# GENOTYPING AND PARTIAL FUNCTIONAL CHARACTERIZATION OF AN OBESITY GWAS LOCI FAS APOPTOTIC INHIBITORY MOLECULE 2 (*FAIM2*) rs7138803

By

**TAN KE EN**

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## ABSTRACT

### GENOTYPING AND PARTIAL FUNCTIONAL CHARACTERIZATION OF AN OBESITY GWAS LOCI FAS APOPTOTIC INHIBITORY MOLECULE 2 (*FAIM2*) rs7138803

**Tan Ke En**

Obesity is defined as excessive fat accumulation that is harmful to a person's health. A combination of environmental and genetic factors contribute to obesity. Previous genome-wide association study (GWAS) has identified gene variant rs7138803 near Fas Apoptotic Inhibitory Molecule 2 (*FAIM2*) as an obesity susceptibility loci. FAIM2 is an anti-apoptotic protein which might be important in adipocyte apoptosis. This study investigated the association of *FAIM2* rs7138803 with obesity among the UTAR students and determined the lipid accumulation in *FAIM2*-overexpressed adipocytes. A total of 502 subjects were recruited and data of demographics, anthropometric measurement, environmental exposure and mouthwash samples were collected from the subjects. Genotyping of rs7138803 was performed by using PCR-RFLP technique. IBM Statistical Package for the Social Sciences 20 was used to performed statistical analysis. The lipid accumulation in 3T3-L1 cells which had been transiently transfected with pcDNA $3.1^{+}$/C-(K)-DYK cloned with *FAIM2* cDNA was assessed by Oil Red O (ORO) assay. The MAF of rs7138803 in the studied population was 0.30. The MAF of risk allele A of rs7138803 in the UTAR population was 0.30 for Chinese and 0.46 for Indians. The distribution of rs7138803 genotypes was in Hardy-Weinberg equilibrium

(HWE). Ethnicity showed significant association with both genotypes and alleles ($p$ = 0.02). Majority of the anthropometric measurements showed no association with rs7138803 genotypes and alleles. Only WHRadjBMI (waist-hip ratio adjusted for BMI) showed significant association with genotype ($p$ = 0.02) and allele ($p$ = 0.03). Stratified analysis showed that WHR was only significantly associated with genotype in males after adjustment for BMI ($p$ = 0.047). ORO assay showed no significant difference between level of lipid accumulation of control and adipocytes with *FAIM2* overexpression ($p$ = 0.51). In conclusion, rs713803 was associated with WHRadjBMI in males and *FAIM2* is proposed to have a role in obesity development.

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## DECLARATION

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

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Tan Ke En

**APPROVAL SHEET**

This project report entitled "**GENOTYPING AND PARTIAL FUNCTIONAL CHARACTERIZATION OF AN OBESITY GWAS LOCI FAS APOPTOTIC INHIBITORY MOLECULE 2 (*FAIM2*) rs7138803**" was prepared by **TAN KE EN** and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) in Biomedical Science at Universiti Tunku Abdul Rahman.

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**PERMISSION SHEET**

It is hereby certified that **TAN KE EN** (ID Number: 13ADB03152) has completed this final year project entitled "**GENOTYPING AND PARTIAL FUNCTIONAL CHARACTERIZATION OF AN OBESITY GWAS LOCI FAS APOPTOTIC INHIBITORY MOLECULE 2 (*FAIM2*) rs7138803**" supervised by Dr Say Yee How from Department of Biomedical Science, Faculty of Science.

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

Yours truly,

……

(TAN KE EN)

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| | |
|---|---|
| AMM | Adipocyte maintenance medium |
| BMI | Body mass index |
| bp | Base pair |
| cDNA | Copy deoxyribonucleic acid |
| $CO_2$ | Carbon dioxide |
| CTNNBL1 | Catenin beta-like 1 |
| Cyt c | Cytochrome C |
| DM | Differentiation medium |
| DMEM | Dulbelcco's Modified Eagle's Medium |
| DNA | Deoxyribonucleic acid |
| dNTPs | Deoxynucleotides |
| *E. coli* | *Escherichia coli* |
| ER | Endoplasmic reticulum |
| FAIM2 | Fas Apoptotic Inhibitory Molecule 2 |
| FasL | Fas ligand |
| FasR | Fas receptor |
| FBS | Fetal bovine serum |
| FTO | Fat mass and obesity associated |
| g | Acceleration of gravity (~9.8 $m/s^2$) |
| GWAS | Genome-wide association study |
| HWE | Hardy-Weinberg Equilibrium |
| hr | hour |
| IL-6 | Interleukin 6 |

