THE ACUTE EFFECT OF BLACK COFFEE ON METHYLENETETRAHYDROFOLATE REDUCTASE (*MTHFR*) GENE EXPRESSION, BLOOD PRESSURE AND HEART RATE IN HABITUAL AND NON-HABITUAL COFFEE DRINKERS

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By

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ABSTRACT

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CHAN MENG HUI

In recent years, coffee has grown to be a well-liked beverage in Malaysia and has become a trend among the young adults. Studies have highlighted on the effect of coffee on genetic activity through modulation of epigenetic mechanisms, increasing the risk of individuals toward certain health conditions such as hypertension. *MTHFR* is a gene that has been closely studied due to its association with increased risk of cardiovascular diseases. However, there was a lack of studies on the association of *MTHFR* gene expression, blood pressure and pulse rate after coffee consumption. Thus, this current study aims to determine the prevalence of habitual coffee drinkers among Universiti Tunku Abdul Rahman (UTAR) students and evaluate the pre-post changes of *MTHFR* expression, blood pressure and pulse rate, shedding light on the impact of coffee towards these physiological responses. A total of 426 students were recruited for the cross-sectional study, where among them, 20 students were recruited for the coffee testing experiment. The prevalence of habitual coffee drinkers (19%) was lesser than non-habitual coffee drinkers (81%). In this study, there was

significant change in systolic and diastolic blood pressure post-coffee consumption in habitual ($p = 0.011$, $p = 0.001$) and non-habitual coffee drinkers ($p = 0.019$, $p = 0.007$) but the acute effect appeared greater in habitual drinkers, while there was no significant change in pulse rate and *MTHFR* expression ($p > 0.05$) post-coffee consumption in both groups. Furthermore, there was no significant difference in *MTHFR* gene expression ($p > 0.05$) between habitual and non-habitual coffee drinkers. From the findings, the *MTHFR* expression, blood pressure and pulse rate were impacted by coffee consumption.

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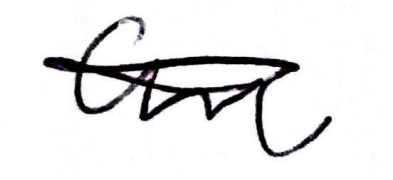
Last but not least, I would like to express my gratitude to my family, who gave me unconditional love and support as well as providing me with daily pictures of my dogs that have become my emotional support.

DECLARATION

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



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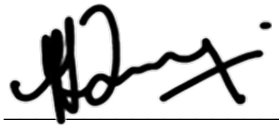
CHAN MENG HUI

APPROVAL SHEET

This final year project report entitled "**THE ACUTE EFFECT OF BLACK COFFEE ON METHYLENETETRAHYDROFOLATE REDUCTASE (*MTHFR*) GENE EXPRESSION, BLOOD PRESSURE AND HEART RATE IN HABITUAL AND NON-HABITUAL COFFEE DRINKERS**" was prepared by CHAN MENG HUI 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:



(Assistant Professor Dr. Phoon Lee Quen) Date: 4 June 2024

Supervisor

Department of Allied Health Science

Faculty of Science

Universiti Tunku Abdul Rahman

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UNIVERSITI TUNKU ABDUL RAHMAN

Date: 25 April 2024

PERMISSION SHEET

It is hereby certified that **CHAN MENG HUI** (ID No: **21ADB00333**) has completed this final year project report entitled "**THE ACUTE EFFECT OF BLACK COFFEE ON METHYLENETETRAHYDROFOLATE REDUCTASE (*MTHFR*) GENE EXPRESSION, BLOOD PRESSURE AND HEART RATE IN HABITUAL AND NON-HABITUAL COFFEE DRINKERS"** under the supervision of DR PHOON LEE QUEN from the Department of Allied Health Sciences, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project report / dissertation / thesis* 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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(CHAN MENG HUI)

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LIST OF ABBREVIATIONS

rRNA	Ribosomal RNA
MTHFR	Methylenetetrahydrofolate reductase
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
CVD	Cardiovascular disease
MetS	Metabolic Syndrome
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
CYP	Cytochrome P-450
CGA	Chlorogenic acid

CHAPTER 1

INTRODUCTION

According to the Ministry of Health Malaysia (2023), three in ten Malaysians were found to be hypertensive. In 2022, hypertensive diseases were one of the ten leading causes of death in Malaysia (Department of Statistic Malaysia, 2023). From the technical report of the National Health and Morbidity Survey 2019, the overall prevalence of hypertension among Malaysians aged 18 years and above was 30% with 5.7% to be among young adults. The World Health Organization (2023) defines hypertension as when an individual has a blood pressure that is 140/90 mmHg or higher. Hypertension is not an isolated condition but it is often interconnected with various health syndromes such as diabetes and dyslipidaemia (Mohamed, et al., 2023). From 2011 to 2019, there was an increasing trend in raised blood glucose and blood cholesterol among Malaysians and the majority were not known to have diabetes and hypercholesterolemia (Institute of Public Health, 2020).

These three healthy conditions were considered as risk factors for non-communicable diseases and are among the essential constituents of metabolic syndrome (MetS), which is an accumulation of metabolic and cardiovascular conditions such as dyslipidaemia, insulin resistance, obesity and hypertension, and could result in higher risk of developing cardiovascular diseases (CVD) and diabetes (Yanai, et al., 2008). Lifestyle and environmental factors like diet and

sedentary lifestyle plays a part in the prevalence and progression of MetS. The overall prevalence of physical inactivity among Malaysian adults was 25.1% and students and non-working adults were reported to be highly physically inactive than those in other occupational categories (Institute of Public Health, 2020). Change in lifestyle is important in controlling the development of MetS (Mohamed, et al., 2023).

Lifestyle and diet can also cause epigenetic effects in humans. Epigenetic is defined as the change in chromosomal components without affecting the underlying DNA sequence, which in turn modify the expression of genes (Liang, 2018; National Human Genome Research Institute, n.d.). Epigenetic regulation is a result of accumulation of daily lifestyle factors and environmental factors over the years and thus leading to the development of chronic diseases (Mochizuki, et al., 2017).

Coffee has always been a popular choice of beverage in Malaysia. In 2021, Statista Research Department recorded the total coffee consumption in Malaysia from 2013 to 2021 and the total coffee consumption peaked in 2019 and has continued to be on the rise since then (Statista Research Department, 2021). Coffee is commonly consumed with the aim of relieving fatigue and staying awake but it can also cause undesirable side effects such as increased blood pressure, upset stomach, and increased urination (Pietrangelo, 2017). However, coffee contains large amounts of polyphenols which bring many beneficial effects. Studies have shown that coffee can provide protective

function against Alzheimer's disease and fatty liver disease (Zelber-Sagi, et al., 2015; M Yelanchezian, et al., 2022). In recent years, there have been emerging studies that highlight the interesting association between habitual coffee consumption and potential benefits in reducing the risk of hypertension (Miranda, et al., 2021).

Methylenetetrahydrofolate reductase (*MTHFR*) is a gene that is associated with blood pressure level and the development of CVD. Many studies have shown the impact of coffee in relation to *MTHFR* polymorphism and the level of homocysteine, which is a substance that is closely related with MTHFR enzyme function and blood pressure (Frosst, 1995; Xuan, et al., 2011). However, there is a lack of study on the association between *MTHFR* gene expression and blood pressure after coffee consumption

1.1 Objectives of Study

The objectives of this present study are listed as below:

i. To determine the prevalence of habitual coffee consumption among UTAR students in a cross-sectional study.

ii. To evaluate the pre-post changes of methylenetetrahydrofolate reductase (*MTHFR*) mRNA expression, blood pressure and pulse rate in a coffee test.

iii. To study the impact of black coffee on *MTHFR* gene expression, blood pressure and pulse rate.

CHAPTER 2

LITERATURE REVIEW

2.1 Metabolic Syndrome

Metabolic syndrome (MetS) is an accumulation of metabolic abnormalities including dyslipidemia, insulin resistance, obesity and hypertension, resulting in an increased risk of developing cardiovascular diseases (CVD) and diabetes (Yanai, et al., 2008).

MetS is influenced by both genetic and environmental factors. Genetic predisposition can play a role in the manifestation and development of MetS through epigenetic mechanisms. The pathogenesis of MetS remains to be fully understood however the main contributors to the development and progression of MetS to CVD are said to be insulin resistance, visceral obesity, oxidative stress and chronic inflammation (Rochlani, et al., 2017; Stanciu, et al., 2023).

2.1.1 Hypertension

Blood pressure is measuring the force used by the heart to pump blood around the body and it is measured in millimeters of mercury (mmHg) (National Health Service, n.d.). According to Ministry of Health Malaysia (2018), 130/85 mmHg or lower is considered to be of normal and when the reading is above 140/90 mmHg, it is a sign of high blood pressure or hypertension.

The prevalence of hypertension in Malaysia has been on continuous rise. As found in a study by Zaki, et al. (2021), the prevalence of hypertension among Malaysian adults was 49.4% in 2021. According to Department of Statistics Malaysia (2023), hypertension was reported to be among one of the top ten leading causes of death among Malaysian community in 2022. This continuous rise of hypertension can be due to many factors such as genetics, lifestyle, and sociodemographic (Ha, et al., 2015; Ismail, et al., 2023). Furthermore, hypertension is a well-established risk factor for CVD and is one of the primary constituents for metabolic syndrome (Mendizábal, et al., 2013).

2.2 Coffee

Coffee is a Rubiaceae coffee plant and its seeds are roasted from different species of the genus *Coffea*. *Coffea arabica* and *Coffea robusta* are the two main sources of coffee beans with commercial importance. However, these two species differ from each other in their chemical content (Saud and Salamatullah, 2021; Socala, et al., 2021). Coffee contains a mixture of thousands of different chemicals, which mostly are biologically active and contribute to its beneficial effect such as antioxidant activity (Godos, et al., 2014). The main constituents of coffee are such as caffeine and chlorogenic acid (CGA).

Caffeine is a trimethylxanthine that is naturally found in coffee beans and has varying concentration in different types of coffee beverages. The structure of caffeine is as shown in Figure 2.1. *Robusta* coffee beans usually have higher content of caffeine than in *Arabica* coffee beans (Godos, et al., 2014).

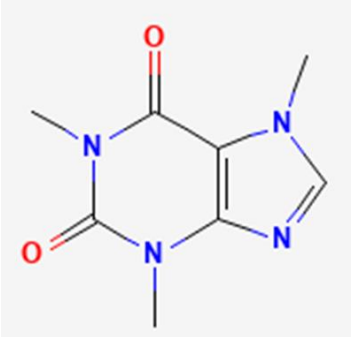

Figure 2.1: Structure of caffeine (National Center for Biotechnology Information, 2023).

After ingestion, caffeine spreads throughout the body and is able to pass through the blood-brain barrier. It is usually metabolized in the liver by cytochrome P-450 (CYP) enzymes, specifically the CYP1A2 enzymes. Caffeine absorption in the stomach and small intestine can be completed within 45 minutes after ingestion and the caffeine level in blood would be the highest after 15 minutes to 2 hours (Grosso, et al., 2017; Nieber, 2017). Caffeine shares structural similarities with adenosine, allowing it to exert its antagonistic effect on adenosine receptors in the cardiovascular system (Cappelletti, et al., 2015). According to Van Dam, et al. (2020), the half-life of caffeine and the sensitivity toward caffeine effect differs between individuals as it depends on factors such as genetic polymorphism, cytochrome P450 metabolism, individual factors and presence of hepatic disease. Individuals with a variant in the CYP1A2 were found to have lower rate of caffeine metabolism and would lead to different cardiovascular changes in response to caffeine ingestion.

CGA is a type of polyphenol in coffee that is commonly studied. Badmos, et al. (2019) found that *Robusta* coffee beans tend to have a higher CGA content as compared with *Arabica* coffee beans. CGAs are strong antioxidant and anti-inflammatory compounds. Such properties may have contributed to the protective effect of coffee against cardiovascular diseases (Godos, et al., 2014). CGA may also play a role in the antihypertensive effects of coffee. Zhao, et al. (2011) have found that there was a decrease of blood pressure in a dose-dependent manner after consumption of coffee with high CGA content.

2.2.1 Coffee Consumption Behavior in Young Adults

In 2019, it was recorded that the total coffee consumption in Malaysia was 850 thousand 60 kg bags of coffee (Statista Research Department, 2021). And in 2022, the Department of Statistics Malaysia recorded that the average Malaysian consumed 2.2 kg of coffee, which was a 5.2% increase from the previous years. Furthermore, there is an increasing number of young adults who are developing habitual coffee consumption, specifically for branded coffees (World Coffee Portal, 2024).

Studies have found that majority of university students were habitual coffee drinkers, both in Malaysia and in other countries. In a study conducted in University of Sarajevo, Bosnia and Herzegovina, it was shown that there was high prevalence of coffee consumption in the targeted population (Serdarevic, et al., 2019). While in another study among Arabian young adults by Lone, et al. (2023), it was reported that more than half of the study population consumed

coffee and that males had a higher frequency of coffee consumption. In a study by Demura, et al. (2013), it was found that males showed a higher coffee consumption frequency than females. Whereas in a study by Chan and Teoh (2021) conducted among first-year medical students in Malaysia, it was reported that the prevalence of coffee consumption was 58%.

2.2.2 Epigenetic Effects of Coffee

Epigenetic is defined as the heritable changes in gene expression by the modification of chromosomal components without change in the underlying DNA sequence. Epigenetic mechanism can affect gene expression through multiple mechanisms such as DNA methylation, histone modification and noncoding RNA (ncRNA) (Zhao, et al., 2022; National Human Genome Research Institute, n.d.). Epigenetic regulation can be influenced by environmental and lifestyle, causing stable and long-lasting impacts (Liang, 2018). Coffee is able to impact the epigenome by modulating DNA methylation, histone modification and ncRNA expression.

DNA methylation in eukaryotic organisms typically occur at cytosine that is located before a guanine in the 5’ to 3’ sequence, which is also known as CpG sites (Feinberg, 2018). It involves the addition of a methyl group to the fifth carbon atom of cytosine, forming 5-methylcytosine (5mC). Increased level of DNA methylation at CpG sites in the promoter region would normally result in the decreased level of gene transcription activity (Liang, 2018).

Histones are an important component of the chromatin complex that help form nucleosomes. The types of histone modification include acetylation, phosphorylation, methylation and more at the amino acid residues, which cause change in the chromatin structure or gene expression level (Zhao, et al., 2022).

NcRNA are the untranslated RNAs and can be divided into two types, namely housekeeping and regulatory ncRNAs. The regulatory ncRNAs are then further divided into two categories based on their size. NcRNAs can cause epigenetic changes and influence expression at gene and chromosome level to control cell differentiation (Wei, et al., 2016).

Many studies have proven the epigenetic effects of coffee due to the interaction of its components with gene, regulating gene expression (de Melo Pereira, et al., 2020). Studies have found that coffee consumption was associated with DNA methylation at multiple CpG sites in humans, including some sites near caffeine metabolizing genes such as *CYP1A2* and Aryl Hydrocarbon Receptor (*AHR*) (Chuang, et al., 2017; Karabegović, et al., 2021). This demonstrates the impact of environmental factors such as diet can impact the gene activity and long-term effects may lead to abnormal activity and thus causing adverse effect on human health.

2.2.3 Effect of Coffee on Blood Pressure

Coffee ingestion has shown to cause an acute increase in blood pressure due to caffeine. Caffeine exposure can increase catecholamine levels which result in

vasoconstriction and rise in blood pressure (Giuseppe, et al., 2019). Other possible mechanisms by caffeine to increase blood pressure include antagonism of adenosine receptor, increased stimulation of norepinephrine release, and activation of the renin-angiotensin system (Geleijnse, 2008; Kujawska, et al., 2021).

However, the blood pressure increase effect of caffeine is found to be short-term and acute. The acute increase of blood pressure after coffee ingestion is due to caffeine but there are other bioactive compounds in coffee, such as chlorogenic acid, that can counterbalance this effect leading to a final neutral-to-positive effect (Van Dam, et al., 2020). Another bioactive component, quercetin may also play a role in the positive vascular effect of coffee (Cicero, et al., 2023). Eumann, et al. (2011) reported contrasting results between acute and long-term effect of caffeine intake. It was observed that there was a profound blood pressure increase in the first 60-minute post-ingestion up to 180 minutes, while two weeks of coffee consumption did not show to increase blood pressure.

Moreover, the effect of coffee on blood pressure is more pronounced among non-habitual coffee drinks. In a study by Hara, et al. (2014), it was found that coffee was capable of acutely increasing blood pressure among normotensive non-habitual coffee drinkers aged 20 to 22 years while the acute pressor effect was blunted in habitual drinkers due to tolerance to caffeine. In another study, individuals that had self-reported a moderate consumption of coffee (2 and >3

cups per day) had a lower systolic blood pressure than non-coffee drinkers. The study also found that regular consumption of coffee showed to have decrease in their blood pressure than non-coffee drinkers (Cicero, et al., 2023).

Furthermore, coffee may have acute pressor effects, however coffee consumption is typically safe, even in individuals with risk of cardiovascular disease. A meta-analysis of six cohort studies had shown that coffee ingestion is associated with a lower risk of hypertension. Consistent consumption of caffeinated coffee was not associated with a higher risk of cardiovascular outcomes in the general population and individuals with cardiovascular history or high blood pressure (Van Dam, et al., 2020) While in a study in 2021, Miranda, et al (2021) found that non-smoker adults in Brazil with moderate consumption of coffee had lower risk of developing hypertension that those who seldom drink. Giorno, et al. (2021) reported that coffee consumption was inversely related with central blood pressure and arterial stiffness among the general population in Switzerland.

2.3 Methylenetetrahydrofolate reductase (*MTHFR*) Gene

MTHFR gene is found on the short arm (p arm) of chromosome 1 at position 36.3 as shown in Figure 2.2 (GeneCards Database, n.d.) and it belongs to the methylenetetrahydrofolate reductase family.

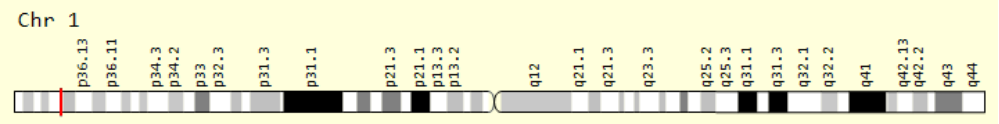

Figure 2.2: Location of *MTHFR* gene on chromosome 1 (GeneCards Database, n.d.)

MTHFR gene encodes for the MTHFR enzyme which is an important regulatory enzyme in the folate and methionine cycles. MTHFR enzyme is also related in the nitric oxide synthesis, affecting blood pressure level (McMahon, et al., 2016).

MTHFR enzyme plays a key function in the one-carbon metabolism, which includes folate and methionine metabolism and synthesis of DNA. For folate to be in its active form, it needs to be reduced to tetrahydrofolate (THF) which will enter the folate cycle. THF will then be converted to 5, 10-methylene-THF, then to 5-methyl-THF by MTHFR enzyme. The 5-methyl-THF then act as the substrate for the conversion of homocysteine to methionine. In the methionine cycle, methionine would be further converted into other molecules which is essential in methylation processes (McMahon et al., 2016). The simplified process of folate cycle and involvement of MTHFR enzyme in the cycle is as depicted in Figure 2.3.

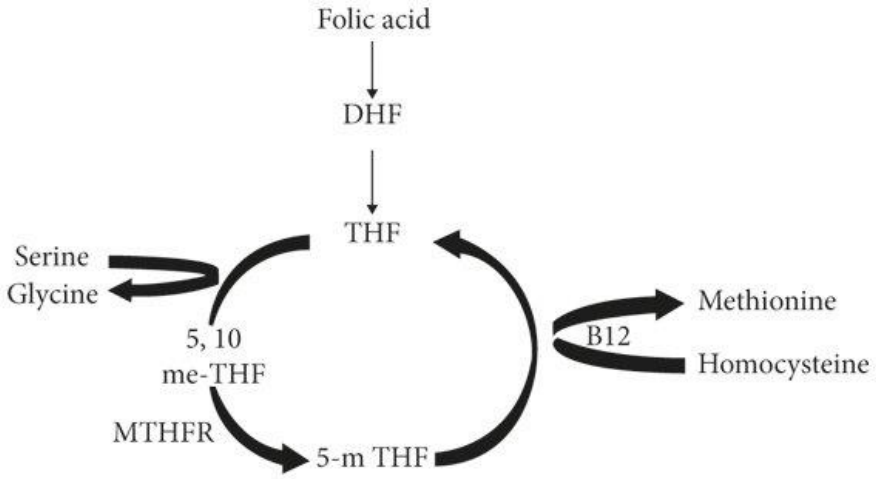

Figure 2.3: Folate cycle (Salameh, et al., 2020).

2.3.1 Association Between *MTHFR* Gene and Blood Pressure

There are two common gene variants of *MTHFR* which are C677T and A1298C. According to Frosst et al. (1995), individuals with *MTHFR* 677TT genotype would have 70% reduction in MTHFR enzyme activity as compared to *MTHFR* 677CC genotype, which in turn would have higher homocysteine levels. Many studies have reported the relationship between *MTHFR* polymorphism, increased plasma homocysteine level, and increased risk for CVD complications. Kluijtmans et al. (1996) and Klerk et al. (2002) found that individuals with *MTHFR* 677TT genotype had a higher risk of developing CVD. While Xuan et al. (2011) reported an association between the *MTHFR* 677TT genotype and risk of myocardial infarction. In a case-control study by Yang et al. (2017), it was reported that increased level of homocysteine was highly associated with hypertension and blood pressure level.

