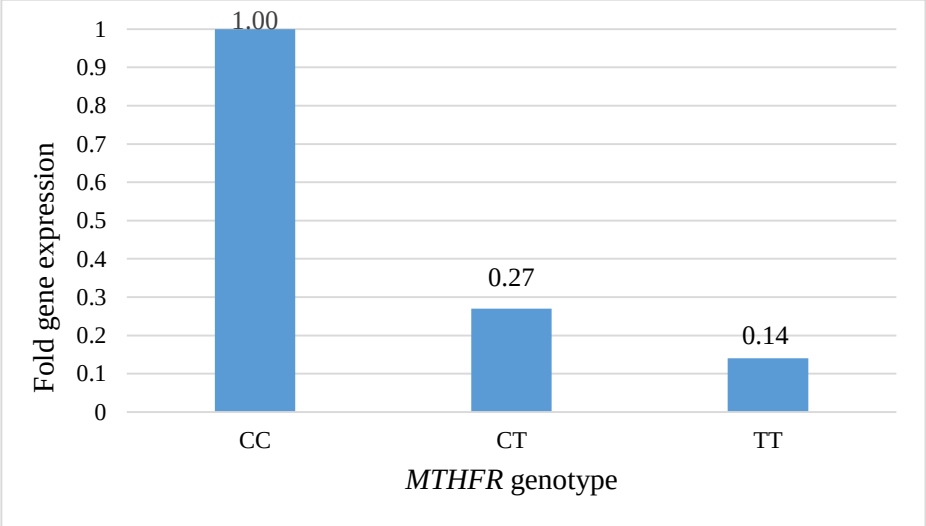

Figure 4.13: Comparison of *MTHFR* gene expression of among CC, CT and TT in prehypertension cases with genetic condition

4.5.4 Comparison of *MTHFR* gene expression among CC, CT and CT in prehypertension cases without genetic condition

The data of gene expression of *MTHFR* in prehypertension cases without genetic condition is demonstrated in Table 4.9 and Figure 4.14. The gene expression of heterozygous CT genotype and homozygous mutant TT genotype were compared to wildtype CC genotype. The fold change of *MTHFR* gene expression of CC genotype was 1.0. The CT genotype was 2.04x higher and TT genotype was 18.77x higher than CC genotype.

Table 4.9: *MTHFR* gene expression of CC, CT and TT in prehypertension cases without genetic condition

	Genotype		
	CC	**CT**	**TT**
Houskeeping gene (18s rRNA) (Ct)			
1	15.29	32.92*	17.31
2	17.18	19.63	15.50
Average	16.24±1.34	19.63±0.00	16.41±1.28
Target gene (*MTHFR*) (Ct)			
1	24.41	-	23.87
2	31.24	30.19	23.67
Average	27.83±4.83	30.19±0.00	23.77±0.14
Gene expression calculation			
ΔCt = Ct (*MTHFR*-18s rRNA)	11.59	10.56	7.36
ΔΔCt = ΔCt(sample-control)	0.00	-1.03	-4.23
$2^{-(\Delta\Delta Ct)}$	1.00	2.04	18.77

Remark: * The data was eliminated because it was suspected as inaccurate reading.

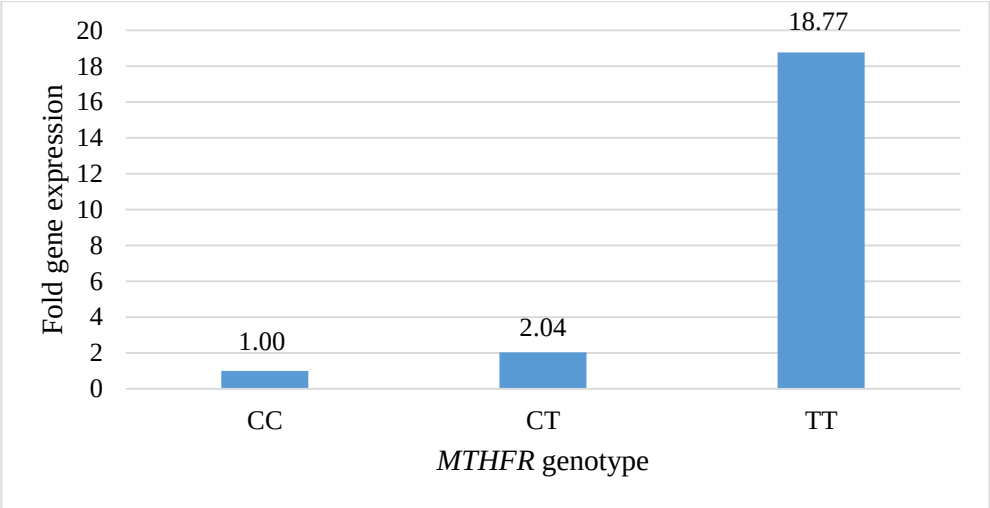

Figure 4.14: Comparison of *MTHFR* gene expression among CC, CT and TT in prehypertension cases without genetic condition

4.6 *MTHFR* gene expression of genotypes in different conditions

Three genotypes which was homozygous wildtype CC, heterozygote CT and homozygous mutant TT genotypes in different conditions were evaluated. The different conditions include normal group without genetic condition (A), normal group with genetic condition (B), prehypertension cases with genetic condition (C) and prehypertension cases without genetic condition (D).

4.6.1 *MTHFR* gene expression of CC in four different groups

Firstly, CC genotypes of the four conditions were assessed. The *MTHFR* gene expression gene was 1.0 in group A. The fold gene expression level of normal group with genetic condition (B) and prehypertension cases with genetic condition (C) was higher than group A which showed 5.21x and 6.32 fold change expression respectively. But, for the prehypertension cases without genetic condition (D), it expressed 0.41 fold only when compared to group A. The

comparison of gene expression among the four groups based on CC genotype is presented in Table 4.10 and Figure 4.15.

Table 4.10: *MTHFR* gene expression of CC genotype in different conditions

Group	**Sample**	**Genotype (CC)**				
		Housekeeping gene (18s rRNA)	***MTHFR* gene**	**ΔCt**	**ΔΔCt**	**$2^{-(\Delta\Delta Ct)}$**
A (normal without genetic condition)	1	28.92	-	-		
	2	16.90	27.21	10.31		
	Average			**10.31**	0	1.0
B (normal with genetic condition)	1	-	23.80*	-		
	2	20.50	28.43	7.93		
	Average			**7.93**	-2.38	5.21
C (Prehypertension with genetic condition)	1	16.24	23.92	7.68		
	2	18.99	26.60	7.61		
	Average			**7.65**	-2.66	6.32
D (Prehypertension without genetic condition)	1	15.29	24.41	9.12		
	2	17.18	31.24	14.06		
	Average			**11.59**	1.28	0.41

Remark: * The data was eliminated because it was suspected as inaccurate reading.