| | |
|---|---|
| kb | Kilobase |
| LB | Luria-Bertani |
| LFG2 | Lifeguard protein 2 |
| MAF | Minor allele frequency |
| $Mg^{2+}$ | Magnesium ions |
| min | Minutes |
| mmHg | Millimeter of mercury |
| ORO | Oil Red O |
| PBS | Phosphate buffer saline |
| PCR | Polymerase Chain Reaction |
| PEM | Pre-adipocytes Expansion medium |
| PI3K-AKT | Phosphatidylinositol 3 kinase-Akt |
| RFLP | Restriction Fragment Length Polymorphism |
| RM | Resting metabolism |
| ROS | Reactive oxygen species |
| rpm | Rotation per minute |
| sec | Seconds |
| SF | Subcutaneous fat |
| SM | Skeleton muscle |
| SNP | Single nucleotide polymorphism |
| TBF | Total body fat |
| TMBIM | Transmembrane Bax inhibitor motif |
| TNF-α | Tumor necrosis factor alpha |
| UTAR | Universiti Tunku Abdul Rahman |
| VFL | Visceral fat level |

| | |
|---|---|
| WC | Weight circumference |
| WHO | World Health Organization |
| WHR | Waist-hip ratio |
| WHRadjBMI | Waist-hip ratio adjusted for body mass index |

# CHAPTER 1

## INTRODUCTION

Obesity and overweight are the main culprits in causing death worldwide as compared to underweight. Obesity is one of the major risk factor for various common chronic diseases including type 2 diabetes mellitus, cardiovascular diseases and cancers. Obesity is defined as a medical condition that poses a threat to a person's health due to excessive accumulation of fat in the body. Body mass index (BMI) is a commonly used parameter that involve weight and height to classify overweight and obesity in adults. Generally, a person with BMI equals to or more than 25 $kg/m^2$ is considered overweight, whereas an obese person is someone with BMI equals to or more than 30 $kg/m^2$ (World Health Organization, 2016).

Prevalence of obesity has been increased quickly worldwide, in both developed and developing countries, becoming a public health concern. South East Asia has the lowest prevalence of obesity (3%) and overweight (14%) for both males and females (Ng, et al., 2014). Although the lowest prevalence was found in South East Asia, but the prevalence is still increase in an alarming rate. Malaysia was ranked as the fattest country within the South East Asia as almost half of the Malaysian population were overweight and obese, which were 43.8% of men and 48.6% of women (Ng, et al., 2014).

A combination of multiple factors such as genetics and environment contributed to the chronic disease obesity. Unhealthy eating behavior and sedentary lifestyle which lack of physical activity are the major environmental factors contributing to obesity. Obesity is a result of energy imbalance when the energy intake is not proportional to the energy expenditure (Claussnitzer, et al., 2015). Excess energy will increase fat accumulation as it increases the storage of triacylglycerol and creation of new adipocytes (Sorisky, Magun and Gagnon, 2000). The possibility of overweight and obesity being inherited is high. Obesity can be due to defect in a single gene (monogenic) or presence of variants in multiple genes (polygenic). Carrier of a genotype that is associated with obesity might further increase the risk of being obese and the susceptibility to obesity-related diseases (Hinney, Vogel and Hebebrand, 2010).

Recently, the role of genetic factors is well supported by extensive genome-wide association studies (GWAS) and candidate gene studies that have identified numerous single nucleotide polymorphisms (SNPs) related to obesity (Liu, et al., 2015). rs7138803 SNP was detected as an obesity susceptibility loci. It is located at the intergenic region near the Fas Apoptotic Inhibitory Molecular 2 (*FAIM2*) gene. The gene variant rs7138803 showed association with BMI and obesity (Ensembl, 2017). There are nine GWAS studies which involved *FAIM2* rs7138803 (National Human Genome Research Institute, 2017a). A GWAS study that involved 32 SNPs concluded that *FAIM2* rs7138803 was significantly associated with BMI (Speliotes, et al., 2010). Not only that, another GWAS study performed among Japanese population showed that rs7138803 was marginally associated with obesity (Hotta, et al., 2009).