2.3.2 Association Between *MTHFR* and Coffee

In a study by Strandhagen, et al. (2004), it was stated that the increase of homocysteine level due to coffee was more obvious in individuals with *MTHFR* 677TT genotype than individuals with *MTHFR* 677CC genotype. Coffee consumption has also been found to be associated with higher plasma homocysteine levels (Panagiotakos, et al., 2004). Verhoef, et al. (2002) stated that caffeine is the main component in coffee that causes the increase in homocysteine level.

Numerous studies have investigated the effect of coffee in relation to blood pressure, *MTHFR* polymorphism and plasma homocysteine level. However, there is a lack of study on the association between *MTHFR* gene expression and blood pressure after coffee consumption. Therefore, the present study aims to evaluate the pre-post changes of *MTHFR* expression, blood pressure and pulse rate in a coffee test.

CHAPTER 3

MATERIAL AND METHODS

3.1 Materials

3.1.1 Biological Sample

This study has been approved by the UTAR Scientific and Ethical Review Committee (SERC) with the ethical approval code number (U/SERC/287/2023) which can be found in Appendix A. The study was carried out from October 2023 until January 2024, and the target population for this study was UTAR students aged 18 to 30 years old. The sample size was calculated as below:

$$n = [t^2 \times p(1-p)] / m^2$$

$$n = [1.962 \times (0.35 \times 0.65)] / 0.05^2$$

$$n = 350$$

Where:

n = required sample size

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

p = estimated prevalence of habitual coffee drinkers at UTAR students based on the previous study at UTAR is about 35%

m = margin of error at 5%

*A total of 420 respondents were targeted to allow 20% drop out.

3.1.2 Chemicals and Materials

The lists of chemicals and materials used in this study were compiled in the Appendix B.

3.1.3 Overview of Methodology

The overview of the project is showed in Figure 3.1.

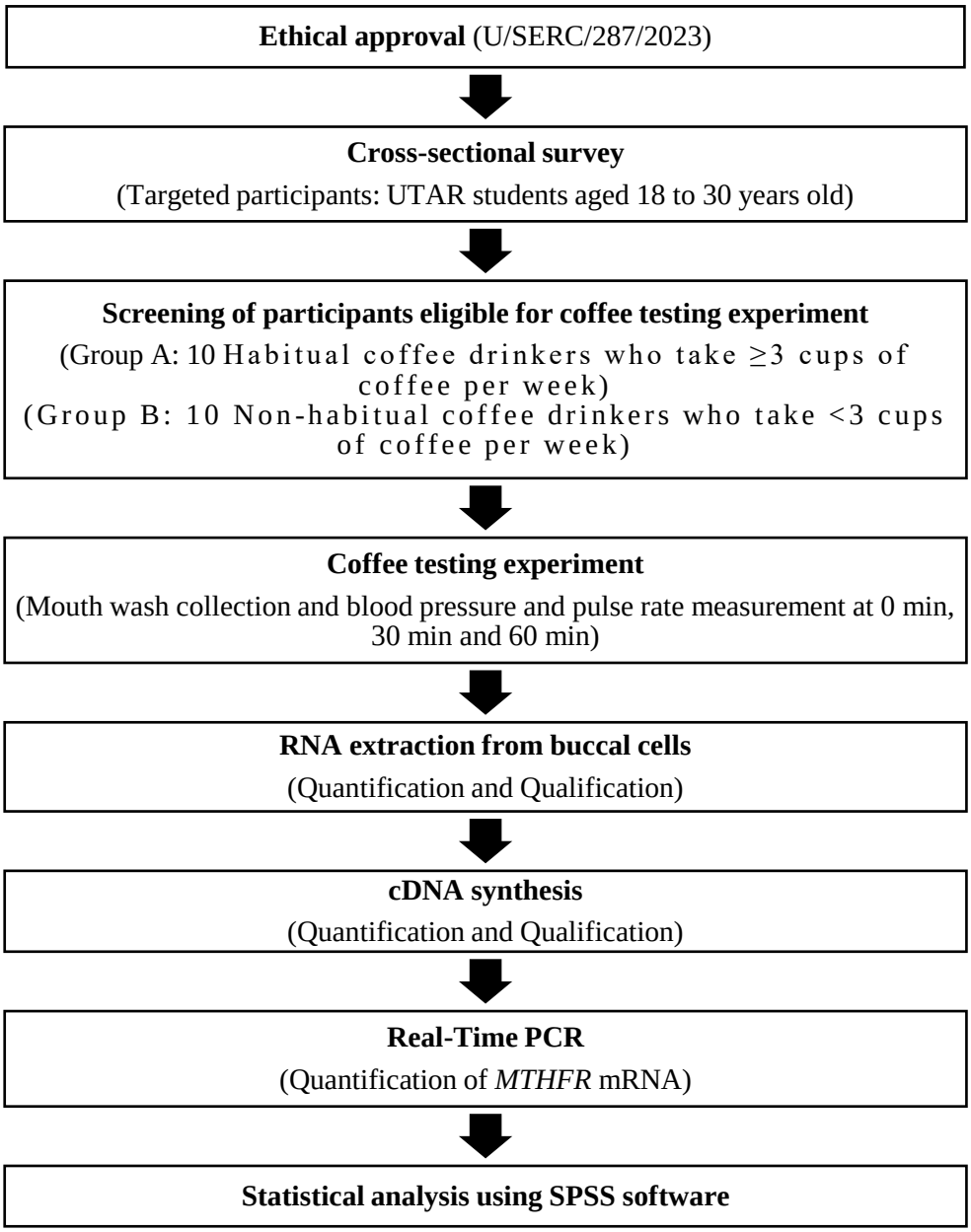

Figure 3.1: Overview of methodology of the study.

3.2 Data Collection

3.2.1 Cross-sectional Survey

The questionnaire was done using online Google Forms and consisted a total of 11 sections. Before answering the questionnaire, participants were informed of the confidentiality and consent of the study. Section A of the questionnaire contained questions regarding participants' demographic characteristics such as their name, gender, ethnicity and contact number. Anthropometric measures, including body weight (kg) and height (m) were obtained in the same section as well.

Next, Section B consisted of questions regarding participants' personal and family history. In Section C, the habits and preferences in coffee consumption, including respondents' frequency of coffee consumption were questioned. While in Section D, the attitude and perceptions on coffee consumption were questioned.

Following that, Section E and F included questions on eating habit and preference as well as food intake to assess the respondents' dietary patterns. Section G included questions regarding food habits which collected information on the processed food and Malaysian/Asian dishes intake frequency per day, week and month.

Besides that, questions about respondents' intake of vitamin or mineral supplement and intake of food supplement were included in Section H and Section I respectively. While Section J questioned the physical activity level of respondents. Lastly, Section K assessed the cognitive performance of respondents.

The questionnaire was distributed to the targeted participants of this study and the responses were recorded in a Google Sheet template. The questions used in this study were compiled in Appendix C.

3.3 Coffee Testing Experiment

Following the inclusion and exclusion criteria that is listed in Table 3.1, 20 eligible participants from the questionnaire were selected for the study. The participants were grouped into two groups: Group A which was made up of ten habitual coffee drinkers who consume more than 3 cups of coffee per week, and Group B which was made up of ten non-habitual coffee drinkers who consume less than 3 cups of coffee per week.

Table 3.1: Inclusion and Exclusion Criteria for selection of participants.

Criteria	Description
Inclusion criteria	Malaysian UTAR students that are aged 18 to 30 years old
Exclusion criteria	Chronic disease (diabetes, hypertension, coronary heart disease, cerebrovascular disease, hypothyroidism or other major diseases except of overweight or obesity), smoker (>1 pack/week), on medication treatment which will affect taste appetite, body weight, menstruating, pregnant, and lactating

Twenty participants (10 habitual and 10 non-habitual coffee drinkers) of equal distribution of gender were selected in the coffee experimental study. Participants were explained of the procedures of the coffee testing and their consent was obtained. Participants were asked attend two separate sessions. In session 1, habitual coffee drinkers underwent the coffee test by consuming coffee, while non-habitual coffee drinkers were given water only. In session 2, habitual coffee drinkers were given water only, while non-habitual coffee drinkers underwent the coffee test by consuming coffee. The pre-post blood pressure and pulse rate measurement and buccal cell collection of each participant were obtained at intervals of 0 minute, 30 minutes, and 60 minutes after consumption of water and coffee at both sessions.

3.3.1 Coffee Preparation

The black coffee powder used was extracted from Robusta and Arabica coffee beans. The material composition declaration and product certificate of analysis of the black coffee powder used were compiled in Appendix D. The black coffee was prepared by dissolving 3 g of coffee powder in 150 mL of water in a cup and it was given to the participants.

3.3.2 Blood Pressure and Pulse Rate Measurement

Subjects were asked to rest for 15 minutes before commencement of coffee testing. Resting brachial blood pressure was measured on the participant's left arm and the systolic blood pressure (SBP), diastolic blood pressure (DBP) and pulse rate were obtained using Omron Blood Pressure Monitor. Blood pressure and pulse rate was measured before and after coffee consumption at 0 minute, 30 minutes, and 60 minutes. The blood pressure and pulse measurement were done three times for each interval and the average value was calculated.

3.3.3 Buccal Cell Collection

Participants were advised to abstain from consuming any caffeinated food and beverages for 2 hours prior to the sample collection. Participants were also asked to rinse their mouth with mineral water to remove food particles. Participants were given a paper cup with 15 mL of saline water (0.9% sodium chloride (NaCl) to gargle for about 1 minute and the saline containing buccal cells was dispensed back into the cup which were then transferred into a sterile

15 mL collection tube. Then the collected buccal cell samples were ready for RNA extraction.

3.4 RNA Extraction

3.4.1 Preparation for RNA Extraction

Prior to RNA extraction, all equipment and consumables were treated with Diethylpyrocarbonate (DEPC) water to inhibit RNase enzyme activity. Equipment and consumables such as microcentrifuge tubes and micropipette tips were immersed in 0.1 % of DEPC water overnight, dried in the oven and then autoclaved.

Cetyltrimethyl ammonium bromide (CTAB) buffer was prepared before RNA extraction. 2 g of CTAB, 28 mL of 1.4 M of NaCl, 10 mL of 100 mM of Tris-HCl (pH 8.0), and 4 mL of 20 mM of EDTA were mixed together and brought to a volume of 88 mL with double distilled water (ddH_2O) and then autoclaved.

3.4.2 RNA Extraction from Buccal Cell Samples

At initial step, 20 mg Polyvinylpyrrolidone (PVP 40) was added into 600 µL of CTAB buffer then mixed well and pre-warmed in a dry bath to 55 °C. Then, 6 µL of β-mercaptoethanol was added into the CTAB buffer. In a 15 mL collection tube, the saline containing buccal cells was centrifuged for 20 minutes at 10,000 rpm at 18 °C. The supernatant was then discarded and CTAB buffer was added to resuspend the pellet. The resuspended pellet was transferred

to a 1.5 mL microcentrifuge tube. Next, 600 µL of chloroform was added then the mixture was vortexed briefly and centrifuged at 16,000 x *g* for 2 minutes. The supernatant was transferred to a new 1.5 mL microcentrifuge tube and the previous step was repeated.

After transferring the supernatant to a new 1.5 mL microcentrifuge tube, an equal volume of isopropanol was added to the tube. The mixture was then subjected to 15 minutes centrifugation at 16,000 x *g*. Following that, the supernatant was discarded and 600 µL of 96% ethanol was added and the mixture was vortex briefly then centrifuged at 16,000 x *g* for 5 minutes. The supernatant was removed and 90 µL of water was added. The mixture was incubated in a 65 °C water bath for 15 minutes to dissolve the pellet.

Next, the mixture was centrifuged at 16,000 x *g* for 5 minutes to remove the debris and the supernatant was transferred into a new 1.5 mL microcentrifuge tube. A volume of 30 µL of 8 M lithium chloride (LiCl) was added and the mixture was incubated at -20 °C for 20 minutes. Following that, the mixture was centrifuged at 16,000 x *g* for 30 minutes and the supernatant was discarded. Then, the pellet was added with 100 µL of 70% ethanol and centrifuged at 16,000 x *g* for 2 minutes. After the centrifugation, the supernatant was discarded. Lastly, the pellet was dissolved in 20 µL distilled water for 15 minutes at 65 °C. The total RNA was labelled properly and stored in -20 °C for further use.

3.4.3 DNase treatment

In a new 1.5 mL microcentrifuge tube, 9 µL of total RNA and 1 µL of diluted DNase I were added then incubated at 37 °C for 30 minutes. Next, 1 µL of 50 mM of ethylenediamine tetraacetic acid (EDTA) was added into the mixture and incubated at 65 °C for 10 minutes. The DNase treated RNA stored in -20 °C for further use.

3.4.4 Qualification and Quantification of RNA

The RNA samples were quantified by measuring the concentration and purity of RNA at wavelength of 260 nm and 280 nm using the NanoDrop Spectrophotometer. The measurement surface and lid were cleaned with ethanol sprayed Kimwipe paper before the samples were loaded onto the optical measurement surface. Next, a volume of 1 µL nuclease-free water was used as blank. Then, 1 µL of RNA sample was carefully pipetted onto the centre of optical measurement surface and the lid was closed. The sample was analysed, and the concentration and purity readings were recorded. Both the optical measurement surface and lid were cleaned before it was used for the next RNA sample.

The RNA sample purity was assessed by the absorbance ratio at 260 nm and 280 nm (A_{260}/A_{280}). The RNA sample with ratio values of 1.8 to 2.0 was generally accepted as pure RNA (Gallagher and Desjardins, 2007).

The quality of the extracted total RNA samples was determined by gel electrophoresis. Approximately 0.2 g of agarose powder was poured into 20 mL of 1X Tris-Borate-EDTA (TBE) buffer in a conical flask, mixed well by swirling and heated in a microwave to dissolve the agarose powder completely. A clean 0.75 mm comb was placed in a gel casting tray and the cooled mixture was poured into the gel casting tray and was left at room temperature for around 30 minutes to solidify. Next, the comb was gently removed and the gel was put in the gel electrophoresis tank. Then, 1X TBE buffer was added into the tank till it covered the whole gel to a depth of about 1 mm.

Before loading the RNA samples into the wells. 1 µL of loading dye was mixed with 5 µL of RNA samples on a clean parafilm. A volume of 2 µL 1 kb DNA ladder was also mixed with 1 µL of loading dye and loaded into the first well of the gel. The gel was then run at 90 V for 35 minutes and viewed under UV transilluminator. The presence of 28S and 18S rRNA bands were observed and RNA gel image with the presence of the two bands were indicative of good RNA integrity (Schroeder, et al., 2006).

3.5 Complementary DNA (cDNA) synthesis

OneScript Plus Reverse Transcriptase with cDNA Synthesis Kit was used for cDNA synthesis of the extracted RNA. First, 10 µM oligo (dT), 10 mM dNTP mix, and nuclease-free water were added into a microcentrifuge tube followed by the extracted RNA. The mixture was then incubated at 65˚C for 5 minutes and chilled on ice for 1 minute. The mixture was gently spun down.

Next, 5X RT buffer, RNase OFF ribonuclease inhibitor and OneScript Plus reverse transcriptase was added into the sample mixture. The mixture was then gently mixed and spun down. After that, the mixture was incubated for 15 minutes at 50 °C to perform the cDNA synthesis. The reaction was stopped by heating the mixture at 85 °C for 5 minutes then chilled on ice. The concentration and purity of the cDNA was measured by Nanophotometer. The volume of each component was as seen in Table 3.2.

Table 3.2: Components of cDNA synthesis.

Components	**Volume (µL)**
10 µM oligo (dT)	1
10 mM dNTP mix	1
Nuclease-free water	11.5
Extracted RNA	1
5X RT buffer	4
RNase OFF ribonuclease inhibitor	0.5
OneScript Plus reverse transcriptase	1
Total	20

3.6 Gradient PCR

Primers of *MTHFR* and a housekeeping gene, 18S ribosomal RNA (*18S rRNA*) were used in this study. The forward and reverse nucleotide sequences of the primers are listed in Table 3.3. The master mix assay for all reactions was prepared as showed in Table 3.4 and were briefly spun down. The condition for

PCR amplification of cDNA is demonstrated in Table 3.5 and the temperature for each well of the gradient PCR is showed in Table 3.6. Gradient PCR was used to optimize the annealing temperature for amplifying DNA fragments. The optimized annealing temperature was 60 °C.

The PCR products were then loaded with 1 µL loading dye on a 2% agarose gel and electrophoresis was performed at 90 V for 35 minutes to confirm the presence of the desired PCR amplicon size. The gel image was viewed and captured under UV transilluminator.

Table 3.3: Nucleotide sequence of primers used in qPCR

Pair wise primer		**Primers (5' to 3')**	**PCR amplicon (bp)**
18S rRNA	Forward	AACTTTCGATGGTAGTCGCCG	104
	Reverse	CCTTGGATGTGGTAGCCGTTT	
MTHFR	Forward	GAACGAAGCCAGAGGAAACA	163
	Reverse	GGGTGGAACATCTCGAACTATC	

Table 3.4: Concentration and volume of PCR components used for PCR amplification

PCR components	Stock concentration	Final concentration	Volume (µL)
2x PCR 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 3.5: Conditions for gradient PCR amplification of cDNA

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

Table 3.6: Temperature of each well of the gradient PCR

Row	Temperature (°C)
A	66.0
B	64.9
C	63.2
D	60.5
E	57.3
F	54.7
G	53.0
H	52.0

3.7 Quantification of MTHFR mRNA Gene Expression

3.7.1 Real time PCR

The *MTHFR* gene expression was qualified using real time PCR with Bright Green 5X qPCR mix. In this study, *18S rRNA* was used as the housekeeping gene, serving as a reference for normalization in gene expression measurement. *18S rRNA* is ubiquitously expressed and its expression would not be affected by the experimental treatments. First, qPCR strips, cover caps, and micropipette tips were sent for autoclave prior to qPCR performance. The master mix assay was prepared as shown in Table 3.7 then all reaction materials were pipetted into qPCR tubes which were then sealed with the caps and spin down briefly. Then, all samples were placed on the heat block of the Real-Time PCR machine for amplification. The qPCR thermal cycling is as shown in Table 3.8.

Table 3.7: Concentration and volume of the qPCR components used for real-time PCR amplification

qPCR components	Stock concentration	Final concentration	Volume (µL)
Bright Green 5X qPCR mix	5x	1x	2.00
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	-	-	6.20
Total			10.00

Table 3.8: Thermal cycling for the real-time PCR amplification

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

3.7.2 Data Analysis of qPCR

The Cq values obtained from the qPCR output was used to quantify the relative gene expression of *MTHFR* (Livak and Schmittgen, 2001; Bonacorsi, et al., 2021). The double delta Ct method ($2^{-\Delta\Delta Cq}$ method) was used in this present study to calculate the fold gene expression of *MTHFR* that was normalized to *18S rRNA*. The formula is listed as following:

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

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

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

3.8 Statistical Analysis

3.8.1 Analysis of Survey

The prevalence of coffee consumption was determined. The types and preferences of coffee as well as the attitude and perception towards coffee consumption was determined. The results from the survey were presented in frequency and percentage.

3.8.2 Comparison Between Habitual and Non-Habitual Coffee Drinkers

The blood pressure, pulse rate and *MTHFR* mRNA expression level were compared between habitual and non-habitual groups using Mann-Whitney U test. The null hypothesis and alternative hypothesis were as followed:

H_0 = There is no difference in blood pressure, pulse rate and *MTHFR* mRNA expression level between habitual coffee drinkers and non-habitual coffee drinkers post-coffee treatment.

H_1 = There is a difference in blood pressure, pulse rate and *MTHFR* mRNA expression level between habitual coffee drinkers and non-habitual coffee drinkers post-coffee treatment.

The difference in blood pressure, pulse rate and *MTHFR* expression level of each group at different time points (0 min, 30 min and 60 min) were compared using the Wilcoxon Signed-Rank Test and the null hypothesis and alternative hypothesis for habitual coffee drinkers were as followed:

H_0 = There is no difference in blood pressure, pulse rate and *MTHFR* expression level between different time points post-coffee treatment among the habitual coffee drinkers.

H_1 = There is a difference in blood pressure, pulse rate and *MTHFR* expression level between different time points post-coffee treatment among the habitual coffee drinkers.

While the hypotheses for non-habitual coffee drinkers were as followed:

H_0 = There is no difference in blood pressure, pulse rate and *MTHFR* expression level between different time points post-coffee treatment among the non-habitual coffee drinkers.

H_1 = There is a difference in blood pressure, pulse rate and *MTHFR* expression level between different time points post-coffee treatment among the non-habitual coffee drinkers.