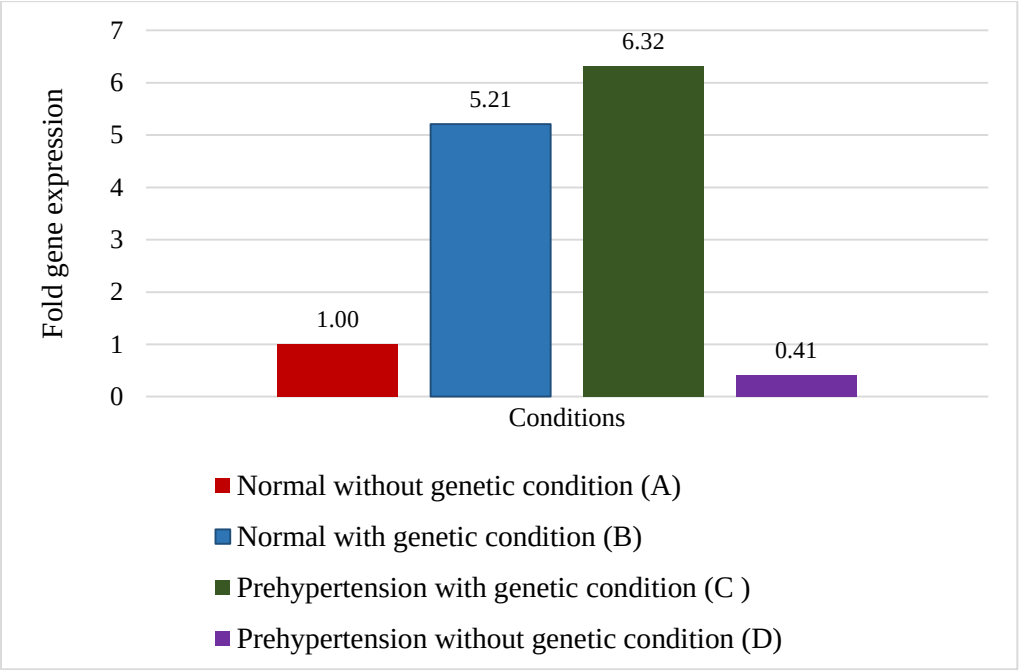

Figure 4.15: *MTHFR* gene expression of CC genotypes of different conditions

4.6.2 *MTHFR* gene expression of CT genotype in four different groups

Secondly, the gene expression of CT genotypes in different conditions was evaluated. The group of normal without genetic condition (A) was used as the control and expressed as 1.0 fold gene expression. The normal group with genetic condition (B) had the lowest gene expression which only at 0.49 fold change level or decrease almost 50% of the gene expression level when compared to group A. For the other two groups which was prehypertension cases with genetic condition (C) and prehypertension cases without genetic condition (D) expressed at 7.78x and 3.84x, respectively as compared to group A. The *MTHFR* gene expression of CT genotype in the four groups was assessed and compared in Table 4.11 and Figure 4.16.

Table 4.11: *MTHFR* gene expression of CT genotype in different conditions

Group	Sample	Genotype (CT)				
		Housekeeping gene (18s rRNA)	***MTHFR* gene**	**ΔCt**	**ΔΔCt**	**$2^{-(\Delta\Delta Ct)}$**
A (normal without genetic condition)	1	10.30	23.10	12.80		
	2	10.82	23.02	12.20		
	Average			**12.50**	0	1.0
B (normal with genetic condition)	1	11.51	25.68	14.17		
	2	11.40	24.30	12.90		
	Average			**13.54**	1.04	0.49
C (Prehypertension with genetic condition)	1	17.86	24.24	6.38		
	2	10.11	22.81	12.70		
	Average			**9.54**	-2.96	7.78
D (Prehypertension without genetic condition)	1	32.92*	-	-		
	2	19.63	30.19	10.56		
	Average			**10.56**	-1.94	3.84

Remark: * The data was eliminated because it was suspected as inaccurate reading.

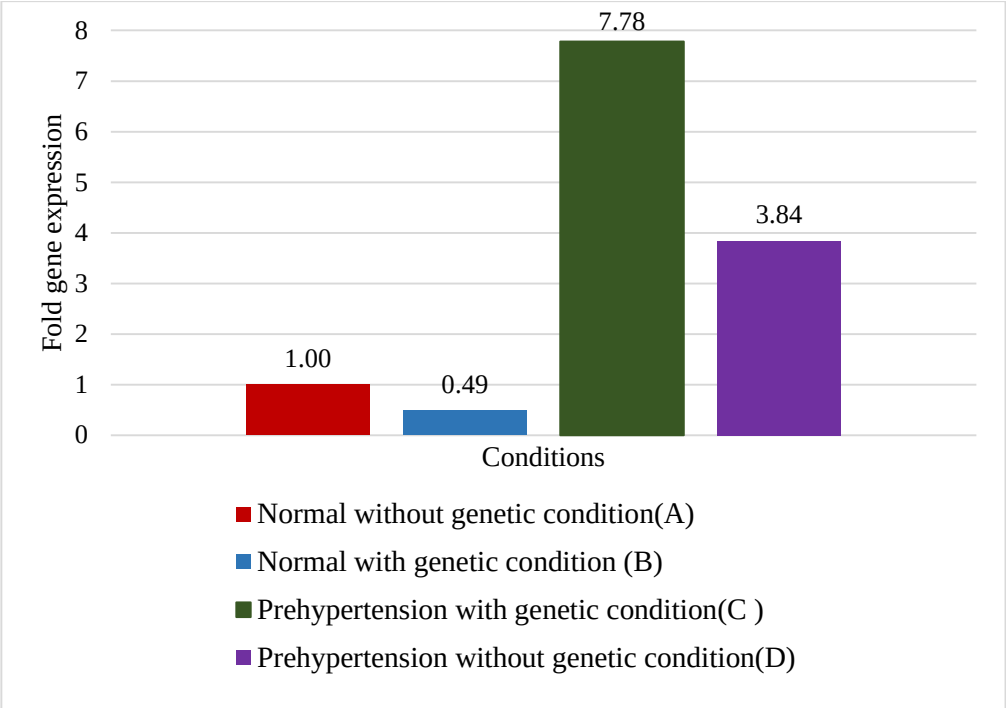

Figure 4.16: *MTHFR* gene expression of CT genotypes of different conditions

4.6.3 *MTHFR* gene expression of TT genotype in four different groups

MTHFR gene expression of TT in the four different groups was compared. The group of normal without genetic condition (A) used as control and expressed as 1.0 fold gene expression. The group of normal with genetic condition (B) and prehypertension cases with genetic condition (C) showed lower *MTHFR* gene expression which was only at 0.08 and 0.59 fold change, respectively. However, the group of prehypertension cases without genetic condition (D) expressed higher at 5.03 fold change compared to group A. The data of *MTHFR* gene expression in different conditions are summarized in Table 4.12 and Figure 4.17.