FAIM2 protein is involved in cell apoptosis and proliferation as it plays a role in the cell metabolism pathways related to long term obesity development. *FAIM2* encodes for a conserved protein that antagonizes with the apoptotic pathway by binding to the Fas receptor (Corella, et al., 2014). Furthermore, *FAIM2* is remarkably highly expressed in the brain, especially in hippocampus region which might affect the neural development of feeding (Li, et al., 2013). Previous studies also found out that *FAIM2* plays different roles in different cells such as human breast cancer cells and neuroblastoma cells (Bucan, et al., 2010; Planells-Ferrer, et al., 2016).

However, there is limited studies which provide evidence for the role of *FAIM2* in the lipid and metabolic traits. It is hypothesized that overexpression of *FAIM2* will increase the lipid accumulation in the adipocytes. In addition, there are numerous evidence which showed that rs7138803 is associated with obesity.

Therefore, the objectives of this study were:

1. To perform genotyping for obesity GWAS loci *FAIM2* rs7138803 among UTAR Kampar Campus students.
2. To study the association of *FAIM2* rs7138803 with obesity and related anthropometric measurements among UTAR Kampar Campus students.
3. To characterize the role of *FAIM2* in lipid accumulation by overexpression transfection in 3T3-L1 adipocytes.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Definition of obesity

Overweight and obesity are defined as a medical condition that is characterized by accumulation of body fat which poses a negative impact to health (World Health Organization, 2016). Fat is accumulated in the form of triacylglycerol in the adipocytes as an energy reservoir (Cohen and Spiegelman, 2016). BMI is universally applied in the classification of overweight and obesity as it can be calculated through a simple calculation. BMI can be obtained by dividing a person's weight in kilogram with the square of the height of a person in meter. Classification of obese and overweight based on BMI is summarized in the Table 2.1. Different ethnic groups have different cut-off points for obesity and overweight as they have different prevalence of developing BMI-related diseases. Asian population tends to have lower BMI cut-off points for overweight and obesity as it is more harmful to Asian population with the increase in weight over time compared to other ethnic groups (Harvard T.H. Chan School of Public Health, 2017a).

**Table 2.1:** Classification of overweight and obesity.

| Nutritional status | BMI cut-off ($kg/m^2$) | | Risk of co-morbidities |
|---|---|---|---|
| | WHO criteria | Asian criteria | |
| Underweight | <18.5 | <18.5 | Low |
| Normal | 18.5 - 24.9 | 18.5 – 22.9 | Average |
| Overweight | 25.0 – 29.9 | 23.0 – 24.9 | Increase |
| Obese | >30.0 | >25.0 | High |

(Wen, et al., 2009; World Health Organization, 2016)

BMI gives the overall indication about overweight and obesity but it does not indicate another important group of obesity, which is abdominal obesity. Abdominal obesity, also known as central obesity, is due to intra-abdominal adiposity where the excessive fat accumulate around the waist (Després, 2008). The waist circumference (WC) cut-off points for central obesity in South Asians are 90 cm for males and 80 cm for females (Harvard T.H. Chan School of Public Health, 2017b). The cut-off points of waist–hip ratio (WHR) for Asians are 0.90 for males and 0.80 for females. WC is still a better choice for indication of central obesity as compared to WHR (World Health Organization, 2008).

## 2.2 Prevalence of obesity

Overweight and obesity has becoming a global pandemic due to its alarming rising prevalence in several countries including Malaysia. The number of overweight and obesity has been increased for 2.5 times from year 1980 (857 million) to year 2013 (2.1 billion) (World Health Organization, 2016). Figure 2.1 illustrates the prevalence of age-standardized obesity on map in 2013.