Next, the association between habitual and non-habitual coffee drinkers were studied by comparing between male and female drinkers of each groups using the Kruskal-Wallis H Test and the hypothesis was as follow:

H_0 = There is no difference between male habitual coffee drinkers, female habitual coffee drinkers, male non-habitual coffee drinkers and female non-habitual coffee drinkers post-coffee treatment.

H_1 = There is a difference between male habitual coffee drinkers, female habitual coffee drinkers, male non-habitual coffee drinkers and female non-habitual coffee drinkers post-coffee treatment.

When significant difference was found, further test was done to compare between the genders within each group.

3.8.3 Comparison Between Gender Within Each Group

The blood pressure, pulse rate and *MTHFR* gene expression were compared between male and female and the hypothesis for habitual drinkers was as listed below. The comparison was done using Mann-Whitney U test.

H_0 = There is no difference in blood pressure, pulse rate and *MTHFR* gene expression between male and female habitual coffee drinkers post-coffee treatment.

H_1 = There is a difference in blood pressure, pulse rate and *MTHFR* gene expression between male and female habitual coffee drinkers post-coffee treatment.

While the hypotheses for non-habitual coffee drinkers were as followed:

H_0 = There is no difference in blood pressure, pulse rate and *MTHFR* gene expression between male and female non-habitual coffee drinkers post-coffee treatment.

H_1 = There is a difference in blood pressure, pulse rate and *MTHFR* gene expression between male and female non-habitual coffee drinkers post-coffee treatment.

The difference in blood pressure, pulse rate and *MTHFR* expression level of each group at different time points (0 min, 30 min and 60 min) were also compared using the Wilcoxon Signed-Rank Test.

For all the statistical analysis, a p-value that was larger than 0.05 indicated that the observed differences were considered statistically significant.

CHAPTER 4

RESULTS

4.1 Distribution of Habitual and Non-Habitual Coffee Drinkers

A total of 426 participants were recruited for the questionnaire. The number of male participants in this study was 175 (41%) and the number of female participants was 251 (59%).

According to the questionnaire responses, less than half of the participants (19%) were considered as habitual coffee drinkers as they consumed coffee three or more than three times a week. While majority of the participants (81%) was non-habitual coffee drinkers as their coffee consumption frequency was less than three times per week. The prevalence of habitual and non-habitual coffee drinkers in UTAR, Kampar has been summarized in Figure 4.1 below. While the gender distribution and classification of coffee consumption is as seen in Table 4.1. It could be observed that majority of the habitual coffee drinkers were males (30%) while majority of the females were non-habitual coffee drinkers (88%). The BMI status of participants was illustrated in Table 4.2. BMI was classified into underweight (<18.5 kg/m^2), normal (18.5-24.9 kg/m^2), overweight (25-29.9 kg/m^2) and obese (>30 kg/m^2). Majority of the participants fell under normal range (57.5%) while 27.9% of the respondents were underweight, followed by overweight (10.6%) and obese (4%).

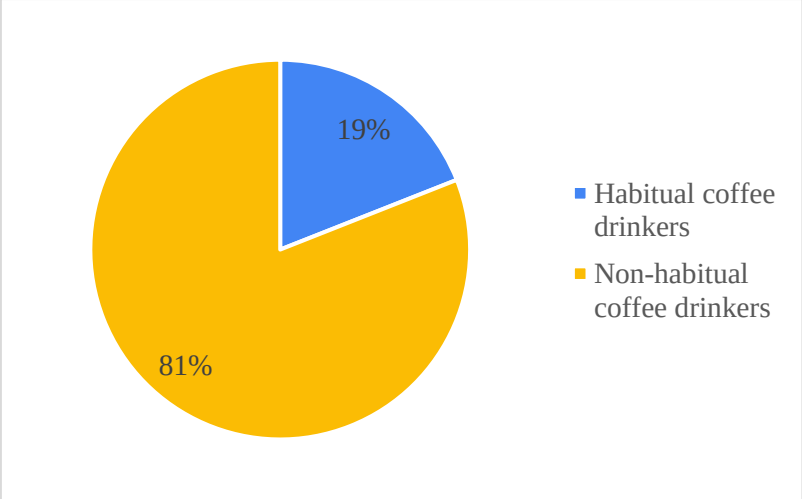

Figure 4.1: The distribution of habitual and non-habitual coffee drinkers in UTAR, Kampar. (n = 426)

Table 4.1: A summary of classification of coffee consumption and gender distribution in UTAR.

Variables	**Subjects (n = 426)**		
	Male	**Female**	**Total**
Gender distribution	175 (41%)	251 (59%)	426 (100%)
Coffee consumption			
Habitual coffee drinkers (consume three or more than three times per week)	52 (30%)	29 (12%)	81 (19%)
Non-habitual coffee drinkers (consume less than three times per week)	123 (70%)	222 (88%)	345 (81%)

Table 4.2: BMI status of participants

BMI (Mean ± S.D.)	**Frequency (n = 426)**
Underweight (<18.5 kg/m^2)	119 (27.9%)
Normal (18.5 - 24.9 kg/m^2)	245 (57.5%)
Overweight (25 - 29.9 kg/m^2)	45 (10.6%)
Obese (>30 kg/m^2)	17 (4.0%)

4.2 Analysis of Questionnaire

4.2.1 Types and Preferences of Coffees of The Coffee Drinkers

The choices of coffee types of coffee drinker that have consumed coffee in the past six months in percentage were recorded. The reason of choosing the used-most and the reason the use most would differ from the prefer-most coffee types of the respondents were also recorded in the questionnaire.

Based on Figure 4.2, instant coffee was found to be the most consumed and preferred coffee type at 38%, followed by ground or filter coffee at 18%. According to the findings that is summarized in Table 4.3 and Table 4.4, the most important reason for respondents when choosing their most used coffee type was based on their taste (17.8%), convenience and availability of the coffee type (16.7%). Respondents also choose their most used coffee based on their personal preference (16.0%)). While the reason the used-most coffee type and preferred-most coffee type would differ is highly due to unavailability in their work place and study place (30.8%).

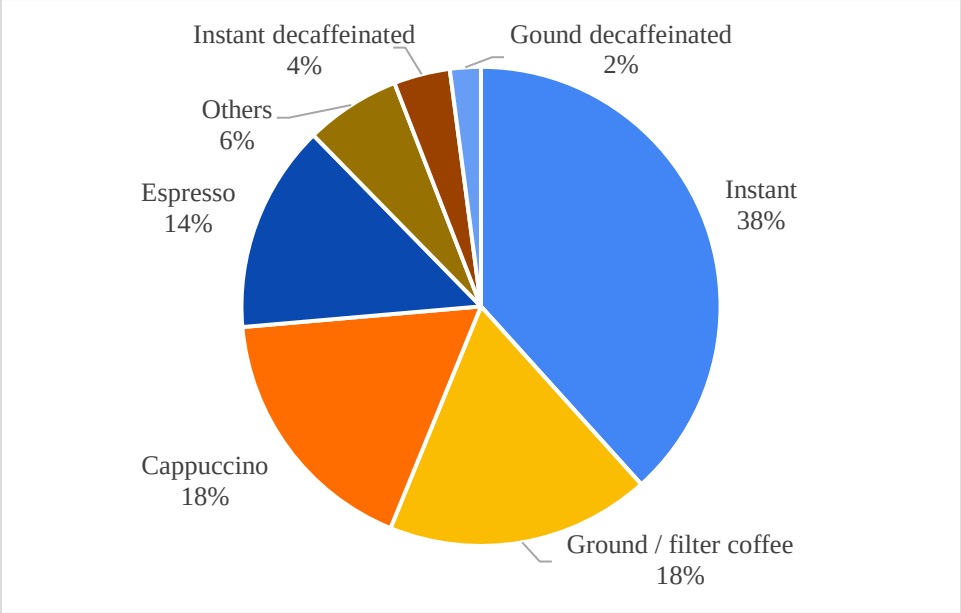

Figure 4.2: The coffee types that are most used and preferred by coffee drinkers who consumed coffee in the past six months.

Table 4.3: Reasons for coffee drinkers to choose the used most coffee type.

Reasons	Subject (n=426)
It is the type I prefer.	68 (16.0%)
It tastes best.	76 (17.8%)
It is the most affordable.	48 (11.3%)
It is the most readily available.	71 (16.7%)
The place where I work/ study provides the most used type.	22 (5.2%)
Other members in my household prefer most used brand.	23 (5.4%)
Others	118 (27.6%)

Table 4.4: Reasons for the used most coffee type to differ from the preferred most coffee type by coffee drinkers.

Reasons	Subject (n=426)
I cannot afford my preferred type.	103 (24.2%)
The place where I work/ study does not use the preferred type.	131 (30.8%)
Other members in my household prefer another type of coffee.	69 (16.2%)
Others	123 (28.8%)

4.2.2 Attitude and Perception Regarding Coffee Consumption

The attitude and perception of respondents regarding coffee consumption were also recorded and the findings, expressed in frequency and percentage were summarized in Table 4.5.

Table 4.5: Attitude and perceptions regarding coffee consumption.

Variables	**Subject (n = 426)**				
Attitude and Perception regarding coffee consumption	**1**	**2**	**3**	**4**	**5**
Drinking coffee increases my risk of getting cancer.	77 (18.1%)	141 (33.1%)	174 (40.8%)	27 (6.3%)	7 (1.6%)
Drinking coffee increases my risk of heart disease.	44 (10.3%)	121 (28.4%)	151 (35.4%)	96 (22.5%)	14 (3.3%)
I cannot fall asleep when I drink coffee during the day.	74 (17.4%)	104 (24.4%)	104 (24.4%)	91 (21.4%)	53 (12.4%)
I prefer drinking tea to drinking decaffeinated coffee.	39 (9.2%)	64 (15.0%)	117 (27.5%)	108 (25.4%)	98 (23.0%))
Drinking coffee gives me an energy boost.	29 (6.8%)	60 (14.1%)	134 (31.5%)	132 (31.0%)	71 (16.7%))
I drink coffee because my friends do.	167 (39.2%)	126 (29.6%)	91 (21.4%)	29 (6.8%)	13 (3.1%)

Table 4.5: (continued)

Variables	**Subject (n = 426)**				
Attitude and Perception regarding coffee consumption	**1**	**2**	**3**	**4**	**5**
I drink coffee for the taste.	40 (9.4%)	59 (13.8%)	116 (27.2%)	145 (34.0%)	66 (15.5%)
I drink coffee because it is fashionable.	163 (38.3%)	139 (32.6%)	94 (22.1%)	21 (4.9%)	9 (2.1%)
I prefer drinking tea to drinking any coffee.	44 (10.3%)	81 (19.0%)	116 (27.2%)	91 (21.4%)	94 (22.1%)
I cannot fall asleep when drinking coffee in the evening.	82 (19.2%)	99 (23.2%)	99 (23.2%)	77 (18.1%)	69 (16.2%)
I drink coffee to help me stay awake.	51 (12.0%)	73 (17.1%)	106 (24.9%)	108 (25.4%)	88 (20.7%)
I prefer local brands of coffee to imported ones.	71 (16.7%)	67 (15.7%)	232 (54.5%)	40 (9.4%)	16 (3.8%)
Drinking coffee makes me feel important.	125 (29.3%)	103 (24.2%)	141 (33.1%)	36 (8.5%)	21 (4.9%)

*1 represents strongly disagree, 2 represents disagree, 3 represents neutral, 4 represents agree and 5 represents strongly agree

4.3 Coffee Test Experiment

4.3.1 Blood Pressure and Pulse Rate Measurements for Habitual and Non-Habitual Coffee Drinkers

Blood pressure and pulse rate were measured before and after treatment with water and coffee at 0 min, 30 min and 60 min and three times at each interval. The average of each participant's measurements was calculated as shown in Appendix E.

4.4 *MTHFR* mRNA Expression

4.4.1 Qualification and Quantification of Extracted RNA Samples

The total RNA was extracted from all samples and the quality of the extracted RNA samples were examined using gel electrophoresis. The gel image obtained is shown in Figure 4.3.

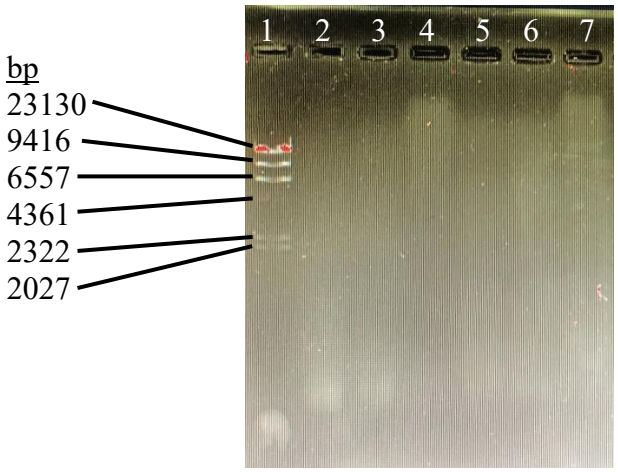

Figure 4.3: Extracted RNA samples (1% agarose gel). Lane 1 is HindIII ladder. Lane 2 to 7 is the extracted RNA from six different samples. The two fragments (28S rRNA and 18S rRNA) were not observed and the bands were smeared as there was too low concentration of total RNAs.

The extracted RNA samples were quantified using nanodrop spectrophotometer to obtain the concertation and purity which are shown in Appendix F. The average concentration total RNA samples and purity were 36.88 ng/µL and 1.82, respectively

4.5 Evaluation of cDNA Samples

The cDNAs were synthesized from the extracted RNA samples and the concentration and purity of the cDNA samples were measured using nanodrop spectrophotometer. The average concentration of cDNA samples and purity were1154 ng/µL and 1.66, respectively as shown in Appendix G.

4.6 Real-time PCR Amplification

cDNAs were standardized to 100 ng/µL prior to real-time PCR amplification. In this study, *MTHFR* gene expression level of the 20 participants were evaluated. The *MTHFR* expression of habitual and non-habitual coffee drinkers pre-post coffee and water treatment is summarized in Table 4.6.

Table 4.6: Gene expression of *MTHFR* among habitual and non-habitual coffee drinkers pre-post coffee and water consumption.

	Treated (coffee)			**Untreated (water)**		
	0 min	**30 min**	**60 min**	**0 min**	**30 min**	**60 min**
Habitual coffee drinkers:						
Average ΔCt [1]	5.52	6.94	6.67	6.17	6.18	5.83
ΔΔCt [2]	0.00	1.42	1.16	0.00	0.00	-0.35

Table 4.6: (continued)

	Treated (coffee)			Untreated (water)		
	0 min	**30 min**	**60 min**	**0 min**	**30 min**	**60 min**
Fold gene expression [3]	1.00	0.37	0.45	1.00	1.00	1.27
Non-habitual coffee drinkers:						
Average ΔCt [1]	6.00	5.78	5.76	5.58	5.53	5.69
ΔΔCt [2]	0.00	-0.22	-0.24	0.00	-0.04	0.12
Fold gene expression [3]	1.00	1.17	1.18	1.00	1.03	0.92

[1] ΔCt = Ct (*MTHFR*) – Ct (*18S*)
[2] ΔΔCt (within treatment group) = ΔCt (average of time interval) - ΔCt (average of origin time)
[3] Compared with 0 min (within treatment group) = $2^{-(\Delta\Delta Ct)}$

4.7 Comparison Between Habitual and Non-Habitual Coffee Drinkers Post-Treatment with Coffee

4.7.1 Blood Pressure and Pulse Rate Measurements Post-Coffee Treatment

Figure 4.4, Figure 4.5 and Figure 4.6 visualized the change in the systolic blood pressure, diastolic blood pressure and pulse rate respectively. It can be observed in the figures that habitual coffee consumers would show an increase in the systolic and diastolic blood pressure at 30 minutes post-coffee consumption. However, at 60 minutes post-coffee treatment, it can be seen that the systolic blood pressure would drop while the diastolic blood pressure continues to rise. While in non-habitual coffee drinkers, it shows a continuous rise in the measurements even at 30 and 60 minutes for blood pressure measurements. Conversely, the pulse rate of habitual and non-habitual coffee drinkers would decrease at 30 minutes. Habitual coffee drinkers showed a greater acute change

in blood pressure while non-habitual coffee drinkers showed a greater acute change in pulse rate.

The findings from the statistical analysis are summarized in Table 4.7. The null hypothesis was failed to be rejected as it is found that there was no significant difference in the cardiometabolic measurements between habitual and non-habitual coffee drinkers after coffee treatment.

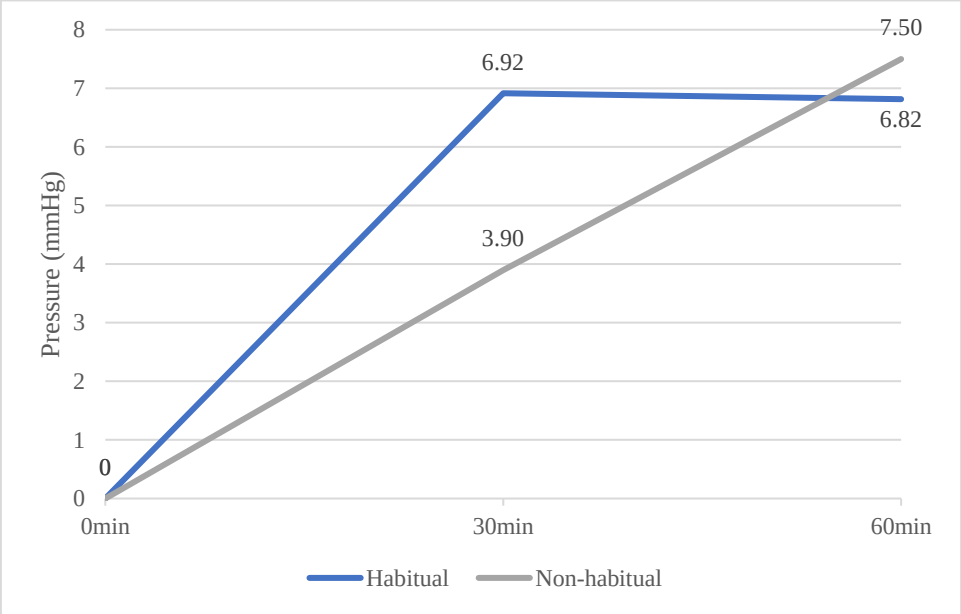

Figure 4.4: Changes in systolic blood pressure of habitual and non-habitual coffee drinkers at 0 min, 30 min, and 60 min.

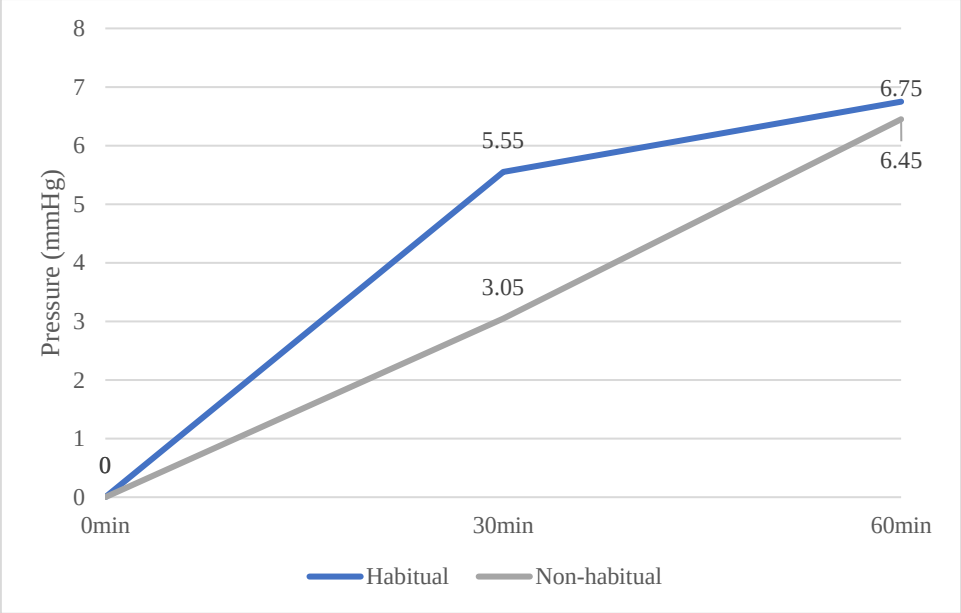

Figure 4.5: Changes in diastolic blood pressure after coffee treatment at 0 min, 30 min, and 60 min.