Table 4.12: *MTHFR* gene expression of TT genotype in different conditions

Group	Sample	Genotype (TT)				
		Housekeeping gene (18s rRNA)	***MTHFR* gene**	**ΔCt**	**ΔΔCt**	**$2^{-(\Delta\Delta Ct)}$**
A (normal without genetic condition)	1	13.60	24.54	10.94		
	2	23.03	31.49	8.46		
	Average			**9.70**	0	1.0
B (normal with genetic condition)	1	14.87	26.42	11.55		
	2	15.82	31.06	15.24		
	Average			**13.40**	3.70	0.08
C (Prehypertension with genetic condition)	1	19.89	27.47	7.58		
	2	15.70	29.06	13.36		
	Average			**10.47**	0.77	0.59
D (Prehypertension without genetic condition)	1	17.31	23.87	6.56		
	2	15.50	23.67	8.17		
	Average			**7.37**	-2.33	5.03

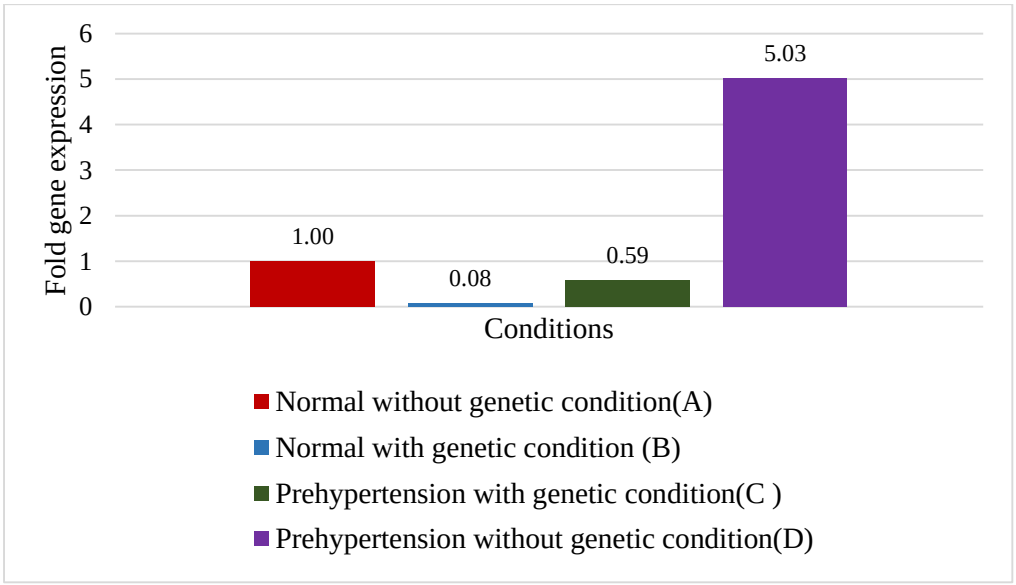

Figure 4.17: *MTHFR* gene expression of TT in different conditions

CHAPTER 5

DISCUSSION

5.1 Prevalence of prehypertension among a cohort of UTAR Kampar students and different genders

In this study, the prevalence of the normal blood pressure of the students was 75% and 25% for the prehypertension students. Prehypertension and hypertension students were classified as prehypertension group. In Malaysia, a similar study that had done in Universiti Putra Malaysia (UPM) showed 30.1% of the prevalence of prehypertension among undergraduate students (Balami et al., 2014). However, the study of Peltzer et al. (2017) reported that the prevalence of prehypertension and hypertension students was 25.7%, which was closer to this study.

In this study, the prevalence of male was higher than females. This can be supported by the study that was conducted by Roger et al. (2011) stated that the prevalence of prehypertension in men was higher than women. Generally, both prehypertension and hypertension were significantly more prevalent in males than the female. This may due to the difference of hormonal activity between genders in the early aged (Gyamfi et al., 2018).

5.2 Genomic DNA and total RNA purity

Genomic DNA was extracted from buccal cells. The reason to choose buccal cell mouthwash sampling is simple, non-invasive and cost-effective. The study showed that the high acceptability of the buccal cell method and the quality of DNA that isolated from the mouthwash samples was sufficient to reliably support the PCR amplification (Lum and Marchand, 1998). A good purity of the DNA is 1.8 while RNA is 2.0. If the value lower than 1.8~2.0, it may due to presence of phenol, protein or other contaminant that absorbs strongly at 280 nm. If greater than the range of purity of DNA, it may contain with the RNA contamination (Hercuvan Lab System, 2018).

5.3 *MTHFR* C677T genotypic and allelic frequencies of the population

Most of the genotype frequency observe in the population was higher in homozygous wildtype CC genotype, followed by heterozygote CT genotype and the homozygous mutant TT genotype had the lowest frequency. In India, the prevalence of the CT genotype was 11.5% in normal and 18.2% in prehypertension group. For the TT genotype, the genotype frequency was 1.5% in normal group and 2.8% in the prehypertension groups (Tripathi et al., 2010). A study of Morocco populations showed that the genotype frequency of CC genotype in normal and prehypertension cases groups were 52.9% and 6.5% respectively. For the CT genotype, the genotype frequency in normal and prehypertension groups were 44.2% and 39.6% respectively. The genotype frequency of TT in normal was 2.9% and prehypertension groups was 13.9% (Nassereddine et al., 2015). The genotypic frequency for each population may

vary due to the geographical and ethnicity differences. The genotype frequency of the TT genotype was higher in prehypertension group compared to normal group in this study. This findings is similar with the study of Nassereddine at al. (2015).

In general, the allele frequency in this study of C and T allele was differenced when compared to other population studies. This may due to the geographical and ethnic factors. Mutchinick et al. (1999) showed that in cases group, the allele frequency of C allele was 0.42 and T allele was 0.58 for Mexican population. In Italian population, the allele frequency of C and T allele was 0.495 and 0.505 respectively while for the TT genotype frequency was 25% (Zappocosta et al., 2008). However, another study in the Wisconsin population showed that the C and T allele frequency were 0.76 and 0.24 respectively and the frequency of TT homozygote genotype was 8% which almost similar to this study (Qi et al., 2003).

The mathematical concept that was developed by Godfrey Hardy and Wilhelm Weinberg is known as Hardy-Weinberg Equilibrium (HWE). This equation was used to calculate the genetic variation of a population at equilibrium (Natural Education, 2014). For a population to be in a state of equilibrium, it is assumed that no mutation is occurring and random mating is present. Beside, the population is large and there is no gene flow by migration in or out of the population. However, since it is highly unlikely that for these conditions to be apply in reality due to evolution occur (O'Neil, 2012). The allele frequency of the normal (control) and prehypertension (cases) group in this study did not deviate from the Hardy-Weinberg Equilibrium. The genetic variant of C677T

was associated with prehypertension. With the support of some publications, the C667T gene variant was showed significant associated with hypertension. The study that carried out by Ghogomu et al. (2015) showed that the T allele predisposes for the hypertension.