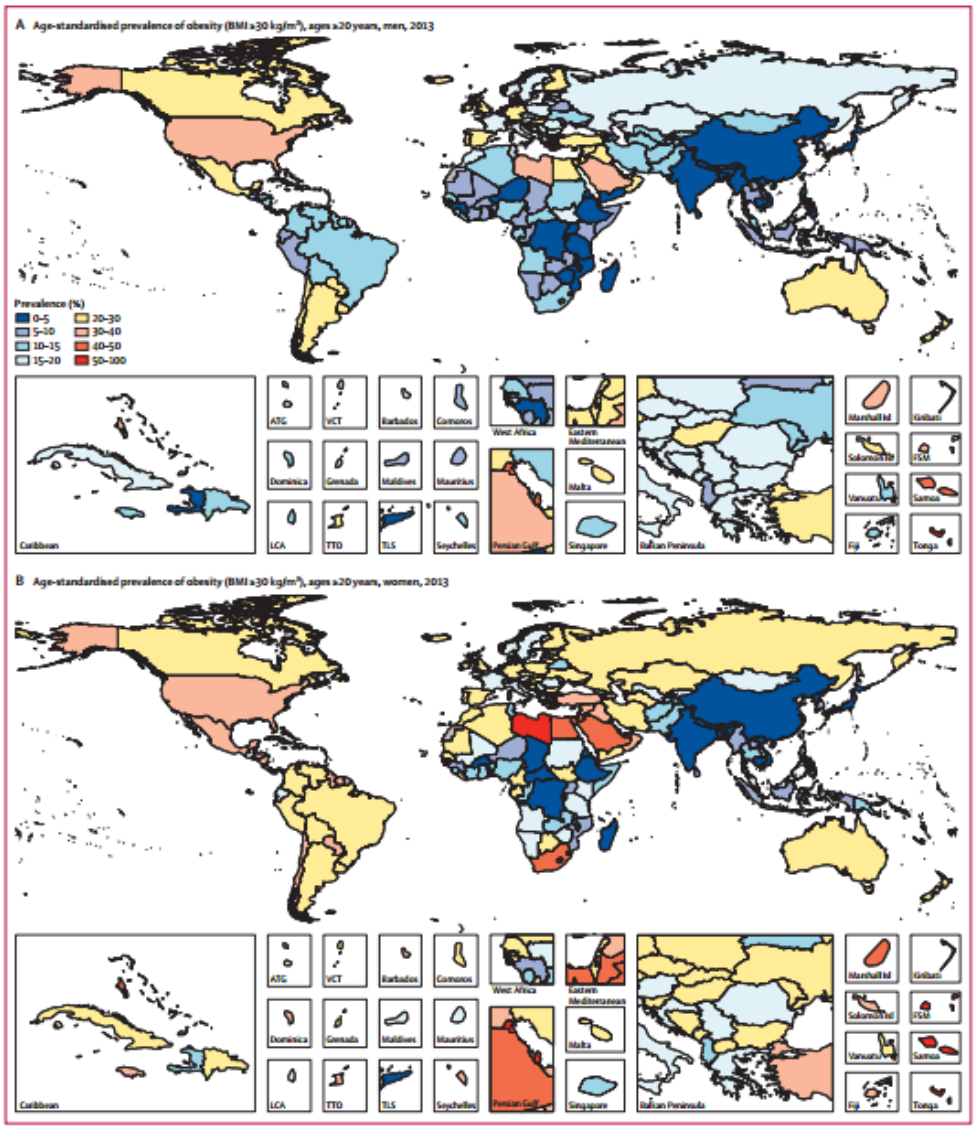


**Figure 2.1:** Prevalence of age-standardized obesity on map in 2013. **(A)** Adult males aged more than 20 years old with BMI more than 30 kg/m$^2$. **(B)** Adult females aged more than 20 years old with BMI more than 30 kg/m$^2$ (Ng, et al., 2014).

South East Asia has the lowest prevalence of obesity (3%) and overweight (14%) for both male and female, but the prevalence still in a rising state (Ng, et al., 2014). Among all the countries in Asia Pacific, India and Vietnam have the lowest prevalence of obesity which are 1.9% and 1.7%, respectively. In South East Asia region, Malaysia has the highest prevalence of obesity (14%), which then followed by Thailand (8.8%) (Cheong, 2014). According to National Health and Morbidity Survey of 2015, Malaysia was ranked as the fattest country within South East Asia as half of the Malaysian population are overweight and obese. In Malaysia, the prevalence of overweight and obesity was lower in men (43.8%) than in women (48.6%). The prevalence of obesity was 16.7% in women, which was greater than in men (11.4%) (Institute of Public Health, 2015).

Two guidelines are used during the classification of overweight and obesity based in BMI, which are World Health Organization (2008) and Malaysian Clinical Practice Guidelines of Obesity (2004). According to World Health Organization (2008) and Malaysian Clinical Practice Guidelines of Obesity (2004), WP Putrajaya had the highest prevalence in obesity which were 25.8% and 30.6%, respectively. Based on World Health Organization (2008) classification, the national prevalence of obesity and overweight were 17.7% and 30.0%. Males had higher prevalence of overweight which was 31.6%. Meanwhile, women (20.6%) had significantly higher prevalence of obesity as compared to men (15.0%). When Malaysian Clinical Practice Guidelines of Obesity (2004) was used to classify BMI, the national prevalence of obesity and overweight were increased to 30.6% and 33.4%, respectively. For obesity,

males (27.8%) had lower prevalence as compared to females (33.6%). Meanwhile, females (30.9%) had significantly lower prevalence of overweight than males (35.8%) (Institute of Public Health, 2015).