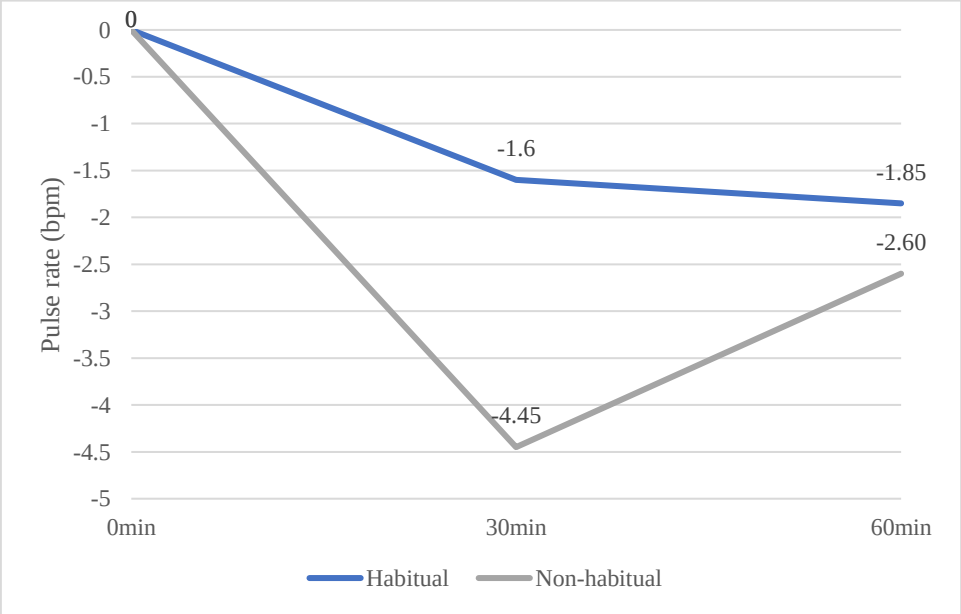

Figure 4.6: Changes in pulse rate after coffee treatment at 0 min, 30 min, and 60 min.

Table 4.7: The blood pressure and pulse rate measurements among habitual and non-habitual coffee drinkers pre-post coffee consumption.

Blood pressure and pulse rate measurements (Mean ± S.D.)			
	Habitual coffee drinkers	**Non-habitual coffee drinkers**	**p-value**
0 minutes			
SBP [1]	102.8 ± 11.3	102.3 ± 11.6	
DBP [2]	67.5 ± 8.1	66.8 ± 8.1	
Pulse rate	66.8 ± 10.7	70.2 ± 7.0	
30 minutes			
SBP [1]	109.8 ± 9.3	106.2 ± 15.6	0.496
DBP [2]	73.1 ± 6.2	69.9 ± 8.9	0.344
Pulse rate	65.2 ± 6.6	65.7 ± 6.2	0.677
60 minutes			
SBP [1]	109.7 ± 12.3	109.8 ± 16.1	0.762
DBP [2]	74.3 ± 6.9	73.3 ± 11.3	0.545
Pulse rate	64.9 ± 9.0	67.6 ± 9.4	0.450

*Significant difference is proven when p-value < 0.05
[1]Systolic blood pressure
[2]Diastolic blood pressure

However, when compared between the intervals within each group, it is found that habitual coffee drinkers showed a significant difference in the systolic and diastolic blood pressure before and after coffee treatment at post-30 minutes and post-60 minutes. Whereas for non-habitual coffee drinkers, there is a significant difference in diastolic blood pressure before and after treatment as well as difference between 30 and 60 minutes. Systolic blood pressure only showed significant difference 60 minutes after treatment. Both habitual and non-habitual coffee drinkers showed no significant difference in pulse rate before and after treatment with coffee. The findings of the statistical analysis were summarized in Table 4.8 and Table 4.9.

Table 4.8: Difference of blood pressure and pulse rate measurements between different time points among habitual coffee drinkers.

Habitual coffee drinkers (n = 10)		
Variable	**Mean difference**	**p-value**
SBP [1]:		
0 – 30 min [2]	7.39 ± 4.48	**0.008***
30 – 60 min [3]	-0.10 ± 6.16	0.889
0 – 60 min [4]	6.50 ± 7.28	**0.011***
DBP [5]:		
0 – 30 min [2]	5.83 ± 3.55	**0.008***
30 – 60 min [3]	1.20 ± 3.63	0.406
0 – 60 min [4]	7.17 ± 4.52	**0.011***
Pulse rate:		
0 – 30 min [2]	-2.03 ± 6.27	0.674
30 – 60 min [3]	-0.25 ± 5.18	0.386
0 – 60 min [4]	-0.81 ± 6.46	0.953

*Significant difference is proven when p-value < 0.05
[1]Systolic blood pressure
[2]Difference between 0 min and 30 min
[3]Difference between 30 min and 60 min
[4]Difference between 0 min and 60 min
[5]Diastolic blood pressure

Table 4.9: Difference of blood pressure and pulse rate measurements between different time points among non-habitual coffee drinkers.

Non-habitual coffee drinkers (n = 10)		
Variable	**Mean difference**	**p-value**
SBP [1]:		
0 – 30 min [2]	3.90 ± 7.37	0.074
30 – 60 min [3]	3.60 ± 6.54	0.085
0 – 60 min [4]	7.50 ± 7.46	**0.019***
DBP [5]:		
0 – 30 min [2]	3.05 ± 3.50	**0.016***
30 – 60 min [3]	3.40 ± 3.84	**0.019***
0 – 60 min [4]	6.45 ± 4.97	**0.007***
Pulse rate:		
0 – 30 min [2]	-4.45 ± 6.67	0.092
30 – 60 min [3]	1.85 ± 5.64	0.474
0 – 60 min [4]	-2.60 ± 8.84	0.358

*Significant difference is proven when p-value < 0.05
[1]Systolic blood pressure
[2]Difference between 0 min and 30 min
[3]Difference between 30 min and 60 min
[4]Difference between 0 min and 60 min
[5]Diastolic blood pressure

4.7.2 Expression of *MTHFR* Post-Coffee Treatment

In this study, the fold gene expression was compared with 0 min within each treatment group as shown in Figure 4.7. After treatment with coffee, it can be observed that the expression level of *MTHFR* among habitual coffee drinkers is downregulated at 30 min and 60 min. This is the opposite that is observed for the non-habitual coffee drinkers as they showed upregulation in *MTHFR* expression post-coffee treatment with a fold change of 1.17 at post-30 minutes and a fold change of 1.18 at post-60 minutes.

The statistical findings are summarized in Table 4.10 and it is found that there is no significant difference between habitual and non-habitual coffee drinkers after coffee treatment. Thus, the null hypothesis was failed to be rejected.

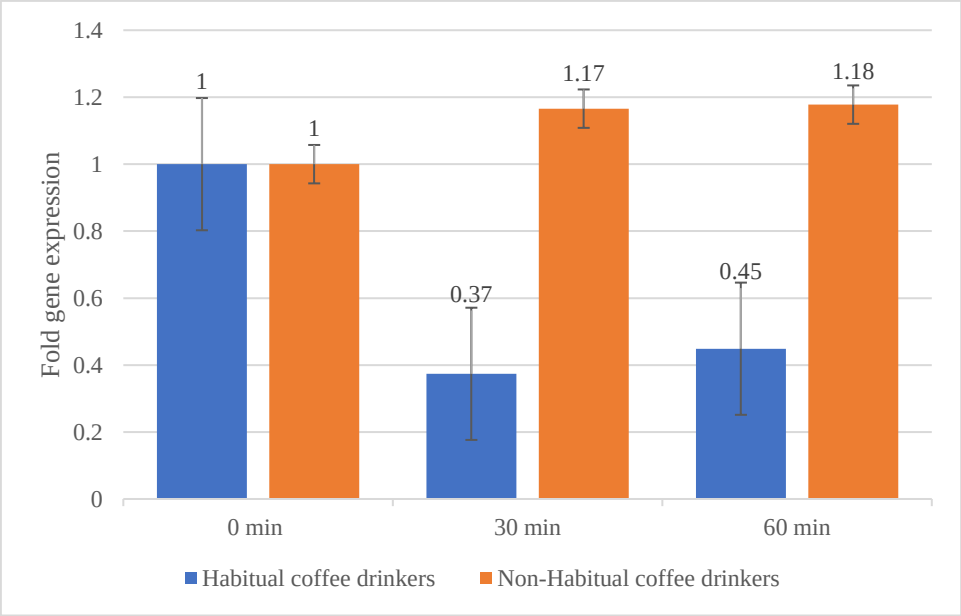

Figure 4.7: Fold gene expression of *MTHFR* gene expression of habitual coffee drinkers and non -habitual coffee drinkers.

Table 4.10: Gene expression of *MTHFR* among habitual and non-habitual coffee drinkers pre-post coffee consumption.

	Gene expression (Mean ± S.D.)		
	Habitual coffee drinkers	**Non-habitual coffee drinkers**	**p-value**
0 min:			
ΔCt	5.52 ± 1.66	6.00 ± 2.08	
ΔΔCt	0.00	0.00	
$2^{-(\Delta\Delta Ct)}$	1.00	1.00	
30 min:			
ΔCt	6.94 ± 2.72	5.78 ± 1.65	0.174
ΔΔCt	1.42	-0.22	
$2^{-(\Delta\Delta Ct)}$	0.37	1.17	
60 min:			
ΔCt	6.67 ± 3.28	5.76 ± 1.74	0.199
ΔΔCt	1.16	-0.24	
$2^{-(\Delta\Delta Ct)}$	0.45	1.18	

*Significant difference is proven when p-value < 0.05

There was also no significant difference in *MTHFR* expression at different timepoints for both habitual and non-habitual coffee drinkers (p > 0.05). The findings of the analysis were summarized in Table 4.11 and Table 4.12.

Table 4.11: Difference of *MTHFR* expression level between different time points among habitual coffee drinkers.

	Habitual coffee drinkers (n = 10)	
ΔCt	**Mean difference**	**p-value**
0 – 30 min [1]	1.29 ± 3.22	0.441
30 – 60 min [2]	0.94 ± 2.48	0.878
0 – 60 min [3]	-0.26 ± 3.61	0.110

*Significant difference is proven when p-value < 0.05
[1]Difference between 0 min and 30 min
[2]Difference between 30 min and 60 min
[3]Difference between 0 min and 60 min

Table 4.12: Difference of *MTHFR* expression level between different time points among non-habitual coffee drinkers.

Non-habitual coffee drinkers (n = 10)		
ΔCt	**Mean difference**	**p-value**
0 – 30 min [1]	-0.22 ± 2.97	0.646
30 – 60 min [2]	-0.24 ± 2.00	0.959
0 – 60 min [3]	-0.01 ± 1.65	0.878

*Significant difference is proven when p-value < 0.05
[1]Difference between 0 min and 30 min
[2]Difference between 30 min and 60 min
[3]Difference between 0 min and 60 min

4.7.3 Comparison Between Male and Female Habitual Coffee Drinkers and Male and Female Non-Habitual Coffee Drinkers

The association between habitual and non-habitual coffee drinkers were evaluated further by comparing between male and female habitual coffee drinkers as well as male and female non-habitual coffee drinkers. It was found that systolic blood pressure, diastolic blood pressure and *MTHFR* gene expression post-60 minutes showed significant difference and thus further test were done to compare between male and female within each group. The statistical findings are summarized in Table 4.13.

Table 4.13: Average blood pressure and pulse rate and ΔCt of habitual and non-habitual coffee drinkers (male and female) pre-post coffee consumption.

Variable	**Grouping (n = 20)**				**p-value**
	Habitual coffee drinkers		**Non-habitual coffee drinkers**		
	Male	**Female**	**Male**	**Female**	
0 min:					
SBP [1]	113.3 ± 7.2	94.5 ± 4.8	109.8 ± 8.0	94.7 ± 9.7	
DBP [2]	72.3 ± 3.3	63.7 ± 9.0	72.4 ± 7.5	61.2 ± 3.5	

Table 4.13: (continued)

Variable	Grouping (n = 20)				p-value
	Habitual coffee drinkers		Non-habitual coffee drinkers		
	Male	Female	Male	Female	
Pulse rate	66.4 ± 6.6	67.0 ± 14.0	71.8 ± 9.9	68.5 ± 2.5	
ΔCt	4.21 ± 1.22	6.57 ± 1.15	5.83 ± 2.24	6.17 ± 2.15	
30 min:					
SBP [1]	115.9 ± 7.2	103.6 ± 7.0	115.0 ± 13.0	97.3 ± 13.6	0.092
DBP [2]	75.6 ± 4.6	70.5 ± 7.0	74.9 ± 8.9	64.8 ± 5.9	0.057
Pulse rate	65.6 ± 2.8	64.8 ± 9.5	65.1 ± 7.2	66.3 ± 5.8	0.458
ΔCt	6.89 ± 3.18	6.99 ± 2.56	5.61 ± 2.03	5.95 ± 1.40	0.494
60 min:					
SBP [1]	119.8 ± 8.2	99.5 ± 3.8	118.9 ± 17.7	100.6 ± 7.6	**0.032***
DBP [2]	79.1 ± 3.6	69.4 ± 5.8	78.8 ± 13.3	67.7 ± 5.8	**0.016***
Pulse rate	66.3 ± 12.3	66.3 ± 12.3	69.3 ± 12.8	65.8 ± 5.3	0.709
ΔCt	4.83 ± 3.34	8.52 ± 2.12	5.07 ± 2.03	6.46 ± 1.24	**0.030***

*Significant difference is proven when p-value<0.05
[1]Systolic blood pressure
[2]Diastolic blood pressure

4.8 Comparison Between Male and Female

4.8.1 Blood Pressure and Pulse Rate Changes of Male and Female Habitual Coffee Drinkers

The changes in the blood pressure measurements of habitual coffee drinkers can be observed in Figure 4.8 and Figure 4.9. In the first 30 minutes, it can be observed both genders showed an increased in systolic and diastolic blood

pressure while there was a decreased in pulse rate. In the second 30 minutes, female drinkers showed a decrease in systolic and diastolic blood pressure while the pulse rate increased.

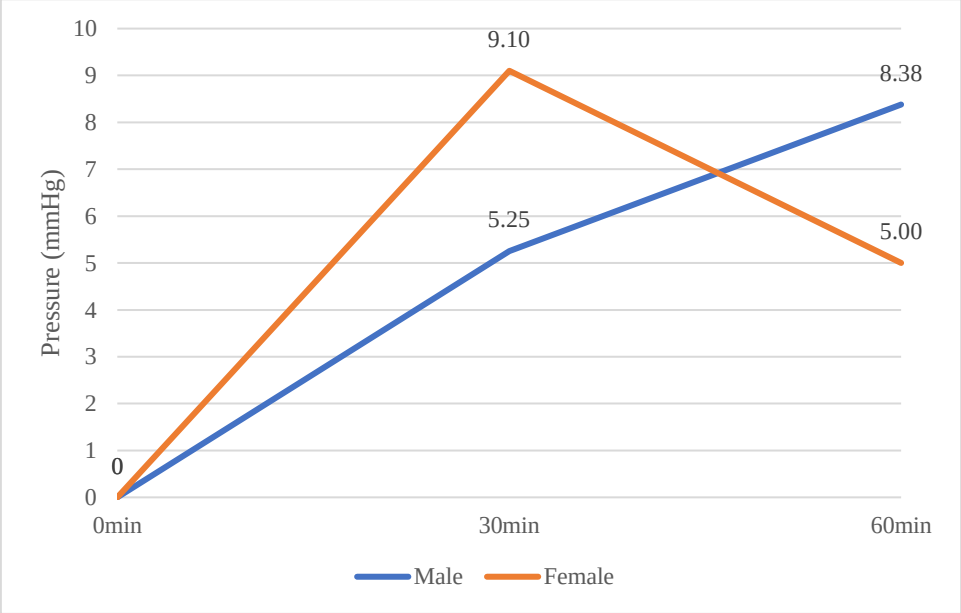

Figure 4.8: Changes in systolic blood pressure of habitual coffee drinkers at 0 min, 30 min, and 60 min (male and female).

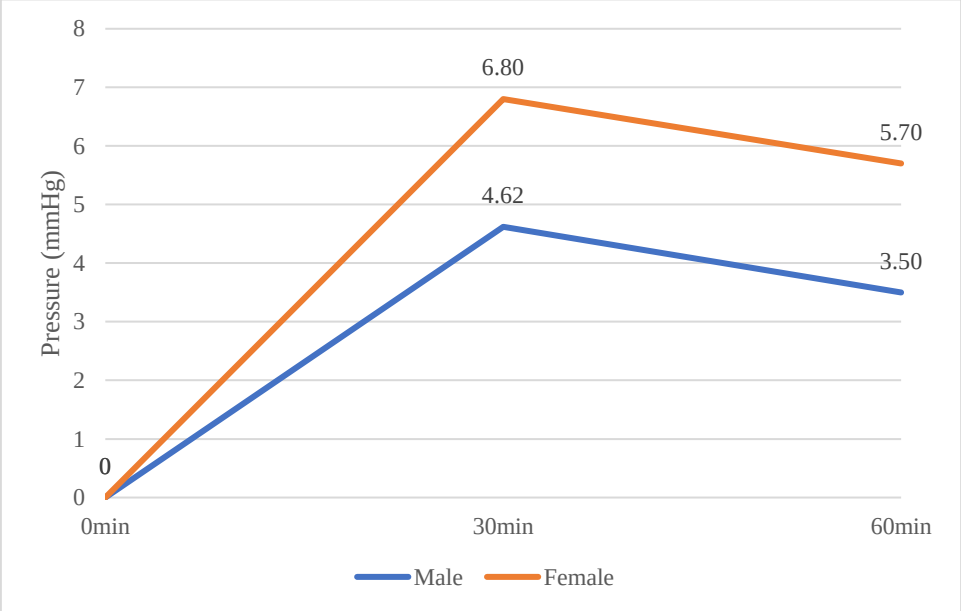

Figure 4.9: Changes in diastolic blood pressure of habitual coffee drinkers at 0 min, 30 min, and 60 min (male and female).

As for male habitual drinkers, the systolic blood pressure continued to rise at 60 min while diastolic blood pressure and pulse rate decreased at 60 min (Figure 4.10). Overall, female drinkers showed a greater change in blood pressure and pulse rate post coffee consumption than male drinkers.

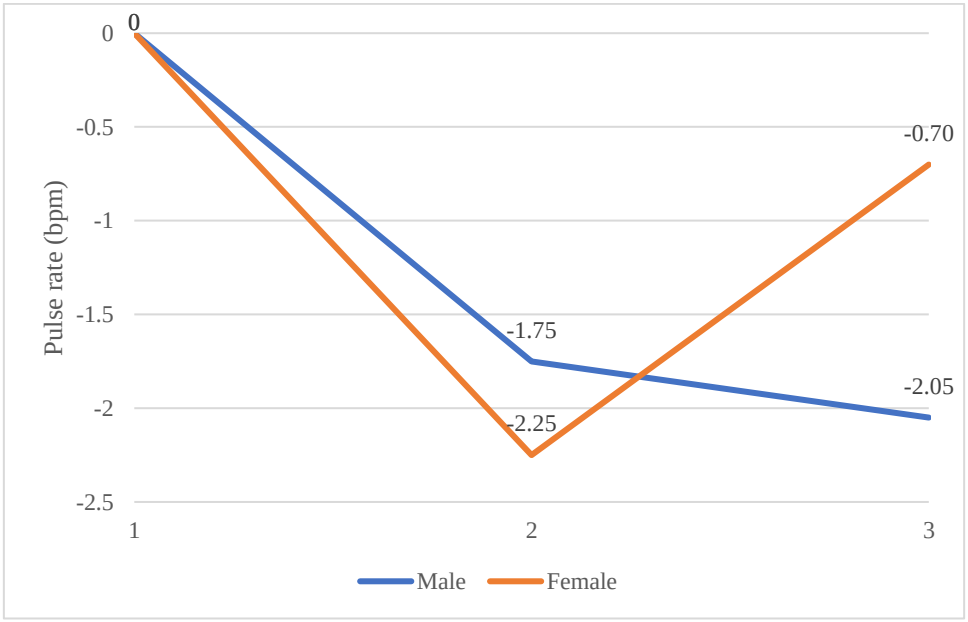

Figure 4.10: Changes in pulse rate of habitual coffee drinkers at 0 min, 30 min, and 60 min (male and female).

From the statistical analysis, it was found that there was a significant difference of the systolic blood pressure at post-30 and post-60 minutes between male and female drinkers while the diastolic was only significantly different post-60 minutes (Table 4.14).

Table 4.14: Changes in blood pressure and pulse rate of habitual coffee drinkers (male and female) pre-post coffee consumption

	Habitual coffee drinkers (n = 10)		
	Male	**Female**	**p-value**
0 min:			
SBP [1]	113.3 ± 7.2	94.5 ± 4.8	
DBP [2]	72.3 ± 3.3	63.7 ± 9.0	
Pulse rate	66.4 ± 6.6	67.0 ± 14.0	
30 min:			
SBP [1]	115.9 ± 7.2	103.6 ± 7.0	**0.028***
DBP [2]	75.6 ± 4.6	70.5 ± 7.0	0.209
Pulse rate	65.6 ± 2.8	64.8 ± 9.5	0.754
60 min:			
SBP [1]	119.8 ± 8.2	99.5 ± 3.8	**0.009***
DBP [2]	79.1 ± 3.6	69.4 ± 5.8	**0.016***
Pulse rate	66.3 ± 12.3	66.3 ± 12.3	0.917

*Significant difference is proven when p-value<0.05
[1]Systolic blood pressure
[2]Diastolic blood pressure

However, when compared between the intervals, there was no significant difference in blood pressure and pulse rate for male habitual coffee drinkers between different timepoints (Table 4.15). While for female habitual drinkers, there was significant difference in the systolic and diastolic blood pressure at 0 min and 30 min. The systolic blood pressure was also found to be significantly different between 0 min and 60 min (Table 4.16).

Table 4.15: Difference of blood pressure and pulse rate between different time points for male habitual coffee drinkers.