5.3.1 *MTHFR* C677T polymorphism associated with prehypertension

In this study, the *MTHFR* polymorphism showed positive association with prehypertension. To our knowledge, there is limit source about *MTHFR* polymorphism associated with prehypertension. Most of the study was related to hypertension condition. Prehypertension condition has 2x higher risk of developing hypertension and many hypotheses was proposed to explain the pathophysiology of prehypertension which involve reactive oxygen species, vascular endothelium, the inflammatory cytokines and C-reactive protein which was almost similar with hypertension pathogenesis (Albarwani et al., 2014). That is the reason why the researchers focus on the hypertension and prehypertension is not a disease. Prehypertension can be recovered by the changes of living habit and dietary behaviour. Most of the study showed that *MTHFR* polymorphism was associated with hypertension in Qassim and Morocco region (Alghasham et al., 2012; Nassereddine et al., 2015). However, a study in the Algerian population demonstrate that *MTHFR* gene polymorphism was not associated with the risk of hypertension (Midoun et al., 2016).

5.4 *MTHFR* gene mRNA expression of prehypertension

MTHFR enzyme is a main regulatory enzyme in folate and homocysteine metabolism by catalysing the conversion of 5-10, methylenetetrahydrofolate to 5-methyltetrahydrofolate. The 5-methytetrahydrofolate is the substrate that will convert the homocysteine to methionine (Leclerc et al., 2013). The researcher showed that MTHFR deficiencies will lead to hyperhomocysteinemia and the gene variant of *MTHFR* C677T was associated with the decreased enzyme activity (Weisberg et al., 1998).

MTHFR gene mutation will reduced of *MTHFR* enzyme activity. The heterozygote of *MTHFR* (C677T) showed decreased of 50% enzyme activity *in vitro* and *in vivo*. Garilli (2012) stated that approximately 70% reduction of the enzyme activity was found in individual with homozygous mutant TT genotype while 40% reduction of enzyme activity for individual with heterozygous CT genotype. Some publications showed that heterozygotes CT have 60% of the enzyme activity while homozygous mutant TT have 30% *MTHFR* enzyme activity only (Rozen, 1997; Bianca, 2012). When people have deficiency of MTHFR enzyme, the level of the folate will decreased and the level of homocysteine in the plasma will be increased which will lead to hyperhomocysteinemia (Leclerc et al., 2013). Furthermore, the elevation of homocysteine level may cause of hypertension (Tyrrell, et al., 1997; Catena, et al., 2014). Since there is lack of study on the *MTHFR* gene expression and the prehypertension, therefore, the gene expression of the *MTHFR* was studied in this study.

The housekeeping gene used in this study was 18s ribosomal RNA and target gene was *MTHFR* gene. The 18s rRNA was chosen because it will show stable expression under a variety of experiment conditions and showed minimal variability in its expression between tissues and physiological states of the organism (Kozera and Rapacz, 2013). Some studies showed that mRNA expression levels of 18s rRNA was the most stable housekeeping gene for normalization and even also stable in the virus expression (Schmid et al., 2003; Bas et al., 2004; Kuchipudi et al., 2012).

5.5 *MTHFR* gene expression in prehypertension cases group with different genotypes compared to normal group in same condition

The *MTHFR* gene expression of three genotypes were homozygous wildtype (CC), heterozygote (CT) and homozygous mutant (TT). The assessment of *MTHFR* gene expression was characterized into four groups which was normal without genetic condition (A), normal with genetic condition (B), prehypertension with genetic condition (C) and prehypertension without genetic condition (D). Normal or control group was refer to the students with normal blood pressure while prehypertension cases group include students with prehypertension and hypertension.

5.5.1 Comparison of *MTHFR* gene expression among CC, CT and TT in normal group with genetic condition

The trend of the gene expression level for each genotype in this study was not similar as like other study. Firstly, the gene expression level of homozygous wildtype CC, heterozygote CT and homozygous mutant TT genotypes in normal group with genetic condition were compared (Table 4.6 and Figure 4.11). In this study, genetic factor and environmental factor will affected the level of *MTHFR* gene expression. The subjects with CT genotype have similar gene expression level with TT genotype but lower than CC genotype. The subjects in this group had the predisposition factor. Therefore, the subjects were had the risk of getting prehypertension in future. *MTHFR* enzyme activity was associated with the environmental factor such as dietary behaviour. According to the observation, lower of enzyme expression level in CT and TT genotype may due to subjects consumed less of folate and Docosahexaenoic acid (DHA) foods such as tuna, spaghetti, nuts and milks compared to the CC genotype subjects. This findings was supported by Nagele et al. (2011) who concluded that the population with the folate fortification showed a significant effect of genetic *MTHFR* variants. A study also showed that the DHA group is significant increase the mRNA expression of the *MTHFR* gene (Huang et al., 2012).

5.5.2 Comparison of *MTHFR* gene expression among CC, CT and TT in normal group without genetic condition

The *MTHFR* gene expression levels of homozygous wildtype CC genotype, heterozygote CT genotype and homozygous mutant TT genotype in normal

group but without genetic condition were compared (Table 4.7 and Figure 4.12). The gene expression in each genotype with or without genetic condition varied. Furthermore, different dietary behaviours may cause the gene expression varied. When compared to the CC genotype, the gene expression level of CT genotype was low. This is because it contains one copy of T allele which may lead to the lower of the *MTHFR* enzyme activity (Rozen, 1997; Bianca, 2012). However, the gene expression level of TT genotype was higher than CC genotype in this study. The subjects was found actively drinking milk that rich in docosahexaenoic acid (DHA) and taking foods which high DHA content such as ham, spaghetti, tuna and mayonnaise. This is suspected that DHA could induce MTHFR enzyme activity. The similar observation was stated by Huang et al. (2012).

5.5.3 Comparison of *MTHFR* gene expression among CC, CT and TT in prehypertension cases with genetic condition

Table 4.8 and Figure 4.13 showed that the *MTHFR* gene expression level of homozygous wildtype CC, heterozygote CT and homozygous mutant TT genotypes of prehypertension individuals with genetic condition. In this study, the trend of the gene expression level was similar with Garilli (2012). Garilli showed that individual with CT genotype expressed higher than individual with TT genotype. Individuals with predisposition factor will have risk to get prehypertension. The hypothesis was proven in this study. Several studies have demonstrated that family history was significant associated with the pressure level (Hunt et al., 1986; Liu et al., 2015). Besides of predisposition factor, the

MTHFR enzyme activity will be affected by environmental factors such as dietary behaviour. According to the observation, individual with predisposition factor that taking high sodium had higher risk of getting high blood pressure compared to individual with genetic condition only. In this study, many students was taking high sodium foods such as deli ham, chicken, bacon, burgers, french fries and salts nuts daily. These high salt food diet may be the cause of having high blood pressure. In several studies, high sodium intake was associated with blood pressure increased (Frisoli et al., 2012; Zhao et al., 2014; Chrysant, 2016). Therefore, healthy dietary diet with low sodium should be practised among the young generations.