### 2.3 Environmental factors of obesity

Environmental factors are responsible for the development of obesity. Majority of the research or studies proved that environmental changes throughout the past decades are responsible for the increment in the numbers of overweight and obesity. Loss of energy balance is the main culprit for obesity as the calorie intake exceeds the body's requirements (Claussnitzer, et al., 2015). Excessive calorie intake results in the positive energy balance, which directed to the white adipose tissue for the energy storage in the form of triglyceride, eventually increasing the lipid accumulation and creation of new adipocytes in the body. Enlarged adipocytes will then release paracrine factor which may induce growth and differentiation of neighboring pre-adipocytes, and eventually strengthen the adipose tissue reservoir (Sorisky, Magun and Gagnon, 2000).

Sedentary lifestyle and physical inactivity are one of the key determinants that contribute to the development of overweight and obesity. A modern living environment makes people tend to be physically inactive. A person with less or no physical activity has less energy expenditure that contributes to the energy imbalance if the individual maintain or exceed the energy intake (Faith and Kral, 2006). Individual tends to have a sedentary pastime during the leisure time. Video games, movies, television and surfing internet has becoming their preferred pastimes. The screen-based sedentary lifestyles decrease the chances

to be physically active. Viewing television for two hours or more per day has been related to overweight and obesity (National Heart, Lung and Blood Institute, 2012). A study showed that increased television viewing was significantly associated with rise in calorie intake and weight (Epstein, et al., 2008; Francis, Lee and Birch, 2003). Research suggests that being physically active may decrease the chance of getting obesity-related diseases (National Institute of Diabetes and Digestive and Kidney Diseases, 2012).

Moreover, alcohol consumption has becoming a concern to the public regarding the health and social issues. Alcohol can be utilized as the source of energy as it contributes to 10% of the calorie intake of those adult drinkers (National Obesity Observatory, 2012). Various studies showed that consumption of small amount of alcohol beverages is not associated with weight gain. According to study done in 2005, minimum alcohol consumption (one drink per day) had a lower BMI than those heavy drinkers. Meanwhile, heavy drinking is more consistently associated with overweight and obesity (Traversy and Chaput, 2015). However, alcohol intake as a risk factor for obesity and overweight remained uncertainty because approximately equal amount of epidemiologic data showed no, negative or positive association between them (Croezen, et al., 2009; Suter, 2005).

Other than alcohol intake, smoking is one of the preventable causes of death. Smoking poses a negative effect to health but it protects the smoker from being overweight and obesity. Based on a cross-sectional study involving United Kingdom middle-aged adults, current smokers are less likely to develop

overweight and obesity compared to non-smoker. In addition, the prevalence of former smokers to be overweight and obese are higher than both non-smokers and current smokers (Dare, Mackay and Pell, 2015). Obesity is negatively associated with smoking which can be explained by the presence of nicotine in the cigarette smoke. Nicotine tends to enhance body metabolism and suppress appetite, leading to less eating and resulting in the negative net energy (Chiolero, et al., 2008). Smoking cessation tends to increase a person's body weight due to reduction of nicotine (Filozof, Pinilla and Fernández-Cruz, 2004).

### 2.4 Genetic factors of obesity

Other than environmental factors, genetic predisposition also plays a significant role in overweight and obesity by influencing intake and expenditure of energy. Risk of developing a disease or phenotype including obesity is dependent on the genetic composition of a person when exposed to a same environment (Faith and Kral, 2006). According to thrifty gene hypothesis in study of prevalence of obesity proposed by Neel (1962), thrifty genes in the early evolutionary are beneficial to the population. Thrifty genes allow deposition of lipid and their holders are able to survive during shortage of food. However, holding thrifty genes in the modern culture are disadvantageous and promote development of obesity. This is because thrifty genes help to accumulate the fat in preparation for shortage of food which is no longer exist as a problem now (Speakman, 2008).