Male habitual coffee drinkers (n = 5)		
Variable	**Mean difference**	**p-value**
SBP [1]:		
0 – 30 min [2]	5.25 ± 4.97	0.066
30 – 60 min [3]	3.90 ± 5.64	0.144
0 – 60 min [4]	8.38 ± 11.15	0.109
DBP [5]:		
0 – 30 min [2]	4.62 ± 3.71	0.068
30 – 60 min [3]	6.75 ± 3.55	0.080
0 – 60 min [4]	3.50 ± 5.01	0.068
Pulse rate:		
0 – 30 min [2]	-1.75 ± 5.97	0.465
30 – 60 min [3]	-0.94 ± 5.49	0.686
0 – 60 min [4]	-2.05 ± 6.47	0.705

*Significant difference is proven when p-value < 0.05
[1]Systolic blood pressure
[2]Difference between 0 min and 30 min
[3]Difference between 30 min and 60 min
[4]Difference between 0 min and 60 min
[5]Diastolic blood pressure

Table 4.16: Difference of blood pressure and pulse rate between different time points for female habitual coffee drinkers

Female habitual coffee drinkers (n = 5)		
Variable	**Mean difference**	**p-value**
SBP [1]:		
0 – 30 min [2]	9.10 ± 3.65	**0.042***
30 – 60 min [3]	-4.10 ± 3.70	0.068
0 – 60 min [4]	5.00 ± 2.57	**0.042***
DBP [5]:		
0 – 30 min [2]	6.80 ± 3.51	**0.043***

Table 4.16: (continued)

Female habitual coffee drinkers (n = 5)		
Variable	**Mean difference**	**p-value**
30 – 60 min [3]	-1.10 ± 1.95	0.273
0 – 60 min [4]	5.70 ± 4.63	0.080
Pulse rate:		
0 – 30 min [2]	-2.25 ± 7.19	0.715
30 – 60 min [3]	1.55 ± 3.21	0.345
0 – 60 min [4]	-0.70 ± 7.80	0.686

*Significant difference is proven when p-value < 0.05
[1]Systolic blood pressure
[2]Difference between 0 min and 30 min
[3]Difference between 30 min and 60 min
[4]Difference between 0 min and 60 min
[5]Diastolic blood pressure

4.8.2 Blood Pressure and Pulse Rate Changes of Male and Female Non-Habitual Coffee Drinkers

For non-habitual coffee drinkers, the changes in blood pressure and pulse rate are shown in the figures below. From Figure 4.11 and Figure 4.12, it can be observed that the blood pressure of male and female non-habitual drinkers increases after coffee consumption. The pulse rate can be seen to decrease in male and female non-habitual coffee drinkers after coffee treatment (Figure 4.13). Generally, it can be observed that males showed greater changes in blood pressure and pulse rate as compared with females.

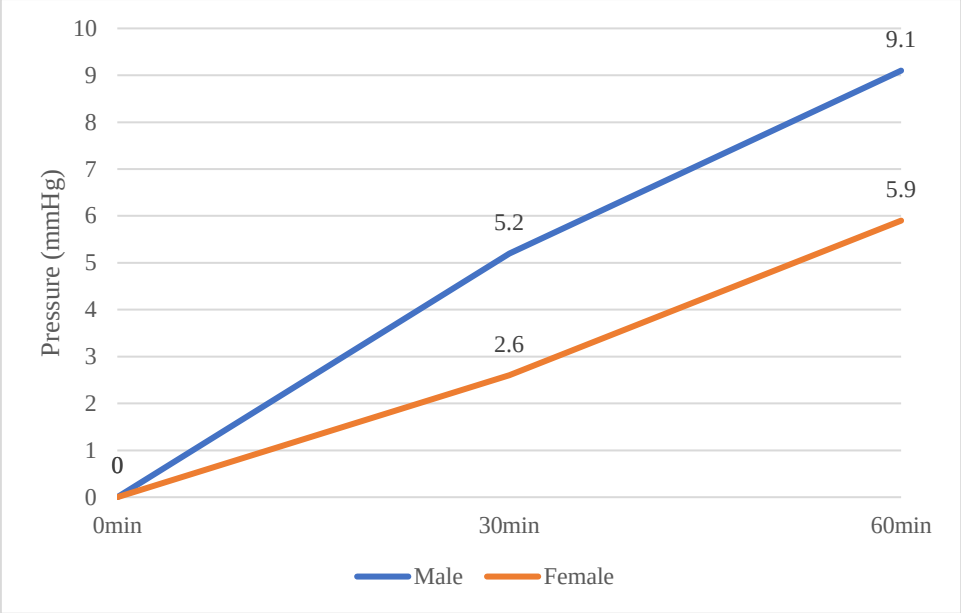

Figure 4.11: Changes in systolic blood pressure of non-habitual coffee drinkers at 0 min, 30 min, and 60 min (male and female).

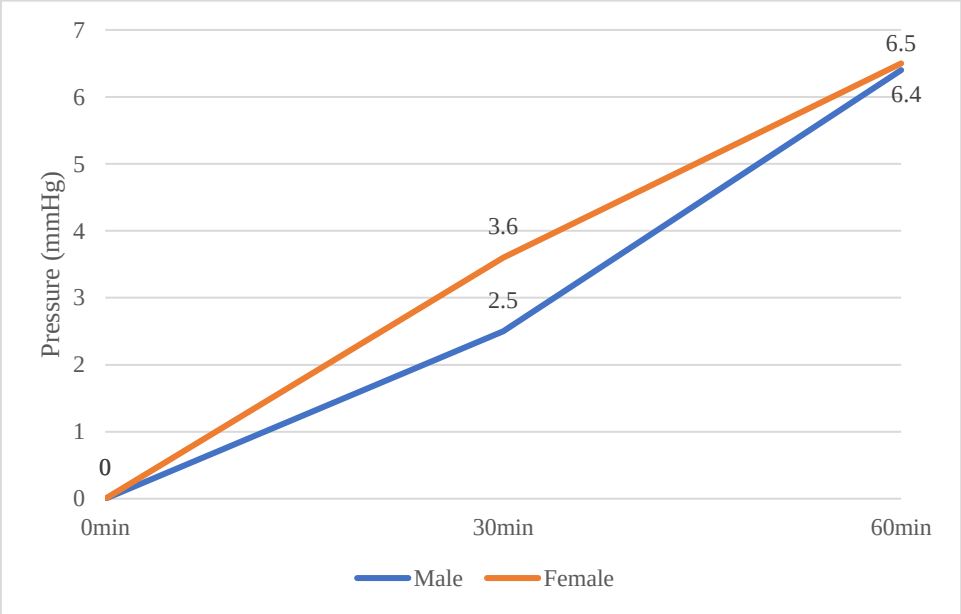

Figure 4.12: Changes in diastolic blood pressure of non-habitual coffee drinkers at 0 min, 30 min, and 60 min (male and female).

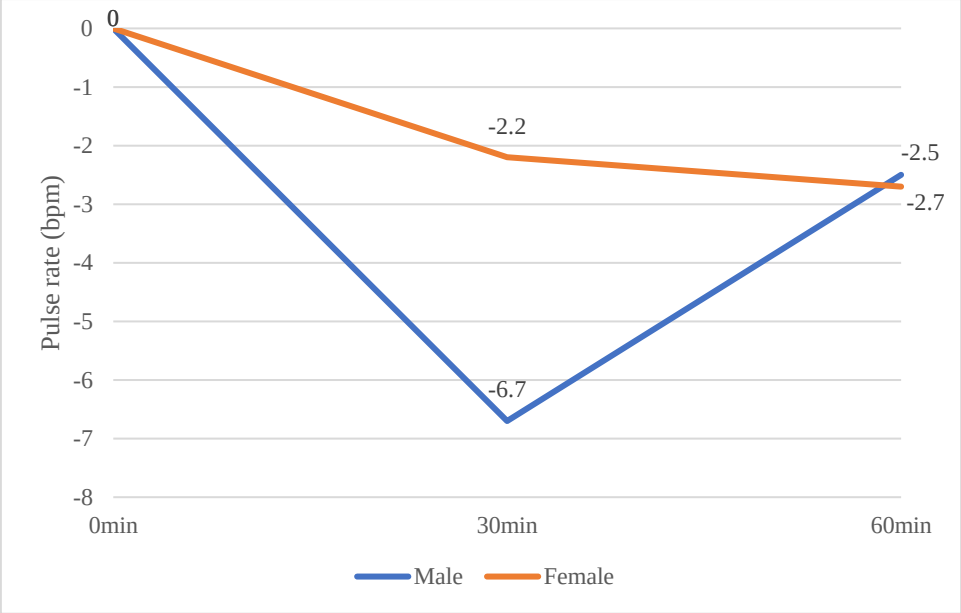

Figure 4.13: Changes in pulse rate of non-habitual coffee drinkers at 0 min, 30 min, and 60 min (male and female).

From the statistical analysis, it was found that there was no significant difference in the blood pressure and pulse rate between male and female non-habitual coffee drinkers and thus the null hypothesis was failed to be rejected (Table 4.17).

Table 4.17: The blood pressure and pulse rate of non-habitual coffee drinkers (male and female) pre-post coffee consumption

	Non-habitual coffee drinkers (n = 10)		
	Male	**Female**	**p-value**
0 min:			
SBP [1]	109.8 ± 8.0	94.7 ± 9.7	
DBP [2]	72.4 ± 7.5	61.2 ± 3.5	
Pulse rate	71.8 ± 9.9	68.5 ± 2.5	
30 min:			
SBP [1]	115.0 ± 13.0	97.3 ± 13.6	0.059

Table 4.17: (continued)

Non-habitual coffee drinkers (n = 10)			
	Male	**Female**	**p-value**
DBP [2]	74.9 ± 8.9	64.8 ± 5.9	0.075
Pulse rate	65.1 ± 7.2	66.3 ± 5.8	0.600
60 min:			
SBP [1]	118.9 ± 17.7	100.6 ± 7.6	0.116
DBP [2]	78.8 ± 13.3	67.7 ± 5.8	0.173
Pulse rate	69.3 ± 12.8	65.8 ± 5.3	0.465

*Significant difference is proven when p-value<0.05
[1]Systolic blood pressure
[2]Diastolic blood pressure

When compared between different time points, male non-habitual coffee drinkers showed a significant difference in pulse rate between 0 min and 30 min as seen in Table 4.18. While for female drinkers, there was a significant difference in the systolic blood pressure between 0 min and 60 min. While there was significant difference in the diastolic blood pressure between 30 min and 60 min as well as between 0 min and 60 min (Table 4.19).

Table 4.18: Difference of blood pressure and pulse rate between different time points for male non-habitual coffee drinkers.

Male non-habitual coffee drinkers (n = 5)		
Variable	**Mean difference**	**p-value**
SBP [1]:		
0 – 30 min [2]	5.20 ± 5.66	0.136
30 – 60 min [3]	3.90 ± 6.88	0.225
0 – 60 min [4]	9.10 ± 10.33	0.078
DBP [5]:		

Table 4.18: (continued)

Male non-habitual coffee drinkers (n = 5)		
Variable	**Mean difference**	**p-value**
0 – 30 min [2]	2.50 ± 3.02	0.104
30 – 60 min [3]	3.90 ± 5.56	0.225
0 – 60 min [4]	6.40 ± 6.22	0.080
Pulse rate:		
0 – 30 min [2]	-6.70 ± 5.76	**0.042***
30 – 60 min [3]	-2.50 ± 11.34	0.104
0 – 60 min [4]	-4.20 ± 7.24	0.498

*Significant difference is proven when p-value < 0.05
[1]Systolic blood pressure
[2]Difference between 0 min and 30 min
[3]Difference between 30 min and 60 min
[4]Difference between 0 min and 60 min
[5]Diastolic blood pressure

Table 4.19: Difference of blood pressure and pulse rate between different time points for female non-habitual coffee drinkers.

Female non-habitual coffee drinkers (n = 5)		
Variable	**Mean difference**	**p-value**
SBP [1]:		
0 – 30 min [2]	2.60 ± 5.35	0.465
30 – 60 min [3]	3.30 ± 6.97	0.197
0 – 60 min [4]	5.90 ± 3.51	**0.042***
DBP [5]:		
0 – 30 min [2]	3.60 ± 4.20	0.080
30 – 60 min [3]	2.90 ± 1.24	**0.042***
0 – 60 min [4]	6.50 ± 4.12	**0.043***
Pulse rate:		
0 – 30 min [2]	-2.20 ± 7.37	0.686
30 – 60 min [3]	-0.50 ± 2.32	0.586
0 – 60 min [4]	-2.70 ± 6.87	0.686

*Significant difference is proven when p-value < 0.05
[1]Systolic blood pressure
[2]Difference between 0 min and 30 min
[3]Difference between 30 min and 60 min
[4]Difference between 0 min and 60 min
[5]Diastolic blood pressure

4.8.3 *MTHFR* Expression of Male and Female Habitual Coffee Drinkers

The fold gene expression of male and female habitual coffee drinkers is depicted in Figure 4.14. The expression of *MTHFR* gene is seen to be downregulated by 0.16-fold in 30 minutes after coffee consumption in male drinkers while it was downregulated with a 75% expression relative to before consumption in the female drinkers. However, by 60 minutes, the *MTHFR* expression in male habitual drinkers showed an increase with a 65% expression relative to the basepoint. The expression level in female drinkers is shown to be lowered at 60 minutes with a 26% expression relative to before consumption.

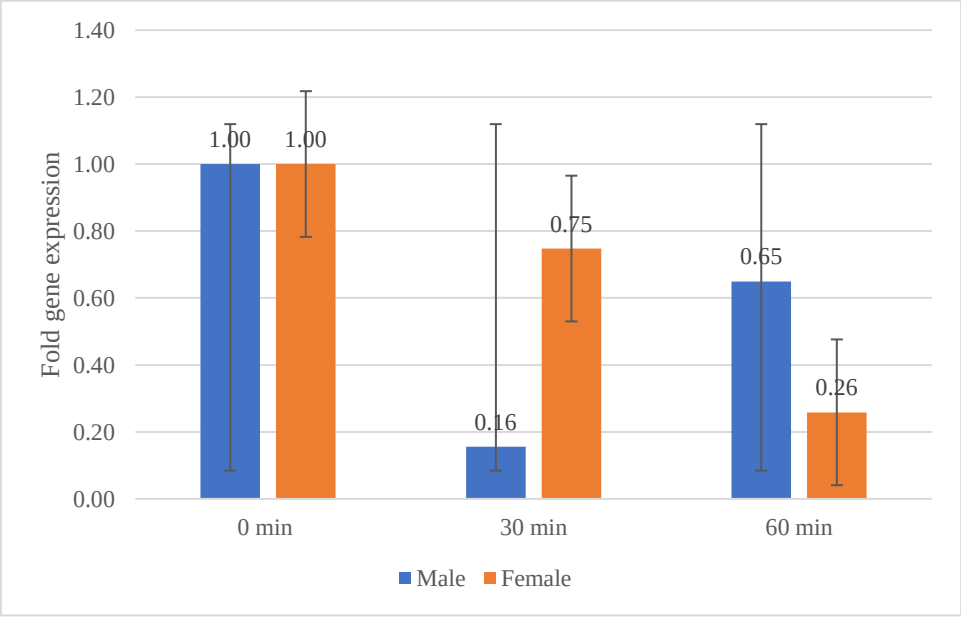

Figure 4.14: Fold gene expression of *MTHFR* among male and female habitual coffee drinkers at 0 min, 30 min, and 60 min.

However, from the statistical analysis, there was no significant difference in *MTHFR* gene expression between male and female habitual coffee drinkers pre-post coffee treatment (Table 4.20).

Table 4.20: Gene expression of *MTHFR* of habitual coffee drinkers (male and female) pre-post coffee consumption.

	Habitual coffee drinkers (n = 10)		
Variable	**Male**	**Female**	**p-value**
0 min:			
ΔCt	4.21 ± 1.22	6.57 ± 1.15	
ΔΔCt	0.00	0.00	
$2^{-(\Delta\Delta Ct)}$	1.00	1.00	
30 min:			
ΔCt	6.89 ± 3.18	6.99 ± 2.56	0.754
ΔΔCt	2.68	0.42	
$2^{-(\Delta\Delta Ct)}$	0.16	0.75	
60 min:			
ΔCt	4.83 ± 3.34	8.52 ± 2.12	0.117
ΔΔCt	0.62	1.95	
$2^{-(\Delta\Delta Ct)}$	0.65	0.26	

*Significant difference is proven when p-value < 0.05

When compared between different timepoints, male showed no significant difference between the intervals (Table 4.21) and only female habitual coffee drinkers showed a significant difference in *MTHFR* gene expression between 0 min and 60 min (Table 4.22).

Table 4.21: Difference of *MTHFR* expression level between different time points for male habitual coffee drinkers

Male habitual coffee drinkers (n = 5)		
ΔCt	**Mean difference**	**p-value**
0 – 30 min [1]	2.38 ± 3.25	0.273
30 – 60 min [2]	-2.06 ± 3.23	0.345
0 – 60 min [3]	-0.33 ± 2.93	0.715

*Significant difference is proven when p-value < 0.05
[1]Difference between 0 min and 30 min
[2]Difference between 30 min and 60 min
[3]Difference between 0 min and 60 min

Table 4.22: Difference of *MTHFR* expression level between different time points for female habitual coffee drinkers

Female habitual coffee drinkers (n = 5)		
ΔCt	**Mean difference**	**p-value**
0 – 30 min [1]	0.42 ± 3.27	0.893
30 – 60 min [2]	1.53 ± 3.28	0.225
0 – 60 min [3]	1.95 ± 1.72	**0.043***

*Significant difference is proven when p-value < 0.05
[1]Difference between 0 min and 30 min
[2]Difference between 30 min and 60 min
[3]Difference between 0 min and 60 min

4.8.4 *MTHFR* Expression of Male and Female Non-Habitual Coffee Drinkers

While the fold gene expression of male and female non-habitual coffee drinkers is depicted in Figure 4.15. The expression of *MTHFR* gene is seen to be upregulated in 30 minutes after coffee consumption in male and female drinkers. However, by 60 minutes, the *MTHFR* expression in male habitual drinkers was higher with a 69% expression relative to the basepoint. The expression level in female drinkers is shown to be lowered at 60 minutes with an 82% expression relative to before consumption.

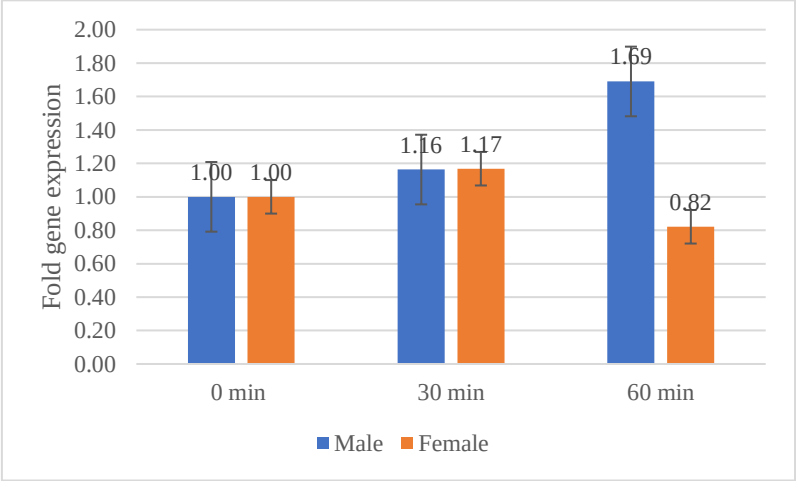

Figure 4.15: Fold gene expression of *MTHFR* among male and female non-habitual coffee drinkers.

From the statistical analysis, it was found that there was no significant difference in *MTHFR* gene expression between male and female non-habitual coffee drinkers pre-post coffee treatment (Table 4.23).

Table 4.23: Gene expression of *MTHFR* non-habitual coffee drinkers (male and female) pre-post coffee consumption.

Non-habitual coffee drinkers (n = 10)			
Variable	**Male**	**Female**	**p-value**
0 min:			
ΔCt	5.83 ± 2.24	6.17 ± 2.15	
ΔΔCt	0.00	0.00	
$2^{-(\Delta\Delta Ct)}$	1.00	1.00	
30 min:			
ΔCt	5.61 ± 2.03	5.95 ± 1.40	0.917
ΔΔCt	-0.22	-0.22	
$2^{-(\Delta\Delta Ct)}$	1.16	1.17	
60 min:			
ΔCt	5.07 ± 2.03	6.46 ± 1.24	0.175
ΔΔCt	-0.76	0.28	
$2^{-(\Delta\Delta Ct)}$	1.69	0.82	

*Significant difference is proven when p-value < 0.05

When comparing the *MTHFR* expression between different timepoints, both male and female non-habitual coffee drinkers showed no significant difference. The statistical findings are summarized in Table 4.24 and Table 4.25.