5.5.4 Comparison of *MTHFR* gene expression among CC, CT and TT in prehypertension cases without genetic condition

Finally, the *MTHFR* gene expressions of homozygous wildtype CC, homozygote CT and homozygous mutant TT genotypes in prehypertension group without genetic condition were compared and evaluated (Table 4.9 and Figure 4.14). These individuals that were diagnosed with prehypertension were due to environmental factors such as dietary behaviour. Predisposition factor was absent in this group. The gene expression of CT genotype was higher than the CC genotype. As discussed in section 5.5.2, heterozygote CT genotype individual that taking high DHA content products such as milk and spaghetti have higher *MTHFR* gene expression level as compared to CC genotype individuals that taking low DHA content products. Huang et al. (2012) showed that DHA induced the expression level of *MTHFR* gene. In this study,

overexpression of MTHFR was detected in TT genotype's individuals. According to this survey, TT genotype individuals taking high DHA content products which can induced the *MTHFR* enzyme expression level. Besides, CT and TT individuals with lower stress leads them had the high expression level of *MTHFR* enzyme compared to CC genotype's individuals. Jimenez et al. (2018) showed that stress was associated with *MTHFR* expression levels. However, TT individuals has higher risk of getting prehypertension as compared to CC and CT genotypes. It is because TT genotype has two copies of T allele which is a risk factor of getting prehypertension. Nassereddine et al. (2015) showed that individuals with TT genotype was strongly associated with the high blood pressure.

5.6 *MTHFR* gene expression of genotypes in different conditions

Subjects with same genotype will show different gene expression levels. This is because prehypertension is a multifactorial disease that consist of influence of genetic and environmental factors. *MTHFR* gene expression of four different condition was studied. They were Group A (normal group without genetic condition), Group B (normal group with genetic condition), Group C (prehypertension cases with genetic condition) and Group D (prehypertension cases without genetic condition).

5.6.1 *MTHFR* gene expression of CC in four different groups

Data in Table 4.10 and Figure 4.15 showed that subjects were expressed differently even though they were in same genotype (CC). The normal blood pressure subjects with genetic condition (B) expressed higher *MTHFR* enzyme level than the individual without genetic condition (A). According to the observation, the subjects in group B were found consume more DHA foods such as milks, ham, spaghetti and nuts frequently per month compared to group A. So, these maybe an addition cause of having higher gene expression. The study also have proved that DHA foods will induce the *MTHFR* gene expression which is similar with the observation done by Huang et al. (2102).

Group C (prehypertension with genetic condition) showed higher *MTHFR* expression when compared to group A (normal without genetic condition). Group C showed overexpression because of the interaction of genetic factor and environmental factor. The individuals with predisposition factor will have the risk to get prehypertension. Liu et al. (2015) showed that family history was significant associated with blood pressure level. As refer to the observation in this study, environmental factors like overweight, consumption of alcohol occasionally, stress and unhealthy dietary behaviour may lead to prehypertension. This findings was supported by the conclusion that was made by Nanchahal et al. (2000), Sachin et al. (2014) and Elzbieta and Krzysztof (2018). High DHA consumption from milk and high folic acid from cereals and vegetables may help to induce the *MTHFR* gene expression as was discussed in section 5.5.2.

Group D (prehypertension without genetic condition) gave lower *MTHFR* gene expression as compared Group A (normal without genetic condition). This comparison proved that environmental variants are significant important to MTHFR enzyme activity. According to the observation, inactive physical activity and high salt intake will reduce the gene expression level. Therefore, dietary and daily activities are playing important role to control the blood pressure.

5.6.2 *MTHFR* gene expression of CT in four different groups

MTHFR gene expression of CT genotypes in different conditions was evaluated. The comparison of *MTHFR* expression was demonstrated in Table 4.11 and Figure 4.16. In general, individual with CT genotype will decrease 40% of enzyme activity as compared to CC genotype. The subjects with CT genotype in group B (normal with genetic condition) have lower *MTHFR* gene expression than group A (normal without genetic condition). The subjects in both groups were have normal blood pressure but Group B carried a predisposition factor. Therefore, it may lead to express lower *MTHFR* gene expression as compared to individual without predisposition factor.

Furthermore, the gene expression of individual with CT genotype in Group C (prehypertension with genetic condition) was higher than group A. The overexpression of the enzyme level in group C may due to the association of genetic and environmental factors. According to the observation, the subjects in group C was found consume high DHA content product such as milk as

compared to the subjects in group A. This may cause overexpression of *MTHFR* gene as was discussed in section 5.5.2. The subjects in group C carried a predisposition factor that have higher risk to get prehypertension compared to the subjects in group A that without predisposition factor. According to the observation, the CT subjects in group C was found that consume high frequency of fast food, inactive activity and overweight had higher risk to get prehypertension. This findings can be supported by Mertens and Gaal (2000) and Ranee (2017).

Group D (prehypertension without genetic condition) showed higher expression level when compared to group A (normal without genetic condition). This comparison showed that the environmental factor are significant to the blood pressure level and *MTHFR* enzyme activity. According to the observation, the subjects in group D was found to have high intake of DHA food and folic acid foods that may induce the enzyme expression level. Nagele et al. (2011) showed high intake of folate foods was significant effect of the *MTHFR* enzyme activity. Besides, the subjects in group D was found headache frequently, stress and lack of sleep. This environmental factor may lead them to have higher risk for getting prehypertension. Liu et al. (2016) showed that stress was highly associated with blood pressure.

5.6.3 *MTHFR* gene expression of TT in four different groups

MTHFR gene expression of the TT genotypes in different conditions was evaluated. The comparison of *MTHFR* expression was demonstrated in Table

4.12 and Figure 4.17. In general, the enzyme activity of individual with TT genotype will decrease to 70% as compared to CC genotype (Bianca, 2012). The *MTHFR* gene expression of TT genotype subjects in group B (normal with genetic condition) was expressed lower than TT subjects in group A(normal without genetic factor). The lower expression of *MTHFR* enzyme due to the individuals in group B carried a predisposition factor. The *MTHFR* enzyme activity can be affected by the environmental factor such as dietary behaviour and exercising. According to the observation, the TT subjects in group B consumed high DHA foods and high folic acid foods that may induce the *MTHFR* gene expression. Huang et al. (2012) and Nagele, et al. (2011) showed that high intake of DHA and folic acid foods was associated with the *MTHFR* enzyme activity.