Obesity-related phenotype can be inherited from one generation to another generation. The heritability of variation in obesity-related phenotypes such as

BMI is approximately 40–70% based on families, twin and adoption studies (Maes, Neale and Eaves, 1997; Atwood, et al., 2002; Wangensteen, et al., 2005). A clear relationship between children and their parents has been proven by various studies including Framingham study, Quebec family study, Canadian fitness study, Norwegian family study and four large family studies. These studies concluded that obese parents gave birth to the obese children in a higher proportion (Scrensen, 1995). Offspring whose parents were obese had double to triple risk of developing moderate obesity and experienced eight-fold increase of risk for severe obesity (Marti, et al., 2004). Twin studies reported that dizygotic twins had lower correlation in BMI as compared to monozygotic twins, despite growing in the similar family environment (Scrensen, 1995).

Obesity can be classified into two groups based on the number of genes involved in developing obesity. Monogenic obesity is a rare and severe form of obesity as defect in one gene is sufficient to show obesity-related traits (Choquet and Meyre, 2011; Farooqi and O'Rahilly, 2007; Hinney, Vogel and Hebebrand, 2010). However, development of polygenic obesity is due to the defect in multiple common and interacting alleles. Each allele might contributes to a minor effect, either additive or non-additive (Reich and Lander, 2001).

### 2.5 Genome-wide association study (GWAS) of obesity-related genes

GWAS involves the rapid screening of phenotype-related markers or genetic variants across a genome. GWAS has been fully utilized to identify disease susceptibility due to the genetic variations (Bush and Moore, 2012). It is a high-throughput methodology in study of polygenic obesity. GWAS provides a better

platform in identification of genetic variants which poses a risk of developing obesity. Detection of polygenic variants can be done within a short period, by using high density SNP-chips that is able to scan millions of SNP markers at one time (Rao, Lal and Giridharan, 2014; Xia and Grant, 2013).

Currently, there are a total of 62 publications regarding GWAS that involved obesity (National Human Genome Research Institute, 2017b). Fat mass and obesity associated (*FTO*) gene was the first identified non-syndromic obesity gene by GWAS. *FTO* gene variant increases the risk of obesity by changing the preference and intake of food (Loos and Yeo, 2014). Catenin, beta-like 1 (*CTNNBL1*) was identified as a novel gene that was strongly associated with BMI and fat mass through a GWAS study involved 1000 unrelated US Caucasian adults. This finding may indicate the involvement of Wnt signalling pathway in development of obesity (Liu, et al., 2008). GWAS approaches also identified two novel genes which are *CDKAL1* and *KLF9* that were associated with BMI in East Asian population (Okada, et al., 2012). GWAS study that involved 339224 subjects had identified 56 novel BMI-related genes ($p<5\times10^{-8}$) and most of them were significantly associated with other metabolic phenotypes (Locke, et al., 2015). Seventeen loci associated with BMI are listed in Table 2.2.

**Table 2.2:** Summary of 17 obesity loci.

| Loci | Chromosomal location | Proposed function |
|---|---|---|
| *NEGR1* | 1p31 | Neuronal outgrowth |
| *SEC16B, RASAL2* | 1q25 | - |
| *TMEM18* | 2p25 | Neural development |
| *ETV5* | 3q27 | - |
| *GNPDA2* | 4p13 | - |
| *PRL* | 6p22.2–p21.3 | - |
| *NCR3, AIF1,BAT2* | 6p21 | - |
| *PTER* | 10p12 | - |
| *BDNF* | 11p14 | Nutritional state and *MC4R* signaling regulate the expression |
| *MTCH2* | 11p11.2 | Cellular apoptosis |
| *FAIM2, BCDIN3D* | 12q13 | Apoptosis of adipocytes |
| *SH2B1, ATP2A1* | 16p11.2 | Neuronal regulation in energy homeostasis |
| *MAF* | 16q22-q23 | Transcription factor involved in insulin-glucagon regulation and adipogenesis |
| *FTO* | 16q22.2 | Neuronal control of appetite |

**Table 2.2** (continued).

| Loci | Chromosomal location | Proposed function |
|---|---|---|
| *NPC1* | 18q11.2 | Transportation of intracellular lipid |
| *MC4R* | 18q22 | Hypothalamic signaling |
| *KCTD15* | 19q13.11 | - |

(Hofker and Wijmenga, 2009)

During this study, Scopus database was utilized as a searching tool to look for the obesity-related articles. The Boolean operator used to search for the related journal articles was GWAS AND (obes*) AND (Chinese OR asia*) AND (association OR gene*). The search was delimited to the field of life sciences and health sciences. A total of 40 journals were found and all of publication in within the year of 2010 to 2015. The obesity-related studies were selected if it involved genetic association study and the population studied were Asian or Chinese. A total of 19 out of 40 studies were included, which seven of them involved Chinese population whereas twelve of them involved Asian population. Based on the 19 publications, a total number of 101 genes were studied in order to determine the association between the related SNPs with obesity in both Chinese and Asian population. Figure 2.2 and Table 2.3 demonstrates the number of studies in obesity of each gene.