Table 4.24: Difference of *MTHFR* expression level between different time points for male non-habitual coffee drinkers

Male non-habitual coffee drinkers (n = 5)		
ΔCt	**Mean difference**	**p-value**
0 – 30 min [1]	-0.22 ± 3.00	0.686
30 – 60 min [2]	-0.54 ± 1.42	0.500
0 – 60 min [3]	-0.76 ± 2.19	0.500

*Significant difference is proven when p-value < 0.05

[1]Difference between 0 min and 30 min
[2]Difference between 30 min and 60 min
[3]Difference between 0 min and 60 min

Table 4.25: Difference of *MTHFR* expression level between different time points for female non-habitual coffee drinkers

Female non-habitual coffee drinkers (n = 5)		
ΔCt	**Mean difference**	**p-value**
0 – 30 min [1]	-0.22 ± 3.30	0.893
30 – 60 min [2]	0.51 ± 1.86	0.500
0 – 60 min [3]	0.28 ± 1.87	0.686

*Significant difference is proven when p-value < 0.05
[1]Difference between 0 min and 30 min
[2]Difference between 30 min and 60 min
[3]Difference between 0 min and 60 min

4.9 Overall Changes of Blood Pressure, Pulse Rate and *MTHFR* Expression Post-Coffee Treatment

Figure 4.16 illustrates the overall changes after consumption of coffee that contains 3.52% caffeine. Generally, it can be observed that coffee consumption would cause an increase in systolic and diastolic blood pressure in both habitual and non-habitual coffee drinkers regardless of gender. Whereas pulse rate would be lowered after coffee consumption in both groups as well. Coffee consumption can also cause an acute effect on *MTHFR* expression as generally *MTHFR* expression is shown to be downregulated in habitual coffee drinkers while it is upregulated in non-habitual coffee drinkers, regardless of gender.

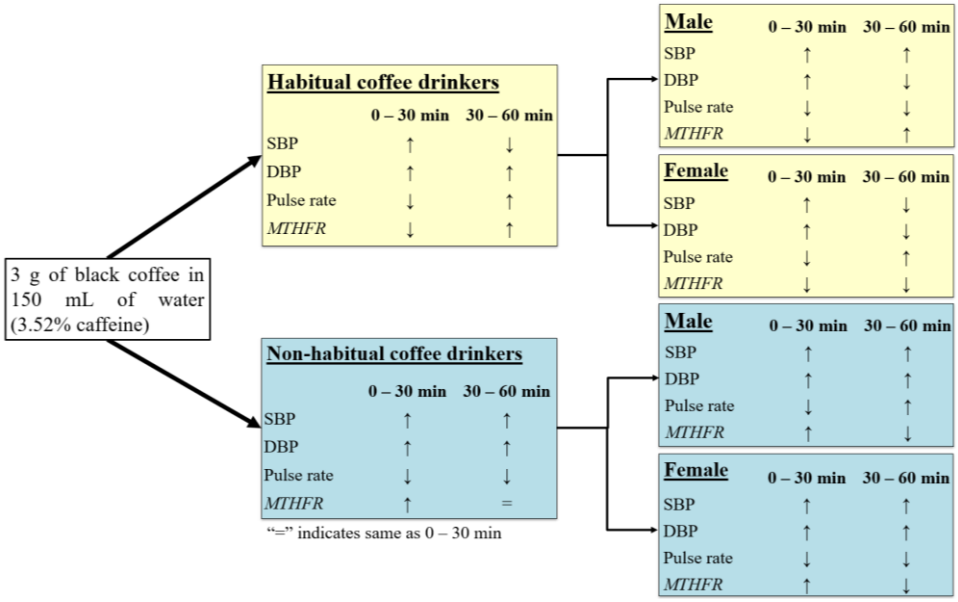

Figure 4.16: The overall changes from 0 min to 30 min and 30 min to 60 min of systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse rate and MTHFR expression pre-post coffee consumption.

CHAPTER 5

DISCUSSION

5.1 Coffee Consumption Status of UTAR Students

According to the findings of coffee consumption frequencies from the cross-sectional survey, the distribution of habitual coffee drinkers in UTAR was approximately 19% while majority (81%) were non-habitual coffee drinkers. On top of that, the habitual coffee drinkers were mostly male. The choice of coffee type of the coffee drinkers was mostly due to their personal preference and taste. Convenience was also a factor they considered when choosing the used most coffee type as the most used coffee type was instant coffee, followed by filter coffee. Furthermore, the possible reason of coffee consumption among the coffee drinkers was the taste of coffee and the energy boost that coffee gives them, helping them to stay awake.

In terms of the prevalence of habitual coffee drinkers, the observation of this study was contradictory to a study by Lone, et al. (2023), among Arabian young adults, found that more than half of the targeted population consumed coffee. Whereas in another study among university students in Bosnia and Herzegovina, it was demonstrated that there was a prevalence of 71.2% (N = 673) coffee consumption among the students (Serdarevic, et al., 2019). While in a study by Riera-Sampol, et al. (2023), it was reported that coffee was the main source of caffeine for 42.7% of the participants.

The contrasting results in prevalence of coffee consumption in this study with other studies could be due to the difference in the ethnicity of the studied population. Ethnicity can play a part in individual perception and sensitivity to bitter taste. In a study by Wang, et al. (2007), it was found that Europeans had a milder sensory sensitivity toward bitter taste as compared with African Americans. Furthermore, Yang, et al. (2020) reported that Chinese were typically found to be more sensitive to bitter taste than Caucasian. The heighten sensitivity of individuals to bitterness may lead to avoidance of certain food and beverages like coffee, which may be perceived as extremely bitter to them (Williams, et al., 2016).

In a cross-sectional study by Chan and Teoh (2021) conducted in Perak, Malaysia, the prevalence of coffee consumption among first-year medical students was more than half. While Annuar, et al., 2023 reported that the prevalence of habitual coffee drinkers among undergraduate pharmacy students was 49.6%. Difference in results may be due to the difference in targeted population as health-science students were highly inclined to consume more coffee to stay awake in order to cope with the higher workload of study (Handino, et al., 2019; Chan and Teoh, 2021; Annuar, et al., 2023).

The purpose of coffee consumption among university students in this study was in similar with the findings by Mahoney, et al. (2019) that reported the main reasons of coffee intake among American university students were to feel more awake and alert, and to enjoy the taste. Furthermore, university students would

normally consume coffee when there is higher academic stress (Kassaw, et al., 2024).

5.1.1 Gender Difference in Coffee Intake

Among the students in UTAR Kampar, the prevalence of habitual coffee consumption was significantly higher in males (30%) than in females (12%). This was in line with the findings reported by Lone, et al. (2023) who reported that there was significantly higher frequency of coffee consumption in male participants (65.04%). In addition to that, a study by Jahrami, et al. (2020) also found than male university students would consume higher amount of coffee as compared to females. While in a cross-sectional study among undergraduate students in a university in Spain, Riera-Sampol, et al. (2022) reported that higher percentage of females consumed coffee while males mainly preferred energy drinks. Demura, et al. (2013) reported that females showed lower coffee consumption than males, and that majority of females did not enjoy the taste of coffee.

A possible reason for higher frequency in coffee intake among males could be due to physiological make up as females were more sensitivity to bitter taste than males (Tepper, et al., 2017). Multiple studies have found that women were reported to have higher bitter taste sensitivity which can influence the preference and frequency of coffee intake of females (Chadwick, et al., 2016; Cavallo, et al., 2019).

5.2 Changes of Blood Pressure and Pulse Rate Post Coffee Treatment

5.2.1 Changes of Blood Pressure and Pulse Rate Measurements Between Habitual and Non-Habitual Coffee Drinkers

In this study, the systolic and diastolic blood pressure showed an increase in both habitual and non-habitual, while the pulse rate showed a decrease post-coffee consumption. It was observed that habitual coffee drinkers showed a greater acute change in blood pressure while non-habitual coffee drinkers showed a greater acute change in pulse rate. However, the difference in changes of blood pressure and pulse rate post coffee consumption between habitual and non-habitual coffee drinkers were not statistically different. When comparing the changes of blood pressure between the intervals within each group, it is found that coffee treatment caused an acute effect on systolic blood pressure for habitual drinkers than in non-habitual drinkers. In terms of diastolic blood pressure, the effect of coffee was acute for both groups and its effect lasted till 60 minutes post treatment. However, even when compared within each group, the pulse rate showed no significant difference between intervals.

Thus, it can be said that coffee consumption would cause a significant increase in blood pressure for both habitual and non-habitual coffee drinkers but the acute effect would appear greater in habitual coffee drinkers. This was contradictory to the findings in a randomized crossover study, in which coffee was found to increase the systolic blood pressure of habitual drinkers while it was decreased for non-habitual drinkers (Zimmermann-Viehoff, et al., 2015).

In another study, it is found that coffee consumption only increased blood pressure in non-habitual drinkers and not in habitual drinkers (Corti, et al., 2002).

The possible causes of this contradictory result in this study with other studies could be due to the effect of other bioactive compounds in coffee that can counterbalance the pressor effect of caffeine, leading to a neutral-to-positive effect (Van Dam, et al. 2020). Coffee contains high amounts of bioactive compounds such as chlorogenic acid and quercetin. Zhao, et al. (2011) had reported that there was a decrease in blood pressure in a dose-dependent manner after consumption of coffee with high amount of chlorogenic acid. Some studies have also reported that chlorogenic acid can decrease blood pressure due to its antioxidant and anti-inflammatory activities (Zhao, et al., 2011; Kajikawa, et al., 2019). Moreover, the amount caffeine in the black coffee used in the coffee test of this study was considerably low (3.52%).

Another possible cause for such contradictory results could be due to individual genetic variation in caffeine metabolizing genes such as *CYP1A2*. CYP1A2 enzyme is one of the main enzymes involved in the metabolism of caffeine. The polymorphism in the *CYP1A2* gene can affect individual caffeine metabolism, leading to classification of “fast metabolizers” and “slow metabolizers”. Individuals with a different variant the *CYP1A2* gene would lead to difference rate of caffeine metabolism and cardiovascular changes in response to acute caffeine ingestion (Soares, et al., 2018; Van Dam, et al., 2020). Furthermore, in a randomized controlled trial study by Hara, et al. (2014), it was reported that

the acute pressor effect of coffee was more significant in non-habitual coffee drinkers as the effect of coffee was blunted in habitual drinkers due to tolerance to caffeine. However, Farag, et al. (2005) had reported that genetic factors, such as polymorphism of caffeine metabolizing genes, may play a role in the development of tolerance to caffeine among habitual coffee drinkers.

In terms of the pulse rate changes, it can be said that coffee consumption would cause an acute decrease in pulse rate regardless of individual coffee consumption frequency. This was similar with the findings by Corti, et al. (2002) that reported that coffee would cause a decrease in heart rate but had no significant difference between habitual and non-habitual coffee drinkers. While in a double-blinded, placebo-controlled trial by Ketelhut, et al. (2022), it was found that caffeine ingestion would cause an acute decrease in heart rate among normotensive individuals. Mikalsen, et al. (2001) suggested that the decrease in heart rate may be due to the increased blood pressure post-coffee consumption in order to counteract the pressor effect of caffeine.

5.2.2 Comparison in The Changes of Blood Pressure and Pulse Rate Measurements Between Male and Female

For habitual coffee drinkers, there was a significant difference in systolic and diastolic blood pressure between male and female. In this study, female habitual drinkers showed a greater and significant change in the blood pressure and pulse rate measurements than male drinkers. While for non-habitual coffee drinkers, males showed a greater change in blood pressure and pulse rate but it was not

statistically significant. While female non-habitual coffee drinkers showed significant difference in blood pressure and pulse rate pre-post coffee consumption.

Therefore, this suggested that the change in blood pressure and pulse rate was more significant in females regardless of their habitual coffee consumption frequency. This was similar to the findings by Temple and Ziegler (2011) where they carried out a double-blind, placebo-controlled trial on adolescents. They found that females showed a greater diastolic blood pressure response after caffeine ingestion while systolic blood pressure was not significantly different between male and female. Furthermore, Lassen, et al. (2022) reported that after caffeine administration, women had a greater change in the systolic and diastolic blood pressure as compared to man. It is found that male and female have significantly different systolic and diastolic blood pressure and usually males have a higher blood pressure (Alhawari, et al., 2018; Sumiya, et al., 2019). Thus, it is suggested that lower resting blood pressure in females may have caused the changes in blood pressure due to coffee consumption to be greater and more obvious than in males.

Furthermore, males metabolize caffeine faster than females as the activity of CYP1A2 has been found to be higher in males (Rasmussen, et al., 2002; Kurokawa, et al., 2023). Thus, this suggests that the difference in metabolism rate of caffeine between male and female may have caused the difference in the changes in blood pressure changes of male and female drinkers.

Pulse rate showed no difference between genders in this study and this was in line with the findings by Kurokawa, et al. (2023) who reported there was no sex difference in the acute effect of caffeine on pulse rate. While Temple and Ziegler (2011) also reported that males showed a greater decrease in heart rate than females post-caffeine administration, and this change in heart rate is similar with the findings of this study were males showed a significant difference in heart rate pre-post coffee consumption.

5.3 Changes of *MTHFR* Expression Post Coffee Treatment Between Habitual and Non-Habitual Coffee Drinkers

The expression of *MTHFR* is observed to be downregulated among the habitual coffee drinkers while the *MTHFR* expression was upregulated in non-habitual coffee drinkers. However, there was no significant difference between habitual and non-habitual coffee drinkers in *MTHFR* expression post coffee treatment. This could be due to the high standard deviation that is observed as the *MTHFR* expression of each individual varied. The difference in *MTHFR* expression could be due to many factors such as genetic variation and genetic polymorphism.

The two common gene variants associated with *MTHFR* are C677T and A1298C. Individuals with *MTHFR* 677TT genotype typically have 70% lower activity of MTHFR enzyme (Frosst, et al., 1995). While in terms of the *MTHFR* A1298C polymorphism, individuals with the CC genotype have lower MTHFR enzyme activity than normal (Moll and Varga, 2015; Li, et al., 2019). It was

also found that individuals with *MTHFR* C677T polymorphism would have decreased *MTHFR* expression as compared with individuals with wildtype (Odin, et al., 2006). Furthermore, in a study by Strandhagen, et al. (2004), the impact of coffee on homocysteine, which is a molecule that would show high levels when there is a decreased in *MTHFR*, was mostly affecting individuals with the *MTHFR* 677TT genotype. Thus, it is possible the variation of *MTHFR* expression of each participant of this study was due to their *MTHFR* genotype.

5.4 Impact of Coffee Consumption on *MTHFR* Expression

Generally, it can be said that coffee consumption would cause an acute downregulation in *MTHFR* expression in habitual coffee drinkers. While it would cause an upregulation in *MTHFR* expression in non-habitual coffee drinkers. Coffee is not only known for its immediate stimulating effects but also for its components and their impact on human health (de Melo Pereira, et al., 2020).

Emerging studies have highlighted on the epigenetic effects of coffee consumption. Coffee can impact the epigenome by modulating DNA methylation, histone modification and ncRNA expression. Studies have shed light on the association of coffee consumption and DNA methylation on several genes in humans including caffeine metabolizing genes such as *CYP1A2* and Aryl Hydrocarbon Receptor (*AHR*) (Chuang, et al., 2017; Karabegović, et al., 2021). These studies have demonstrated how environmental factors like lifestyle and diet can impact gene activity without causing change in the

underlying DNA sequence. Thus, this suggest that long-term habitual coffee consumption may have a negative impact on *MTHFR* expression.

These long-term effects of coffee may lead to abnormal expression of *MTHFR*, causing adverse effects on human health. The abnormal *MTHFR* expression may impact the level of MTHFR enzyme, leading to deficiency in the enzyme. When people have deficiency of MTHFR enzyme, the level of folate will decrease while the level of plasma homocysteine will increase, leading to hyperhomocsyteinemia (Frosst, et al., 1995). Studies have reported the association of elevated homocysteine in plasma, decreased plasma folate levels, and the increased risk of cardiovascular complications (Kluijtmans, et al., 1996; McNulty, et al, 2017). In a study by Yang, et al. (2017), it was found that increased level of homocysteine was highly associated with elevated blood pressure level. Moreover, lowered *MTHFR* expression has been consistently associated with health conditions such as cancer development, increased risk of cardiovascular diseases and higher risk for stroke (Odin, et al., 2006; Yang, et al., 2017; Eldeeb, et al., 2022; Bennett, et al., 2023).

Epidemiological studies have also demonstrated that individuals with *MTHFR* 677TT genotype, which has lowered *MTHFR* expression, were at increased risk of developing one or more metabolic conditions such as obesity and high blood pressure that are components of metabolic syndrome, increasing their risk of developing cardiovascular diseases (Ward, et al., 2011; Zhi, et al., 2016; Wang, et al., 2018).

5.5 Limitations of Study

The current study had utilized a small sample size due to financial constraint thus normalization for the highly varied *MTHFR* expression could not be done. Furthermore, due to time constraint, a clinical study was unable to be carried and thus the long-term changes of blood pressure, pulse rate and *MTHFR* expression and the possible interventions could not be evaluated. The relationship between *MTHFR* expression, coffee consumption, blood pressure and pulse rate can vary from individual to individual due to genetic factors and dietary pattern. And thus, certain collected data might not have followed a specific distribution and so non-parametric methods were used in the analysis.

Furthermore, there was a lack of categorizing participants by their MTHFR polymorphism prior to quantification of *MTHFR* expression and thus variation in the *MTHFR* expression of each individual could have been due to the genetic polymorphism of *MTHFR*. In addition, there was also a lack of genotyping and grouping of participants by their rate of caffeine metabolism as genetic variation in caffeine-metabolizing genes among individuals could have caused variation in the response in each individual's blood pressure and pulse rate.

5.6 Recommendation in future studies

In future studies, a larger sample size could be used to increase the reliability of the results. A pre-clinical study using mouse model can also be carried out to determine the long-term changes of blood pressure, pulse rate and *MTHFR* expression, allowing the study on the possible positive or negative impact of

coffee consumption in the respective genotype. The pre-clinical study using mouse model can also be conducted by administration of varying concentration of caffeine in the coffee in order to examine the impact of coffee with different caffeine amount. Moreover, genotyping and grouping of participants either by their *MTHFR* polymorphism or rate of caffeine metabolism could be done before quantification of *MTHFR* expression to further understand the association of coffee, blood pressure, heart rate and *MTHFR* expression.

CHAPTER 6

CONCLUSION

In this present study, the prevalence of habitual and non-habitual coffee drinkers among UTAR students was explored, which was 19% and 81% respectively. Habitual coffee drinkers were the minority in the studied population and most of them were males.

The acute changes of blood pressure, pulse rate and *MTHFR* expression after coffee consumption was evaluated. There is a significant change in systolic and diastolic blood pressure pre-post coffee consumption in habitual ($p = 0.011$, $p = 0.001$) and non-habitual coffee drinkers ($p = 0.019$, $p = 0.007$), but the acute changes would appear to be greater in habitual coffee drinkers. Whereas for change in pulse rate and *MTHFR* expression, there was no significant difference in pre-post coffee consumption for the two groups. However, there is no significant difference in blood pressure, pulse rate and *MTHFR* expression between the two groups ($p > 0.05$).

When compared between gender, male and female habitual coffee drinkers showed significant difference in systolic ($p = 0.009$) and diastolic ($p = 0.016$) blood pressure while male and female non-habitual coffee drinkers did not ($p > 0.05$). Female coffee drinkers had a more significant change in systolic and diastolic blood pressure pre-post coffee consumption, regardless of their coffee

intake frequency. While the changes in *MTHFR* expression was not significantly different between male and female for both habitual and non-habitual coffee drinkers ($p > 0.05$).

Generally, coffee consumption has an impact on blood pressure, heart rate and *MTHFR* expression. This sheds light on the immediate physiological responses to coffee intake. Long term effect of coffee on blood pressure, pulse rate and *MTHFR* expression may lead to abnormal levels in a person and cause adverse effect to their health. This brings insight on how these effects can determine the impact of coffee on cardiovascular health as well as how genetic factors may affect how each person reacts to coffee consumption. This study can be shared to the public, allowing guidelines for coffee consumption to be developed, contributing to individual dietary recommendations.

In future study, larger sample size should be investigated for higher reliability. Furthermore, the genotyping and classification of individuals by their polymorphism should be done prior to quantification of expression, allowing better understanding on the changes that coffee can bring towards genetic expression.