Group C (prehypertension with genetic condition) showed lower expression as compared to group A (normal without genetic condition). The lower expression of *MTHFR* enzyme activity and high blood pressure of TT subjects in group C will be affected by predisposition factor and environmental factor. Individuals with the predisposition factor will have higher risk to get prehypertension. According to the observation, the subjects in group C taking high sodium foods, inactive physical activity and stress that may lead them to have high blood pressure as was discussed in section 5.6.2.

In this study, the TT subjects in group D (prehypertension without genetic condition) showed higher of *MTHFR* gene expression as compared to TT subjects in group A (normal without genetic condition). The overexpression of

the enzyme activity was significant affected by the environmental factor such as dietary behaviour. According to the observation, individuals that drink high DHA content product such as milk that may induced the *MTHFR* enzyme activity level. However, from the observation, the individuals in group D was found that they was overweight, feeling stress and also consumed high frequency of fast food overweight that may cause prehypertension. Tayem et al. (2012) showed that the unhealthy diet and overweight was associated with the blood pressure level.

5.7 Limitation of study

In this study, the sample size was small. This may due to the time constrained for recruiting volunteers. Besides, the quality of RNA samples was not very good in this study. This is because the purity of RNA samples were less than the value of pure purity which was 2.0. The contamination of DNA and degradation of the RNA samples may affected the accuracy of the expression enzyme level in real-time PCR.

5.8 Future studies

In future study, large sample size in a population can be conducted to increase the statistical power. Furthermore, the extraction of the RNA from the buccal cell sample is a challenging procedure. The procedure can be improve by using the Trizol method to obtain more yield and increase the purity of the RNA. Ridgeway and Timm (2014) reported that Trizol method was commonly used

for the gene expression analysis and produce high quality of RNA. Besides, the other SNPs which was *MTHFR* A1298C can be chosen and test for the association with the prehypertension. This is because the study showed that *MTHFR* 1298 C alleles and the co-occurrence of *MTHFR* C1298C/MTHFR C677T genotypes were associated with the high blood pressure (Alghasham, et al., 2012).

CHAPTER 6

CONCLUSION

In this study, *MTHFR* C677T gene variant is associated with prehypertension in a cohort of study among university student in Universiti Tunku Abdul Rahman (UTAR). There were 80 participants in this study. A total of 25% of the students was prehypertension. The prevalence of prehypertension males was higher than female students (70% vs 30%) respectively.

In the genetic polymorphism study, there are three genotypes of *MTHFR* C677T gene variant which was homozygous wildtype (CC) genotype, heterozygoute (CT) genotype and homozygous mutant (TT) genotype. The genotyping of participants used identified by using PCR-RFLP and result showed CC was 198bp, CT was 198bp and 175bp and TT was 175bp. The prevalence of the wildtype genotype, heterozygote genotype and homozygous mutant genotype was 57.5%, 32.5% and 10.0% respectively. According to Hardy-Weinberg Equilibrium, *MTHFR* C677T gene variant is associated with the prehypertension and T allele is the important allele that may cause a risk of getting prehypertension.

In the comparison study of *MTHFR* gene expression, four groups were included normal without genetic condition (A), normal with genetic condition(B), prehypertension cases with genetic condition (C) and prehypertension cases without genetic condition (D). Comparison between different genotype in same

groups was assessed. The *MTHFR* gene expression of TT genotype was higher than CT genotype in group A, B and D but lowest in group C. Besides, *MTHFR* gene expression of each genotype in different group was also evaluated. It found that the expression of enzyme level varied in different groups. Most of the prehypertension cases groups with CC, CT and TT genotypes were expressed higher than the normal groups with CC, CT and TT genotypes. In this study, the *MTHFR* enzyme activity was significant affected by the genetic factor and environmental factor such as dietary bahavior.

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APPENDICES

Appendix A

Ethnical Approval from SERC

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

Re: U/SERC/96/2018

13 August 2018

Dr Phoon Lee Quen
Department of Biomedical Science
Faculty of Science
Universiti Tunku Abdul Rahman
Jalan Universiti, Bandar Baru Barat,
31900 Kampar
Perak

Dear Dr Phoon,

Ethical Approval For Research Project/Protocol

We refer to your application which was circulated for the consideration of the UTAR Scientific and Ethical Review Committee (SERC). We are pleased to inform that your application for ethical approval for your research project involving human subjects has been approved by SERC.

The details of the project are as follows:

Research Title	Investigation of Methylenetetrahydrofolate Reductase (MTHFR) Gene Expression Level and MTHFR Polymorphism C677T on the Prehypertension Samples
Investigator(s)	Dr Phoon Lee Quen Catherine Siew Lee Yeng (UTAR Postgraduate Student)
Research Area	Science
Research Location	UTAR, Kampar Campus
No of Participants	100 participants (Age: 19 - 29)
Research Costs	Self-funded
Approval Validity	13 August 2018 - 12 August 2019

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.

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 you collect personal data of participants in your study, please have the participants in the research signed the attached Personal Data Protection Statement for your records.

The University wishes you all the best in your research.

Thank you.

Yours sincerely,

Professor Ir Dr Lee Sze Wei
Chairman
UTAR Scientific and Ethical Review Committee

c.c Dean, Faculty of Science
Director, Institute of Postgraduate Studies and 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

Appendix B

Personal Data Protection Statement

PERSONAL DATA PROTECTION STATEMENT

Please be informed that in accordance with Personal Data Protection Act 2010 ("PDPA") which came into force on 15 November 2013, Universiti Tunku Abdul Rahman ("UTAR") is hereby bound to make notice and require consent in relation to collection, recording, storage, usage and retention of personal information.

Notice:

1. The purposes for which your personal data may be used are inclusive but not limited to:-
   - For assessment of any application to UTAR
   - For processing any benefits and services
   - For communication purposes
   - For advertorial and news
   - For general administration and record purposes
   - For enhancing the value of education
   - For educational and related purposes consequential to UTAR
   - For the purpose of our corporate governance
   - For consideration as a guarantor for UTAR staff/ student applying for his/her scholarship/ study loan

2. Your personal data may be transferred and/or disclosed to third party and/or UTAR collaborative partners including but not limited to the respective and appointed outsourcing agents for purpose of fulfilling our obligations to you in respect of the purposes and all such other purposes that are related to the purposes and also in providing integrated services, maintaining and storing records. Your data may be shared when required by laws and when disclosure is necessary to comply with applicable laws.

3. Any personal information retained by UTAR shall be destroyed and/or deleted in accordance with our retention policy applicable for us in the event such information is no longer required.

4. UTAR is committed in ensuring the confidentiality, protection, security and accuracy of your personal information made available to us and it has been our ongoing strict policy to ensure that your personal information is accurate, complete, not misleading and updated. UTAR would also ensure that your personal data shall not be used for political and commercial purposes.