Among 101 genes, 71 of them had only been studied once and 17 of them had been studies twice. *FTO* is the most well studied gene as it had been studied for 10 times in these 19 included publications. Criteria including the gene needs to

be less well studied and had at least three replicative studies, were set in order to select a gene for further studies. Based on the criteria, *FTO*, *MC4R*, *KCTD15*, *GNPDA2* and *SH2B1* were excluded as significant association with obesity had been showed. *FAIM2* was chosen as it fulfill the criteria set. The SNP related to *FAIM2* and to be further studied is rs7138803.

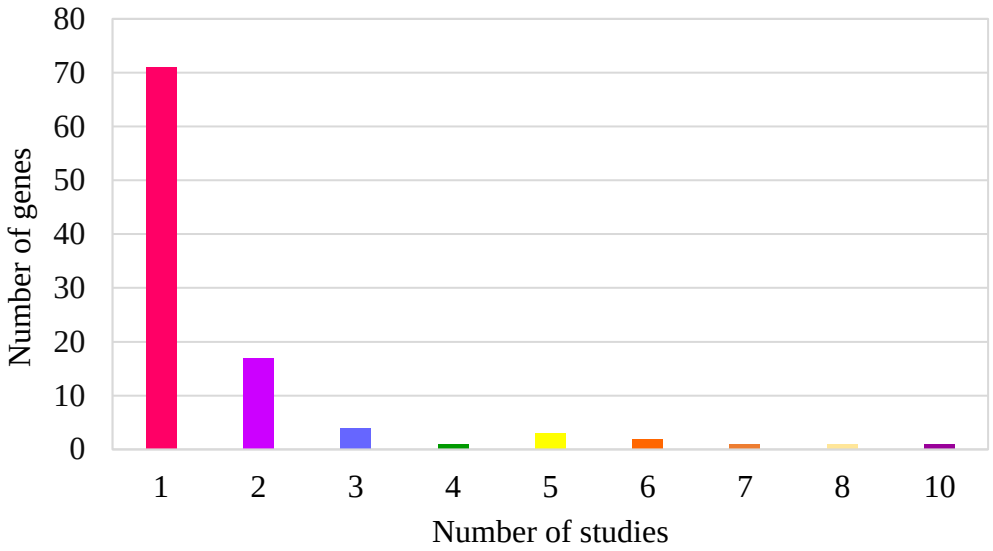


**Figure 2.2**: Bar chart of the number of studies performed for the candidates genes involved in obesity.

**Table 2.3**: Number of studies in obesity of each gene.

| Number of study | Number of Gene | Gene name |
|---|---|---|
| 1 | 71 | *ADAMTS9, ADCY5, ALCAM, APOE-C1-C4-C2, ATP11B, BCL2, BCL11A, BTG1, BUD13-ZNF259, CDC123/CAMK1D3, CDH13, CETP, CELSR2-PSRC1-SORT1 , CENTD2, CMYA5, CNBD1, CTNNBL1, CTSO, CYP7B1, C11orf53, DACH1, DUSP9, ETV5, FAM5C, FLJ38723, GATAD2A, GP2, HNF1A, HNF1B (TCF2) 1, HMGA2, HHEX/IDE2, JAZF13, IGF2BP21, IRS1, KCNJ117, KCNQ1, KIAA1822L, KLF14, LEPR, LOC400120, MAF, MDFIC, MLXIPL, MTIF3, NDUFS3, CUGBP1, NOTCH2, NUDT12, OR4K17, PPARD, PRC1, PTPRD, RBMS1, RBJ, RELN, RPL27A, SLC30A8, SLIT1, SKOR1, TCF7L2, THADA, TLE4 (CHCHD9) ,TMEM160, TNKS, MSRA, TP53INP1, TSPAN8/LGR5, WFS1, ZNF259, ZFAND6, ZBED3* |
| 2 | 17 | *ADCY3/RBJ, BAT2, CDKAL1, CDKN2A/B, DGKB/TMEM1954, FLJ35779, GCKR, GIPR/QPCTL, LRRN6C, NPC1, NUDT3, PPARG, PROX1, PTBP2, QPCTL, TFAP2B, TNNI3K* |
| 3 | 4 | *FAIM2, MAP2K5, MTNR1B, PCSK1* |
| 4 | 1 | *BDNF* |
| 5 | 3 | *MTCH2, NEGR1, SEC16B* |
| 6 | 2 | *GNPDA2, SH2B1* |
| 7 | 1 | *KCTD15* |
| 8 | 1 | *MC4R* |
| 10 | 1 | *FTO* |