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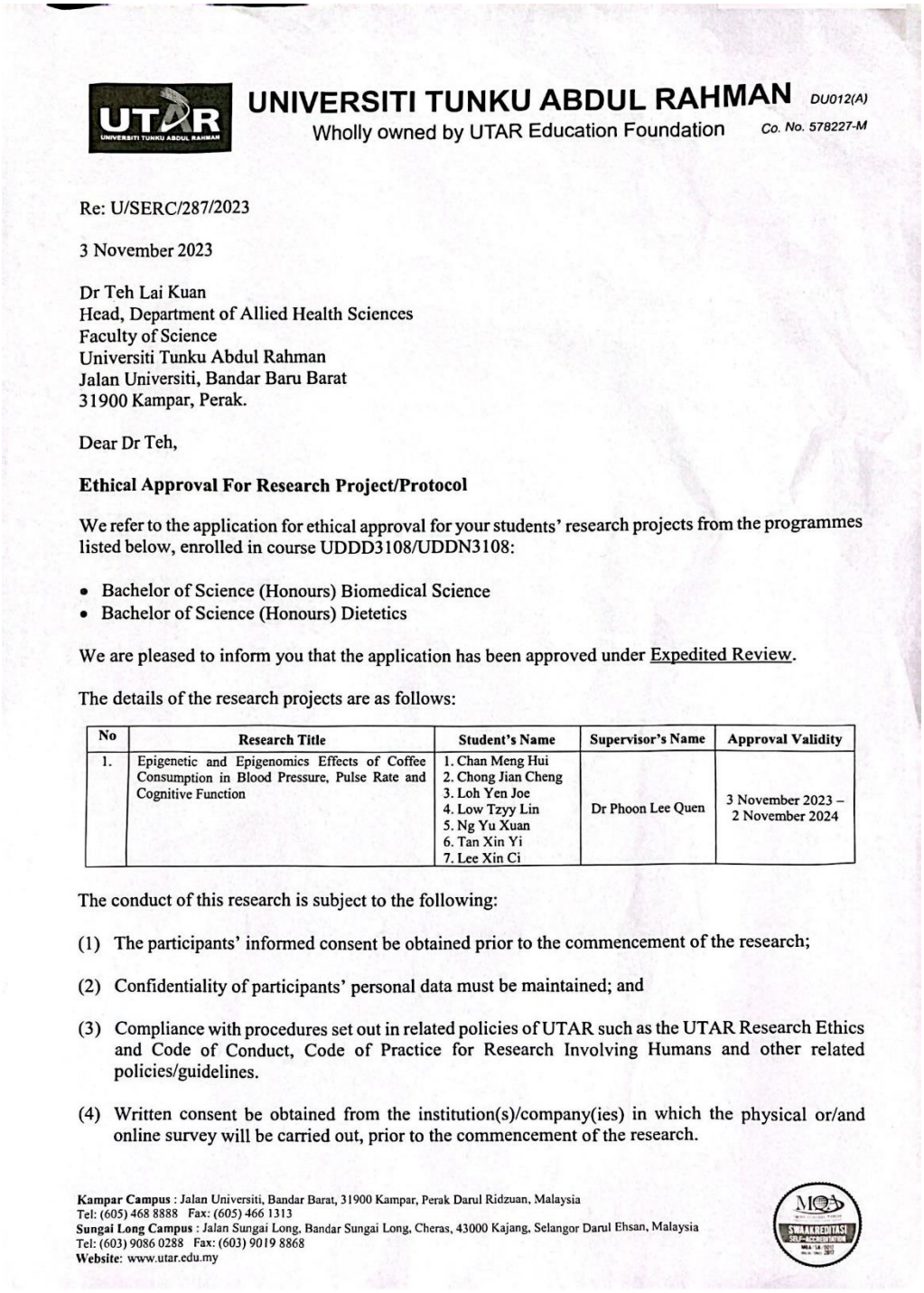
APPENDIX A

ETHICAL APPROVAL FROM SERC



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Re: U/SERC/287/2023

3 November 2023

Dr Teh Lai Kuan
Head, Department of Allied Health Sciences
Faculty of Science
Universiti Tunku Abdul Rahman
Jalan Universiti, Bandar Baru Barat
31900 Kampar, Perak.

Dear Dr Teh,

Ethical Approval For Research Project/Protocol

We refer to the application for ethical approval for your students' research projects from the programmes listed below, enrolled in course UDDD3108/UDDN3108:

- Bachelor of Science (Honours) Biomedical Science
- Bachelor of Science (Honours) Dietetics

We are pleased to inform you that the application has been approved under Expedited Review.

The details of the research projects are as follows:

No	Research Title	Student's Name	Supervisor's Name	Approval Validity
1.	Epigenetic and Epigenomics Effects of Coffee Consumption in Blood Pressure, Pulse Rate and Cognitive Function	1. Chan Meng Hui 2. Chong Jian Cheng 3. Loh Yen Joe 4. Low Tzyy Lin 5. Ng Yu Xuan 6. Tan Xin Yi 7. Lee Xin Ci	Dr Phoon Lee Quen	3 November 2023 – 2 November 2024

The conduct of this research is subject to the following:

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

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

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

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

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

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

Thank you.

Yours sincerely,

Professor Ts Dr Faidz bin Abd Rahman
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
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Website: www.utar.edu.my

APPENDIX B

LIST OF MATERIAL AND CHEMICAL USED

Material / Chemical	Manufacturer
Saline solution (0.9% NaCl)	RinsCap, Malaysia
Diethylpyrocarbonate (DEPC)	BioBasic, Canada
Cetyltrimethyl ammonium bromide (CTAB)	Amresco, United States
Sodium chloride (NaCl)	Fisher, United States
Ethylenediamine tetraacetic acid (EDTA)	OmniPur, Germany
Tris(hydroxymethyl)aminomethane (Tris)	1st Base, Singapore
B-mercaptoethanol	Bio Basic, Canada
Polyvinylpyrrolidone (PVP 40)	Bio Basic, Canada
Chloroform	QReC, Malaysia
Isopropanol	QReC, Malaysia
70% ethanol	Chem Sola, Malaysia
Lithium chloride (LiCl)	Sigma-Aldrich, United States
DNase I	Thermo Scientific, United States
50 mM Ethylenediamine tetraacetic acid (EDTA)	Thermo Scientific, United States
OneScript Plus cDNA synthesis kit	Applied Biological Materials, Canada
BrightGreen 5X qPCR MasterMix	Applied Biological Materials, Canada

APPENDIX C

QUESTIONNAIRE

Epigenetic and Epigenomics Effects of Coffee Consumption in Blood Pressure, Pulse Rate and Cognitive Function

Informed Consent

Aim / Purpose of the Research:

The objectives are:

1. To study the prevalence of habitual coffee consumption among young adults.

2. To compare the distribution of forgetfulness, distractibility, and false triggering among habitual coffee drinkers and non-habitual coffee drinkers.

3. To identify the allelic and genotypic frequencies in different study groups.

4. To compare the distribution of metabolism related genes and cognitive related genes' genotypes in habitual coffee consumption population and non-habitual coffee consumption population.

5. To study the distribution of genotypes in different classification of cognitive performance (forgetfulness, distractibility, and false triggering).

6. To compare the distribution of genotypes with different classification of cognitive performance (forgetfulness, distractibility, and false triggering) in habitual coffee drinkers and non-habitual coffee drinkers.

7. To determine the association between gene variants and habitual coffee consumption.

8. To determine the association between gene variants and cognitive performance.

9. To determine the association between gene variants, habitual coffee consumption and cognitive performance.

10. To evaluate the pre-post changes of mRNA expression, blood pressure, pulse rate and cognitive functions in coffee testing experiment.

11. To study the impact of black coffee on gene expression, blood pressure, pulse rate and cognitive functions via alteration of DNA methylation.

Procedure, Risk and Discomfort:

The project consists of four parts:

(i) survey and questionnaire;

(ii) genotyping
identification;

(iii) mRNA gene expression

(iv) statistical analysis.

A total of 420 subjects will be recruited to respond this questionnaire in order to determine their demographic characteristics, including gender, age and education level. Anthropometric measurements, such as blood pressure (BP) and pulse rate (PR), will be obtained following the collection of mouth wash sample from the participants. BP and PR will be taken by using an automated blood pressure monitor. It will be measured on the left arm after the participant has rested for 8 minutes in a seated position. Anthropometric measurement using an automated BP monitor poses minimal risks and discomforts to participant as it is a non-invasive measurement method. Secondly, you are required to rinse your mouth with drinking water to remove the food particles present in your oral cavity. Then, you will be requested to rinse your mouth with 0.9% saline solution for buccal cell collection. The mouth rinse will be collected in a disposable paper cup and used for genomic DNA extraction. For eligible participants (to be informed), you will be notified to attend the coffee testing experiment and there will be another round of buccal cell collection. For this instance, the buccal cell collections are used for RNA extraction. Buccal cell collection poses minimal risks and discomforts to donor as it is a non-invasive method. The genomic DNA as well as the RNA extraction will be used for further laboratory analysis.

Confidentially

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.

Who to Contact

If you have any questions, you may contact:

1. Chan Meng Hui (01124290902)

2. Chong Jian Cheng (0167150988)

3. Lee Xin Ci (0193966663)

4. Loh Yen Joe (0183723685)

5. Low Tzyy Lin (0126051663)

6. Ng Yu Xuan (0124931325)

7. Tan Xin Yi (0179583963)

This proposal has been reviewed and approved by UTAR Scientific & Ethical Review Committee, which is a committee to confirm that research participants are protect from harm.

Disclosure

Data, samples and specimens obtained from this study will not identify you individually. The data, samples and specimens may be given to the sponsor and/or regulatory authorities and may be published or be reused for research purposes not detailed within this consent form. However, your identity will not be disclosed. The original records will be reviewed by the principal investigator and the research team, the UTAR Scientific and Ethical Review Committee, the sponsor and regulatory authorities for the purpose of verifying research procedures and/or data.

* Indicates required question

1. Email *

The flow of this project you need to know:

1. Part 1 - Questionnaire answering.
2. Part 2 - Coffee Testing experiment.

*Only the eligible participants who fulfill our research requirements will be invited to participate in Part 2, further details will be notified in advance. *

Voluntary Participation

If you are eligible, your participation in this research is entirely voluntary. You understand that participation in this study is voluntary and that if you decide not to participate, you will experience no penalty or loss of benefits to which you would otherwise be entitled. If you decide to participate, you may subsequently change your mind about being in the study, and may stop participating at any time. You understand that you must inform the principal investigator of your decision immediately.

2. **Consent** *

Tick all that apply.

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

Section A: Demographics

3. Name *

_____

4. Contact Number (e.g. 0123456789) *

_____

5. UTAR Email *

_____

6. Age *

_____

7. Gender *

Mark only one oval.

◯ Male
◯ Female

8. Ethnicity *

Mark only one oval.

- ◯ Malay
- ◯ Chinese
- ◯ Indian
- ◯ Other: _____

9. Education Qualification *

Mark only one oval.

- ◯ Foundation
- ◯ Diploma
- ◯ Bachelor degree
- ◯ Master degree
- ◯ Doctor of Philosophy

10. Place of Origin (Which state?) *

_____

11. Residential Area (hometown) *

Mark only one oval.

- ◯ Urban
- ◯ Rural

12. Current height (cm) *

_____

13. Current weight (kg) *

14. Amount of sleeping hours *

15. Usual bedtime *

Example: 8.30 a.m.

16. Usual wakeup time *

Example: 8.30 a.m.

Skip to question 17

Section B: Health History

Part 1: Personal health

Please answer the questions as Yes or No. If "Yes", please further elaborate at the "other" option.

17. 1. Please tick (√) at the appropriate column for each item. *

Mark only one oval per row.

	Yes	No
Do you have diabetes?	◯	◯
Do you have hypertension?	◯	◯
Do you have coronary heart disease?	◯	◯
Do you have cerebrovascular disease?	◯	◯
Do you have hypothyroidism?	◯	◯
Have you had any form of neurological disease? (seizures, epilepsy, etc)	◯	◯
Do you have any form of psychiatric disorder? (depression, anxiety, etc)	◯	◯
Are you pregnant?	◯	◯
Are you lactating?	◯	◯

18. 2. Do you have other major disease? *
(If "Yes", please state at the "other" option)

Mark only one oval.

- ◯ No
- ◯ Other: _____

19. 3. Have you been prescribed any medication? (If "Yes", please state at the "other" option) *

Mark only one oval.

- ◯ No
- ◯ Other: _____

20. 4. Do you smoke? (If "Yes", please state the amount (pack/week) at the "other" option) *

Mark only one oval.

- ◯ No
- ◯ Other: _____

Skip to question 21

Section B: Health History

Part 2: Family health history
Please answer the questions as Yes or No. If "Yes", please state who and/or the disease at the "other" option.

21. 1. Do any of your parents (father/mother) have diabetes? *

If "Yes", Who? (state at the "other")

Mark only one oval.

- ◯ No
- ◯ Other: _____

22. 2. Do any of your parents (father/mother) have hypertension? *

If "Yes", Who? (state at the "other")

Mark only one oval.

- ◯ No
- ◯ Other: _____

23. 3. Do any of your parents (father/mother) have coronary heart disease? *

If "Yes", Who? (state at the "other")

Mark only one oval.

- ◯ No
- ◯ Other: _____

24. 4. Do any of your parents (father/mother) have cerebrovascular disease? *

If "Yes", Who? (state at the "other")

Mark only one oval.

- ◯ No
- ◯ Other: _____

25. 5. Do any of your parents (father/mother) have hypothyroidism? *

If "Yes", Who? (state at the "other")

Mark only one oval.

- ◯ No
- ◯ Other: _____

26. 6. Do any of your parents (father/mother) have other major diseases? *

If "Yes", Who and please state the disease at the "other" option.

Mark only one oval.

- ◯ No
- ◯ Other: _____

27. 7. Do any of your parents (father/mother) have any form of neurological disease? (seizures, epilepsy, etc) *

If "Yes", Who? (state at the "other")

Mark only one oval.

- ◯ No
- ◯ Other: _____

28. 8. Do any of your parents (father/mother) have any form of psychiatric disorder? (depression, anxiety, etc). *

If "Yes", Who? (state at the "other")

Mark only one oval.

- ◯ No
- ◯ Other: _____

Skip to question 29

Section C: Habits and Preferences in Coffee Consumption

29. Have you drunk at least one cup of coffee in the past six months? *

Mark only one oval.

- ◯ Yes *Skip to question 30*
- ◯ No *Skip to question 38*

Skip to question 30

Habits and Preferences to the Coffee Consumption of Coffee Drinkers

30. 1. Coffee consumption frequencies: *

Mark only one oval.

- ≤ 3 times per month
- ≥ 3 times per month
- ≤ 3 times per week
- ≥ 3 times per week
- ≤ 3 times per day
- ≥ 3 times per day

31. 2. In a typical day, how many cups of coffee do you drink? *

Mark only one oval.

- 2 cups or less
- 3 - 4 cups
- 5 - 6 cups
- 7 - 8 cups
- 9 cups or more

32. 3. How often do you drink coffee on each of the following occasions? *

Mark only one oval per row.

	Never	Rarely	Often	Always
First thing after waking up in the morning?				
With breakfast?				
During your morning break?				
With lunch?				
During your afternoon break?				
With dinner?				
In the evening after dinner?				
At bedtime?				
During examination week?				
When visiting friends or family?				
When you are stressed?				

33. 4. Please indicate which one of the following types of coffee you use most and which one you prefer most. *

Tick all that apply.

- [ ] Instant
- [ ] Ground/ filter coffee (e.g. house of coffee)
- [ ] Instant decaffeinated
- [ ] Ground decaffeinated
- [ ] Cappuccino
- [ ] Espresso
- [ ] Other: _____

34. 5. Which of the following is the **most important reason** for **using** the type of coffee specified in **Question 4**? Please tick (√). *

Mark only one oval.

- ◯ It is the type I prefer.
- ◯ It tastes best.
- ◯ It is the most affordable.
- ◯ It is the most readily available.
- ◯ The place where I work/ study provides the most used type.
- ◯ Other members in my household prefer most used brand.
- ◯ Other: _____

35. 6. If the type of coffee you **use most often differs from the type you prefer most**, which is the **most important reason** for this discrepancy? *

Mark only one oval.

- ◯ I cannot afford my preferred type.
- ◯ The place where I work/ study does not use the preferred type.
- ◯ Other members in my household prefer another type of coffee.
- ◯ Other: _____

36. 7. How important is each of the following to you when purchasing/ ordering coffee? *

Mark only one oval per row.

	Totally unimportant	Unimportant	Important	Very important
Low price	◯	◯	◯	◯
Smooth taste	◯	◯	◯	◯
Purchasing/ ordering a specific brand	◯	◯	◯	◯
The strength of the coffee	◯	◯	◯	◯
Purchasing/ ordering a local brand	◯	◯	◯	◯
Purchasing/ ordering an imported brand	◯	◯	◯	◯

37. 9. Do you consider yourself to be a coffee addict? *

Mark only one oval.

◯ Yes

◯ No

Skip to question 39

Habits and Preferences of Non-Coffee Drinkers

38. Please rank, in order of importance, each of the following reasons for **not consuming coffee** in the last 6 months. *

Allocate a rank of "**1**" to the **most important reason** for not consuming coffee in the last 6 months, a rank of "2" to the second most important reason, etc. allocate a rank of "**6**" to the **least important reason** for not consuming coffee in the last 6 months. (**All the numbers from 1 to 6 should be chosen for this question, without equal rank**.)

Mark only one oval per row.

	1	2	3	4	5	6
I do not like the taste of coffee.	◯	◯	◯	◯	◯	◯
I do not like the smell of coffee.	◯	◯	◯	◯	◯	◯
Coffee is expensive.	◯	◯	◯	◯	◯	◯
I do not drink coffee for health reason.	◯	◯	◯	◯	◯	◯
I cannot sleep when drinking coffee	◯	◯	◯	◯	◯	◯
I prefer drinking tea.	◯	◯	◯	◯	◯	◯

Skip to question 39

Section D: Attitude and Perceptions on Coffee Consumption

39. 1. To what extent do you agree with each of the following statements? *

Mark only one oval per row.

	Strongly disagree	Disagree	Neutral	Agree	Strongly agree
Drinking coffee increases my risk of getting cancer.	◯	◯	◯	◯	◯
Drinking coffee increases my risk of heart disease.	◯	◯	◯	◯	◯
I cannot fall asleep when I drink coffee during the day.	◯	◯	◯	◯	◯
I prefer drinking tea to drinking decaffeinated coffee.	◯	◯	◯	◯	◯
Drinking coffee gives me an energy boost.	◯	◯	◯	◯	◯
I drink coffee because my friends do.	◯	◯	◯	◯	◯
I drink coffee for the taste.	◯	◯	◯	◯	◯
I drink coffee because it is fashionable.	◯	◯	◯	◯	◯

Drinking coffee is fashionable.					
Drinking coffee relaxes me.					
I prefer drinking tea to drinking coffee.					
I cannot fall asleep when drinking coffee in the evening.					
I drink coffee to help me stay awake.					
I prefer local brands of coffee to imported ones.					
Drinking coffee makes me feel important.					

Skip to question 40

Section E: Eating Habit and Preferences

Please list the foods and drinks you consume normally. Please state "Nil" if none.

40. Breakfast (1st meal) and snacks: *

41. Tea break (before lunch) *

42. Lunch (2nd meal) and snacks: *

43. Tea break (before dinner)

44. Dinner (3rd meal) and snacks: *

45. Supper: *

Skip to question 46

Section F: Food Intake

46. 1. How many **days in a week** do you eat/drink: *

Mark only one oval per row.

	0	1	2	3	4	5	6	7
Confectionery (local cakes, cakes, ice-cream, ABC, jelly, snacks, etc.)?	◯	◯	◯	◯	◯	◯	◯	◯
Fruits (exclude canned fruits)	◯	◯	◯	◯	◯	◯	◯	◯
Vegetables/ salads	◯	◯	◯	◯	◯	◯	◯	◯
Plain water	◯	◯	◯	◯	◯	◯	◯	◯

47. 2. How many **serving** of the following types of food do you take usually on the day you eat? *

Mark only one oval per row.

	1	2	3	4	5	More than 5
Confectionery (local cakes, cakes, ice-cream, ABC, jelly, snacks, etc.)	◯	◯	◯	◯	◯	◯
Fruits (exclude canned fruits)	◯	◯	◯	◯	◯	◯
Vegetables/ salads	◯	◯	◯	◯	◯	◯

48. 3. Usually on the days you drink plain water, how much of plain water do you drink (mL)? *

Skip to question 49

Section G: Food Habit

Part 1: Processed Foods

49. Please tick at the appropriate columns of the amount of servings **per day.** *

Mark only one oval per row.

	0	1-2	3-4	5-6	More than 6
Any kind of milks					
Cereals					
Deli ham (chicken/beef)					
Sausage					
Bacon					
Burgers					
French fries					
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					
Salted nuts, pumpkin/ sunflower seeds					
Dried fruits					
Red meat					

50. Please tick at the appropriate columns of the amount of servings **per week**. *

Mark only one oval per row.

	0	1-2	3-4	5-6	More than 6
Any kind of milks	◯	◯	◯	◯	◯
Cereals	◯	◯	◯	◯	◯
Deli ham (chicken/beef)	◯	◯	◯	◯	◯
Sausage	◯	◯	◯	◯	◯
Bacon	◯	◯	◯	◯	◯
Burgers	◯	◯	◯	◯	◯
French fries	◯	◯	◯	◯	◯
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					
Salted nuts, pumpkin/ sunflower seeds					
Dried fruits					
Red meat					

51. Please tick at the appropriate columns of the amount of servings **per month**. *

Mark only one oval per row.

	0	1-2	3-4	5-6	More than 6
Any kind of milks	◯	◯	◯	◯	◯
Cereals	◯	◯	◯	◯	◯
Deli ham (chicken/beef)	◯	◯	◯	◯	◯
Sausage	◯	◯	◯	◯	◯
Bacon	◯	◯	◯	◯	◯
Burgers	◯	◯	◯	◯	◯
French fries	◯	◯	◯	◯	◯
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					
Salted nuts, pumpkin/ sunflower seeds					
Dried fruits					
Red meat					

Skip to question 52

Section G: Food Habit

Part 2: Malaysian/Asian Dishes

52. Please tick at the appropriate columns of the amount of servings **per day**. *

Mark only one oval per row.