Consent:

1. By submitting this form you hereby authorise and consent to us processing (including disclosing) your personal data and any updates of your information, for the purposes and/or for any other purposes related to the purpose.

2. If you do not consent or subsequently withdraw your consent to the processing and disclosure of your personal data, UTAR will not be able to fulfill our obligations or to contact you or to assist you in respect of the purposes and/or for any other purposes related to the purpose.

3. You may access and update your personal data by writing to us at _____.

Acknowledgment of Notice

[ ] I have been notified by you and that I hereby understood, consented and agreed per UTAR above notice.

[ ] I disagree, my personal data will not be processed.

.....
Name:
Date:

Appendix C

Consent form and questionnaire of study

UNIVERSITI TUNKU ABDUL RAHMAN
DEPARTMENT OF BIOMEDICAL SCIENCE
(FINAL YEAR PROJECT, January 2018 Trimester)

Name : **ID no :**

CONSENT FORM

Project Title:

Prevalence of prehypertension in UTAR biomedical science students and it's risk factors.

Aim / Purpose of the Research:

You are participating in these research studies to identify the factors associated with prehypertension in a cohort of students in UTAR. The objectives are:

1. To investigate the prevalence of prehypertension among undergraduate students
2. To study the link between psychological factors and prehypertension among undergraduate students
3. To investigate the relationship between BMI, dietary behavior associated with gender disparities among prehypertension participants.

Procedure, Risk and Discomfort:

Blood pressure reading will be taken down by using an automated blood pressure monitor. The blood pressure was measured on the left arm after the participant has rested for 5 minutes in a seated position. Blood pressure measurement poses minimal risk and discomfort to participant as it is a non-invasive method involved in the measurement.

Confidentiality:

Serial number will be assigned as to protect your personal information and the result obtained. The info is used for research purpose only and would not be revealed to any other third parties.

Consent and Signature:

I have read and understood the condition stated above and I agree to voluntarily participate in these research studies.

_____

Signature:

Date :

Section A: Demographic

Please fill in the following informations.

Name:

Contact number:

Email:

Age:

Gender: ☐ male ☐ female

Race: ☐ Malay ☐ Chinese ☐ Indian ☐ others

Current height (cm):

Has your weight changed in the last year? If yes, please state your previous weight.

Measurement readings (filled up by the researcher)

Current weight (kg):

BMI (kg/m^2):

	1st reading		2nd reading		3rd reading		mean
	Systolic	Diastolic	Systolic	Diastolic	Systolic	Diastolic	
Blood pressure (mmHg)							

Health history

Please tick at the appropriate column for each item.

No	Item	Yes	No	Not sure
1	Do you suffer from prehypertension?			
2	Have you been prescribed any medications to lower your blood pressure?			
3	Have you experience any dizziness, fatigue or headache **frequently** in last 3 months?			
4	Have you had any form of vascular disorders? (heart attack, stroke, etc)			
5	Have you had any form of neurological disease? (seizures, epilepsy, etc)			
6	Do you have any form of psychiatric disorder? (depression, anxiety, etc)			
7	Do you have any other health problem? If yes, please state:			
8	Do you have any of your family members (parents & siblings) suffering from hypertension? If yes, please state the relationship:			
9	Do you have any of your relatives suffering from hypertension?			
10	Have you consumed alcohols in the last 3 months? Please circle accordingly: occasionally/frequently			
11	Have you smoked cigarettes in the last 3 months? Please circle accordingly: occasionally/frequently			

Section B: Dietary behaviour

Please list any foods or drinks you had consumed in the past 48 hours.

DAY 1

Breakfast (1st meal): _____

Snack: _____

Lunch (2nd meal): _____

Snack: _____

Dinner (3rd meal): _____

Snack: _____

Supper: _____

DAY 2

Breakfast (1st meal): _____

Snack: _____

Lunch (2nd meal): _____

Snack: _____

Dinner (3rd meal): _____

Snack: _____

Supper: _____

Please tick at the appropriate columns with stating the right amount of servings.

Foods	No	Yes (state the number of servings accordingly)		
		Per day	Per week	Per month
Any kind of milks				
Cereals				
Deli ham (chicken/beef)				
Sausage				
Bacon				
Burgers				
French fries				
Coffee				
Salad dressing				
Mayonnaise				
Frozen pizza				
Pickles				
Canned fruits (cocktails, pineapple, peach, etc)				
Canned vegetables (mushrooms, green peas, etc)				
Canned soups				
Canned tuna				
Canned spaghetti sauce				
Cheese				
Fast foods (Mc Donalds, KFC, pizza,Wingzone, etc)				
Instant noodles				
Instant porridges				
Butter/ margerine				
Salted nuts, pumpkin/ sunflower seeds				
Dried fruits				
Red meat				

5. How much time did you usually spend on one of those days doing **moderate** physical activities as part of your work?

_____ **hours per day**

_____ **minutes per day**

6. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **as part of your work**? Please do not count any walking you did to travel to or from university/work.

☐ Yes ____ **days per week**

☐ No job-related walking

*Skip to **PART II: TRANSPORTATION***

7. How much time did you usually spend on one of those days **walking** as part of your work?

_____ **hours per day**

_____ **minutes per day**

PART II: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like university, work, stores, movies, and so on.

8. During the **last 7 days**, on how many days did you **travel in a motor vehicle** like a train, bus, car, or tram?

☐ Yes ____ **days per week**

☐ No traveling in a motor vehicle

Skip to question 10

9. How much time did you usually spend on one of those days **traveling** in a train, bus, car, tram, or other kind of motor vehicle?

_____ **hours per day**

_____ **minutes per day**

Now think only about the **bicycling** and **walking** you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the **last 7 days**, on how many days did you **bicycle** for at least 10 minutes at a time to go **from place to place**?

☐ Yes ___ **days per week**

☐ No bicycling from place to place

Skip to question 12

11. How much time did you usually spend on one of those days to **bicycle** from place to place?

_____ **hours per day**

_____ **minutes per day**

12. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time to go **from place to place**?

☐ Yes ___ **days per week**

☐ No walking from place to place

Skip to PART III: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

13. How much time did you usually spend on one of those days **walking** from place to place?

_____ **hours per day**

_____ **minutes per day**

PART III: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the **last 7 days** in and around your home/hostel like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, chopping wood, or digging in the garden or yard?

☐ Yes ___ **days per week**

☐ No vigorous activity

Skip to question 16

15. How much time did you usually spend on one of those days doing **vigorous** physical activities in the garden or yard?

____ hours per day

____ minutes per day

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, sweeping, washing clothes and vehicles, and raking in the garden or yard?

☐ Yes **___ days per week**

☐ No moderate activity

Skip to question 18

17. How much time did you usually spend on one of those days doing **moderate** physical activities in the garden or yard?