	0	1-2	3-4	5-6	More than 6
Roti Canai	◯	◯	◯	◯	◯
Nasi Lemak	◯	◯	◯	◯	◯
Char Kuey Teow	◯	◯	◯	◯	◯
Rendang	◯	◯	◯	◯	◯
Boba/Bubble Tea	◯	◯	◯	◯	◯
Teh Tarik	◯	◯	◯	◯	◯
Curry Mee	◯	◯	◯	◯	◯
Mee Goreng/Maggie Goreng	◯	◯	◯	◯	◯
Satay	◯	◯	◯	◯	◯
Fried Chicken	◯	◯	◯	◯	◯
Karipap	◯	◯	◯	◯	◯
Mee Jawa	◯	◯	◯	◯	◯
Naan Cheese	◯	◯	◯	◯	◯
Hokkien Mee	◯	◯	◯	◯	◯
Prawn Mee	◯	◯	◯	◯	◯

53. Please tick at the appropriate columns of the amount of servings **per week**. *

Mark only one oval per row.

	0	1-2	3-4	5-6	More than 6
Roti Canai	◯	◯	◯	◯	◯
Nasi Lemak	◯	◯	◯	◯	◯
Char Kuey Teow	◯	◯	◯	◯	◯
Rendang	◯	◯	◯	◯	◯
Boba/Bubble Tea	◯	◯	◯	◯	◯
Teh Tarik	◯	◯	◯	◯	◯
Curry Mee	◯	◯	◯	◯	◯
Mee Goreng/Maggie Goreng	◯	◯	◯	◯	◯
Satay	◯	◯	◯	◯	◯
Fried Chicken	◯	◯	◯	◯	◯
Karipap	◯	◯	◯	◯	◯
Mee Jawa	◯	◯	◯	◯	◯
Naan Cheese	◯	◯	◯	◯	◯
Hokkien Mee	◯	◯	◯	◯	◯
Prawn Mee	◯	◯	◯	◯	◯

54. Please tick at the appropriate columns of the amount of servings **per month**. *

Mark only one oval per row.

	0	1-2	3-4	5-6	More than 6
Roti Canai	◯	◯	◯	◯	◯
Nasi Lemak	◯	◯	◯	◯	◯
Char Kuey Teow	◯	◯	◯	◯	◯
Rendang	◯	◯	◯	◯	◯
Boba/Bubble Tea	◯	◯	◯	◯	◯
Teh Tarik	◯	◯	◯	◯	◯
Curry Mee	◯	◯	◯	◯	◯
Mee Goreng/Maggie Goreng	◯	◯	◯	◯	◯
Satay	◯	◯	◯	◯	◯
Fried Chicken	◯	◯	◯	◯	◯
Karipap	◯	◯	◯	◯	◯
Mee Jawa	◯	◯	◯	◯	◯
Naan Cheese	◯	◯	◯	◯	◯
Hokkien Mee	◯	◯	◯	◯	◯
Prawn Mee	◯	◯	◯	◯	◯

Skip to question 55

Section H: Vitamin/Mineral Supplement Intake

Usually medicine-like supplements. Example: Multivitamin/ Multimineral, Vitamin A/ Carotenoids, B complex vitamin, Vitamin B12, Vitamin C, Folic acid/ B6, Iron, Calcium, Vitamin E, Zinc, etc.
Exclude animal extracts and herbal supplements.

55. 1. Did you take any vitamin/ mineral supplement within the last 12 months? *

Mark only one oval.

- ◯ Yes *Skip to question 56*
- ◯ No *Skip to question 59*

Skip to question 56

Vitamin/Mineral Supplement Intake

Please list out the supplements taken in your daily routine (or frequently, eg. once per week)

56. 1. What type of vitamin/ mineral supplements do you take? List all. *

_____

57. 2. What was you reason for taking vitamin/ mineral supplement? *

_____

58. 3. How often did you take vitamin/ mineral supplement (**1** as **first type** that you have listed in Q1, **2** as the **second** type that you have listed in Q1, etc.). Please range it and give only one response in the 1 row if only one type of supplement is listed in Q1.

Mark only one oval per row.

	Occasionally	1-3 times per month	Once a week	More than once a week	Everyday
1	◯	◯	◯	◯	◯
2	◯	◯	◯	◯	◯
3	◯	◯	◯	◯	◯
4	◯	◯	◯	◯	◯
5	◯	◯	◯	◯	◯

Skip to question 59

Section I: Food Supplement Intake

Refers to animal extracts and herbal supplements. Example: Fish oil, Evening primrose oil, bird's nest, Essence of chicken, Haruan fish stock, Royal jelly, Collagen, Spirulina, Gingko biloba, Mangosteen extract, Sea cucumber products, Slimming products, Prune essence, Berry essence, Health powder (exclude slimming product), etc.

59. In the last 12 months, have you ever taken any food supplement? *

Mark only one oval.

- ◯ Yes *Skip to question 60*
- ◯ No *Skip to question 63*

Skip to question 60

Food Supplement Intake

Please list out the supplements taken in your daily routine (or frequently, eg. once per week)

60. 1. What type of food supplement do you take? List all. *

_____

61. 2. What was your reason for taking food supplement? *

_____

62. 3. How often did you take food supplement (**1** as **first type** of supplement(s) that you have listed in Q1, **2** as the **second** type that you have listed in Q1, etc.). Please range it and give only one response in the 1 row if only one type of supplement is listed in Q1.

Mark only one oval per row.

	Occasionally	1-3 times per month	Once a week	More than once a week	Everyday
1	◯	◯	◯	◯	◯
2	◯	◯	◯	◯	◯
3	◯	◯	◯	◯	◯
4	◯	◯	◯	◯	◯
5	◯	◯	◯	◯	◯

Skip to question 63

Section J: Physical Activity

Part 1: Recreation, Sport, and Leisure-time Physical Activity

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

Mark only one oval per row.

	No walking in leisure time	1-2	3-4	5-6	7
[ ] days per week	◯	◯	◯	◯	◯

64. 2. How many **hours per day** did you usually spend on one of those days **walking** in your leisure time? State "0" as no walking in leisure time. *

65. 3. How many **minutes per day** did you usually spend on one of those days **walking** in your leisure time? State "0" as no walking in leisure time. *

66. 4. During the **last 7 days**, did you do **vigorous** physical activities like aerobics, running, fast bicycling, or fast swimming in your leisure time? *

Mark only one oval per row.

	No vigorous activity in leisure time	1-2	3-4	5-6	7
[ ] days per week	◯	◯	◯	◯	◯

67. 5. How many **hours per day** did you usually spend on one of those days doing **vigorous** physical activities in your leisure time? State "0" as no vigorous physical activities in leisure time. *

68. 6. How many **minutes per day** did you usually spend on one of those days doing **vigorous** physical activities in your leisure time? State "0" as no vigorous physical activities in leisure time. *

69. 7. During the **last 7 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? *

Mark only one oval per row.

	No	1-2	3-4	5-6	7
[ ] days per week	◯	◯	◯	◯	◯

70. 8. How many **hours per day** did you usually spend on one of those days doing **moderate vigorous** physical activities in your leisure time? State "0" as no moderate vigorous physical activities in leisure time. *

_____

71. 9. How many **minutes per day** did you usually spend on one of those days doing **moderate vigorous** physical activities in your leisure time? State "0" as no moderate vigorous physical activities in leisure time. *

_____

Skip to question 72

Section J: Physical Activity

Part 2: Time Spent Sitting

72. 1. During the **last 7 days**, how many **hours per day** did you usually spend **sitting** while at work, at home, while doing course work and during leisure time. on a **weekday/weekend days?** (exclude sitting in a motor vehicle) *

Mark only one oval per row.

	1-3	4-6	7-9	9-11	12 or more
Weekdays (Monday-Friday)	◯	◯	◯	◯	◯
Weekend days (Saturday, Sunday)	◯	◯	◯	◯	◯

73. 5. During the **last 7 days**, how many **minutes per day** did you usually spend **sitting** while at work, at home, while doing course work and during leisure time. on a **weekday/weekend day?** (exclude sitting in a motor vehicle) *

Mark only one oval per row.

	1-30	31-60	61-90	91-120	121-150	151-180	More than 180
Weekdays (Monday-Friday)	◯	◯	◯	◯	◯	◯	◯
Weekend day (Saturday, Sunday)	◯	◯	◯	◯	◯	◯	◯

Section K: Cognitive Assessment

74. We want to know how often these things have happened to your in the past 6 months. Please choose the appropriate number. *

Mark only one oval per row.

	Always	Often	Occasionally	Very rarely	Never
Do you read something and find you haven't been thinking about it and must read it again?	◯	◯	◯	◯	◯
Do you find you forget why you went from one part of the house to the other?	◯	◯	◯	◯	◯
Do you fail to notice signposts on the road?	◯	◯	◯	◯	◯
Do you find you confuse right and left when giving directions?	◯	◯	◯	◯	◯
Do you bump into people?	◯	◯	◯	◯	◯
Do you find you forget whether you've turned off a light or a fire or locked the door?	◯	◯	◯	◯	◯
Do you fail to listen to people's names when you are meeting them?	◯	◯	◯	◯	◯
Do you say something and realize afterwards that it might be taken as	◯	◯	◯	◯	◯

Do you fail to hear people speaking to you when you are doing something else?	☐	☐	☐	☐	☐
Do you lose your temper and regret it?	☐	☐	☐	☐	☐
Do you leave important letters unanswered for days?	☐	☐	☐	☐	☐
Do you find you forget which way to turn on a road you know well but rarely use?	☐	☐	☐	☐	☐
Do you fail to see what you want in a supermarket (although it's there)?	☐	☐	☐	☐	☐
Do you find yourself suddenly wondering whether you've switched off the cooker?	☐	☐	☐	☐	☐
Do you have trouble making up your mind?	☐	☐	☐	☐	☐
Do you find you forget appointments?	☐	☐	☐	☐	☐
Do you forget where you put something like a book?	☐	☐	☐	☐	☐

book? newspaper or a book?					
Do you find you accidentally throw away the thing you want and keep what you meant to throw away - as in the example of throwing away the matchbox and putting the used match in your pocket?					
Do you find you accidentally throw away the thing you want and keep what you meant to throw away - as in the example of throwing away the matchbox and putting the used match in your pocket?					
Do you daydream when you ought to be listening to something?					
Do you daydream when you ought to be listening to something?					
Do you find you forget people's names?					
Do you find you forget people's names?					
Home and get distracted into doing something else (unintentionally)?					
Home and get distracted into doing something else (unintentionally)?					
Do you find you can't quite remember something although it's "on the tip of your tongue"?					
Do you find you can't quite remember something although it's "on the tip of your tongue"?					
Do you find you forget what you came to the shops to buy?					
Do you find you forget what you came to the shops to buy?					
Do you drop things?					
Do you drop things?					
Do you find you can't think of anything to say?					

Do you find you can't think of anything to say?					

Acknowledgement

Thank you very much for taking the time and effort to participate in this survey.

Your cooperation and support are our greatest encouragement. Without your help, this survey would not be completed as expected. Greatest gratitude and appreciation for helping us in this survey.

Declaration:

Your personal information and the responses are used strictly for research purpose only and would not be revealed to any other third parties. Data, samples and specimens obtained from this study will not identify you individually.

However, the data, samples and specimens may be given to the sponsor and/or regulatory authorities and may be published or be reused for research purposes not detailed within this consent form. Your identity will not be disclosed as the original records will be reviewed by the principal investigator and the research team, the UTAR Scientific and Ethical Review Committee, the sponsor and regulatory authorities for the purpose of verifying research procedures and/or data.

75. Consent *

Tick all that apply.

☐ I agree and understand the statements and declarations made by filling out this questionnaire.

APPENDIX D

MATERIAL COMPOSITION DECLARATION AND PRODUCT CERTIFICATE OF ANALYSIS OF BLACK COFFEE

MATERIAL COMPOSITION DECLARATION

PRODUCT NAME	Instant Coffee Spray Dried Powder SS008-P
PACKAGING	25 kg Carton Box

NO.	INGREDIENTS BREAKDOWN	PERCENTAGES (%)	ALLERGEN ? (YES / NO)
1	Indian Robusta Bean	90%	No
2	Indian Arabica Bean	10%	No
3	-	-	-
4	-	-	-
	TOTAL	100%	NO

*** The Indian Coffee is from 100% coffee beans, there are no other materials being used during the extraction process.*

Product Certificate of Analysis

		SS008P	Microbiological Analysis	
Moisture	-	3.18%	Total Plate Count(IS:5402:1967)	<1000 CFU
Ash	-	8.40%	Mould And Yeast(IS:5403:1999)	<100 CFU
Caffeine on dry basis	-	3.52%	E.Coli(IS:5887(part I)-1976)	Absent
Colour	-	65.9 CTN	Salmonella(IS:5887(partIII-1976)	Absent in 25g
Density	-	0.25 (g/cc)		
Salmonella/25g	-	Absent		
pH value	-	5.01		

The shelf life is 2 years from date of production if the product is stored in a dry and ventilated place.

APPENDIX E

AVERAGE BLOOD PRESSURE AND PULSE RATE READINGS IN COFFEE TEST

	Habitual coffee drinker (N = 10)								
	Treated (coffee)								
	0 min			30 min			60 min		
	SBP	DBP	Pulse	SBP	DBP	Pulse	SBP	DBP	Pulse
M1	105.0	69.5	59.3	117.5	79.0	63.5	129.5	82.5	64.8
M2	121.5	71.5	68.5	123.5	76.0	62.0	123.5	80.0	63.0
M3	-	-	-	105.5	70.5	69.0	112.5	79.5	55.5
M4	116.5	77.0	63.5	121.0	81.0	66.0	123.5	80.5	65.3
M5	110.0	71.0	74.5	112.0	71.5	67.3	110.0	73.0	69.0
F1	100.5	74.5	77.5	111.5	81.0	77.5	102.0	79.0	83.5
F2	92.0	62.0	53.5	103.0	66.0	54.0	99.0	67.0	51.0
F3	93.5	71.0	86.0	96.5	74.0	71.0	96.5	70.0	72.5
F4	98.0	58.5	60.3	110.0	67.5	62.8	104.5	67.5	63.8
F5	88.5	52.5	57.8	97.0	64.0	58.5	95.5	63.5	60.8

"-" indicates missing data that were eliminated due to extreme value

	Non-habitual coffee drinker (N = 10)								
	Treated (coffee)								
	0 min			30 min			60 min		
	SBP	DBP	Pulse	SBP	DBP	Pulse	SBP	DBP	Pulse
M1	118.0	77.5	71.5	124.5	77.0	71.0	138.0	86.5	88.0
M2	98.5	62.5	79.5	95.0	64.0	69.0	90.5	65.0	71.5
M3	108.5	74.5	80.0	118.5	81.5	70.5	121.0	83.0	72.0
M4	117.0	80.5	55.5	127.0	84.5	55.0	127.5	94.5	54.5
M5	107.0	67.0	72.5	110.0	67.5	60.0	117.5	65.0	60.5
F1	91.0	56.5	66.5	95.0	62.0	72.0	95.0	64.5	69.5
F2	88.5	63.0	70.0	92.0	65.0	59.0	99.5	66.0	57.0
F3	96.0	58.5	71.5	91.5	58.0	62.5	100.0	62.0	65.0
F4	87.0	64.0	69.0	87.0	65.0	72.0	95.0	69.0	70.0
F5	111.0	64.0	65.5	121.0	74.0	66.0	113.5	77.0	67.5

APPENDIX F

RNA CONCENTRATION AND PURITY

Concentration of DNA (ng/µL)	Purity (A260/A280)
12.00	2.68
45.80	1.68
55.40	1.79
7.10	1.50
41.00	1.86
6.40	1.42
15.40	2.21
24.20	2.03
20.40	1.59
14.10	1.83
13.00	1.98
10.40	2.11
58.20	1.86
33.40	1.69
46.10	1.74
22.20	1.55
15.00	1.47
15.70	1.56
26.80	1.73
47.20	1.43
80.40	1.88
34.80	1.91
241.60	1.53
19.80	1.90
13.70	1.70
11.50	2.04
12.60	1.70
16.10	1.66
24.30	1.48
11.30	1.97
29.40	1.54
10.70	1.67
10.70	1.37
16.10	1.42
25.00	1.41
10.10	1.35
9.80	1.74
19.30	1.46
8.00	2.03
16.60	1.45

Concentration of DNA (ng/µL)	Purity (A260/A280)
21.10	1.31
14.30	1.47
93.70	1.94
213.10	1.87
226.20	1.93
106.90	1.88
70.50	1.92
167.00	1.71
11.90	1.59
17.00	1.54
12.90	1.56
7.20	-
21.30	1.13
36.40	1.56
61.90	1.56
31.90	1.54
18.00	1.55
38.30	1.67
11.80	1.97
31.70	1.68
59.50	1.76
24.10	1.70
21.20	1.77
63.40	1.87
27.80	2.28
9.30	2.30
26.60	1.48
29.80	1.41
20.10	1.62
24.40	1.64
41.10	1.56
56.60	1.44
37.10	1.86
18.80	1.40
29.00	1.74
18.50	1.68
44.80	1.94
633.00	1.48
9.70	2.22
15.00	1.58
22.40	1.49
18.10	1.20
26.60	1.52
11.60	1.75
114.00	1.49
32.80	1.52

Concentration of DNA (ng/µL)	Purity (A260/A280)
10.80	1.82
17.40	1.31
141.10	1.44
18.40	1.51
16.90	1.58
31.50	1.67
2.30	2.05
9.20	1.67
14.00	1.93
20.50	1.60
20.50	1.90
34.80	1.84
1.40	15.64
53.20	1.51
21.90	1.40
4.70	2.62
4.10	2.46
2.90	2.73
3.00	1.93
13.20	1.31
16.50	1.50
1.80	1.57
6.60	1.52
12.10	1.67
9.80	1.42
9.60	1.82
14.30	1.70
7.90	1.63
26.10	1.51
11.80	1.94
29.70	1.75
12.80	1.69
15.60	1.58
8.80	1.64

APPENDIX G

cDNA CONCENTRATION AND PURITY

Concentration of DNA (ng/µL)	Purity (A260/A280)
997.00	1.65
1115.90	1.64
1183.80	1.70
918.60	1.64
1262.30	1.72
851.60	1.63
1387.00	1.68
1240.30	1.64
1230.90	1.63
1096.70	1.64
911.90	1.64
969.90	1.60
1527.20	1.74
1421.60	1.73
1664.30	1.74
260.00	1.71
335.90	1.67
1191.20	1.64
1546.10	1.64
994.10	1.64
975.50	1.63
1409.30	1.73
1273.10	1.71
1227.70	1.67
1053.70	1.66
1137.40	1.72
1006.60	1.62
452.00	1.68
1170.20	1.65
951.30	1.65
1261.60	1.66
955.10	1.64
965.30	1.63
1242.60	1.65
1151.10	1.61
950.90	1.62
1172.10	1.53
927.40	1.56
1362.90	1.68
1421.30	1.68

Concentration of DNA (ng/µL)	Purity (A260/A280)
1322.20	1.65
1122.90	1.65
969.60	1.61
1288.10	1.71
1197.90	1.70
1319.20	1.73
1001.10	1.63
1289.00	1.73
1003.50	1.66
1411.80	1.69
1021.60	1.65
961.30	1.63
1349.60	1.66
1374.60	1.73
1552.30	1.77
1351.00	1.71
1064.20	1.66
1371.80	1.73
1431.20	1.71
939.30	1.64
980.00	1.71
1304.10	1.67
1224.40	1.63
1302.30	1.71
1292.50	1.65
951.80	1.64
1350.20	1.68
359.90	1.54
1198.50	1.62
1250.10	1.63
1391.10	1.71
1039.30	1.64
1379.30	1.72
1385.80	1.66
1453.80	1.68
1453.00	1.68
1108.60	1.65
2108.20	1.75
2672.30	1.65
541.90	1.61
1407.90	1.69
253.10	1.66
1297.50	1.67
1631.60	1.61
1316.20	1.74
1406.90	1.71

Concentration of DNA (ng/µL)	Purity (A260/A280)
408.70	1.64
1466.40	1.67
1259.20	1.72
1359.10	1.64
319.60	1.64
1346.30	1.74
1241.00	1.67
888.70	1.65
987.80	1.63
1287.40	1.67
1054.30	1.69
1888.80	1.77
1411.20	1.72
1021.00	1.65
1292.40	1.65
1223.30	1.66
1100.00	1.67
1160.40	1.67
1013.10	1.66
962.20	1.63
839.00	1.60
1326.00	1.68
910.20	1.59
986.60	1.62
1009.70	1.64
885.10	1.64
1159.30	1.64
918.20	1.64
1171.10	1.68
1019.70	1.67
881.40	1.66
961.00	1.63
1207.70	1.66
951.80	1.65

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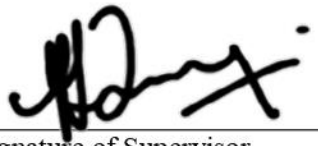

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