____ hours per day

____ minutes per day

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, washing clothes, scrubbing floors and sweeping **inside your home**?

☐ Yes **___ days per week**

☐ No moderate activity inside home

Skip to PART IV: RECREATION,

SPORT AND LEISURE-TIME PHYSICAL ACTIVITY

19. How much time did you usually spend on one of those days doing **moderate** physical activities inside your home?

____ hours per day

____ minutes per day

PART IV: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the **last 7 days** solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time in your leisure time?

☐ Yes ____ **days per week**

☐ No walking in leisure time

Skip to question 22

21. How much time did you usually spend on one of those days **walking** in your leisure time?

_____ **hours per day**

_____ **minutes per day**

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like aerobics, running, fast bicycling, or fast swimming in your leisure time?

☐ Yes ____ **days per week**

☐ No vigorous activity in leisure time

Skip to question 24

23. How much time did you usually spend on one of those days doing **vigorous** physical activities in your leisure time?

_____ **hours per day**

_____ **minutes per day**

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis in your leisure time?

☐ Yes ____ **days per week**

☐ No moderate activity in leisure time

Skip to PART V: TIME SPENT SITTING

25. How much time did you usually spend on one of those days doing **moderate** physical activities in your leisure time?

____ **hours per day**

____ **minutes per day**

PART V: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the **last 7 days**, how much time did you usually spend sitting on a **weekday**?

____ **hours per day**

____ **minutes per day**

27. During the **last 7 days**, how much time did you usually spend sitting on a **weekend day**?

____ **hours per day**

____ **minutes per day**

Section D: Depression, Anxiety and Stress Scales

Please read each statement and circle a number 0, 1, 2 or 3 which indicates how much the statement applied to you *over the past week*. There are no right or wrong answers. Do not spend too much time on any statement.		
The rating scale is as follows: 0 Did not apply to me at all 1 Applied to me to some degree, or some of the time 2 Applied to me to a considerable degree, or a good part of time 3 Applied to me very much, or most of the time		
1	I found it hard to wind down	0 1 2 3

2	I was aware of dryness of my mouth	0 1 2 3
3	I couldn't seem to experience any positive feeling at all	0 1 2 3
4	I experienced breathing difficulty (eg, excessively rapid breathing, breathlessness in the absence of physical exertion)	0 1 2 3
5	I found it difficult to work up the initiative to do things	0 1 2 3
6	I tended to over-react to situations	0 1 2 3
7	I experienced trembling (eg, in the hands)	0 1 2 3
8	I felt that I was using a lot of nervous energy	0 1 2 3
9	I was worried about situations in which I might panic and make a fool of myself	0 1 2 3
10	I felt that I had nothing to look forward to	0 1 2 3
11	I found myself getting agitated	0 1 2 3
12	I found it difficult to relax	0 1 2 3
13	I felt down-hearted and blue	0 1 2 3
14	I was intolerant of anything that kept me from getting on with what I was doing	0 1 2 3
15	I felt I was close to panic	0 1 2 3
16	I was unable to become enthusiastic about anything	0 1 2 3
17	I felt I wasn't worth much as a person	0 1 2 3
18	I felt that I was rather touchy	0 1 2 3
19	I was aware of the action of my heart in the absence of physical exertion (eg, sense of heart rate increase, heart missing a beat)	0 1 2 3
20	I felt scared without any good reason	0 1 2 3
21	I felt that life was meaningless	0 1 2 3

This is the end of the questionnaire, thank you for participating.

Appendix D

Calculation of allele frequency:

Hardy-Weinberg Equiibrium: $p^2 + 2pq + q^2$

'p' as the C allele while 'q' as the T allele

For control group

$$P^2 = \frac{number\ of\ homozygous\ wildtype\ genotype}{total\ number\ of\ genotypes}$$

$= \frac{35}{60}$

$= 0.58$

$p = \sqrt{0.58}$

$= 0.76$

$q = 1 - p$

$= 1 - 0.76$

$= 0.24$

For cases group

$$P^2 = \frac{number\ of\ homozygous\ wildtype\ genotype}{total\ number\ of\ genotypes}$$

$= \frac{11}{20}$

$= 0.55$

$p = \sqrt{0.55}$

$= 0.74$

$q = 1 - p$

$= 1 - 0.74$

$= 0.26$

Appendix E

Concentration and Purity of DNA samples

Sample	Concentration DNA (ng/µL)	A260/A280
1	1050	1.810
2	888	1.636
3	26.2	1.45
4	60	1.846
5	60	1.412
6	285	1.810
7	67.5	1.588
8	490	1.798
9	238	1.696
10	118	1.516
11	60	1.714
12	198	1.837
13	378	1.864
14	265	1.582
15	97.5	1.857
16	102	1.783
17	29.3	1.450
18	52.5	1.000
19	555	1.866
20	298	1.653
21	625	1.623
22	47.5	1.267
23	52.5	1.750
24	595	1.803
25	212	1.735
26	495	1.768
27	230	1.878
28	168	1.825
29	690	1.781
30	75	1.875
31	178	1.972
32	320	1.641
33	70	1.750
34	5	1.725
35	22.5	1.286
36	440	1.709
37	90	1.565
38	235	1.541
39	30	1.512

Samples	Concentration DNA (ng/µL)	A260/A280
40	3705	0.938
41	27.5	2.200
42	363	1.629
43	692	1.689
44	690	1.816
45	1308	1.720
46	745	1.840
47	110	1.913
48	15	3.000
49	298	1.803
50	140	1.436
51	67.5	1.350
52	430	1.811
53	265	1.828
54	505	1.757
55	32.5	1.444
56	810	1.770
57	32.5	1.725
58	27.5	1.802
59	448	1.845
60	92.5	2.056
61	155	1.512
62	52.5	1.725
63	495	1.868
64	57.5	1.865
65	87.5	1.823
66	140	1.769
67	663	1.828
68	90	2.400
69	228	1.596
70	305	1.821
71	70	1.750
72	1403	1.469
73	42.5	1.853
74	50	1.538
75	70	1.556
76	458	1.664
77	495	1.833
78	123	1.581
79	75	1.579
80	365	1.802
Average	**327**	**1.720**

Appendix F

Concentration and Purity of RNA samples

Samples	Concentration RNA (ng/µL)	A260/A280
1	48	2.000
2	22	1.833
3	14	1.440
4	24	1.267
5	12	1.500
6	38	1.333
7	30	1.667
8	34	1.758
9	58	1.958
10	64	1.654
11	64	1.345
12	14	1.495
13	10	1.789
14	20	2.000
15	78	1.950
16	12	2.000
17	28	2.000
18	20	1.667
19	10	1.250
20	12.0	3.000
21	28	1.789
22	22	1.833
Average	**30**	**1.750**