## 2.6 Gene structure of *FAIM2*

As shown in Figure 2.3, *FAIM2* is a protein coding with the cytogenic location of 12q13.12 with exon count of 14 (National Center for Biotechnology Information, 2017). It is also known as lifeguard protein 2 (*LFG2*). *FAIM2* provides unique protection to the cells through regulation of Fas-mediated apoptosis by interfering the activation of caspase-8. It may also play a part in development of cerebellum by affecting the size of cerebellum, thickness of internal granular layer and development of Purkinje cell (UniProt, 2017).


**Figure 2.3:** *FAIM2* gene. The exons are labeled from 1 to 14 (National Center for Biotechnology Information, 2017).

*FAIM2* rs7138803 is an intergenic gene variant (GCTTGGGCATAATGAATTCCACAAG[A/G]TCACCCTGCTGTGCAAA GTATTTCA) found near to the *FAIM2* gene at the chromosome 12:49853685 (National Center for Biotechnology Information, 2017). The ancestor allele for rs7138803 is G allele while the effect allele is A allele. The minor allele frequency for rs7138803 is 0.26. The gene variant is significant associated with BMI, WC, weight, obesity and menarche (Ensembl, 2017). Its dbSNP HGVS names are NC_000012.11:g.50247468G>A and NC_000012.12:g.49853685G>A, meanwhile the Ensembl HGVS name is NC_000012.12:g.49853685G>A. According the F-SNP database, there is no functional information available for rs7138803 yet (Queen University, 2017).

### 2.7 Studies on *FAIM2* and its SNP rs7138803

*FAIM2* is a gene associated with obesity but the mechanism of involvement of *FAIM2* in obesity is not understood. Epigenetic alterations are important in the obesity development. A recent study found out that there is significant association between methylation of *FAIM2* promoter with obesity and dyslipidemia. A cohort study proved the significant association in *FAIM2* promoter methylation with sedentary lifestyle in obese and non-obese children (Wu, et al., 2015a). Fifty-nine obese and thirty-nine lean children were involved and 8 CpG site were examined (Wu, et al., 2015b). Other than that, *FAIM2* showed highest expression in the hypothalamus, which were significantly different from the various fat tissue including mesenterial and subcutaneous fat. By using a Zucker rat model, changes in adipose tissue seem to be liable for the development of phenotype of Zucker diabetic fatty rat due to the differences between *FAIM2* expression in various fat tissue and similarity of the *FAIM2* expression in both fat and lean rat (Schmid, et al., 2012).

GWAS studies were also fully utilized in determination of association between rs7138803 and obesity. Based on the GWAS catalog, there are nine GWAS studies which involved *FAIM2* rs7138803 (National Human Genome Research Institute, 2017a). A GWAS study that involved 32 SNPs, screening 249796 subjects proved that *FAIM2* rs7138803 was significantly associated with BMI at genome-wide significance of $p<5\times10^{-8}$ (Speliotes, et al., 2010). A longitudinal replication studies of GWAS showed significant association between rs7138803 and longitudinal childhood BMI with the *p*-value of 0.004. The risk effects of allele G of rs7138803 was significantly reduced with

increasing age (Mei, et al., 2011). Besides, another GWAS study was also performed involving six BMI-associated loci in Japanese population. SNP rs7138803 was found marginally associated with obesity in Japanese adult population with *p*-value less than 0.05 (Hotta, et al., 2009).

Association of BMI, weight-to-height ratio and WC with rs7138803 in Chinese children population were identified. BMI (adjusted for age and sex) was significantly associated with rs7138803 under additive model after adjusting for multiple testing (*p*-value=0.0097). Weight-to-height ratio and WC were also significantly associated rs7138803 (Wu, et al., 2010). There was significant association between rs7138803 with weight, BMI, and WC for rs7138803 with *p*-values of $4.9\times10^{-7}$, $8.2\times10^{-7}$ and $7.6\times10^{-8}$, respectively (Sandholt, et al., 2011). Study done by Corella and team confirmed the association of obesity for *FAIM2* rs7138803 and a novel and consistent relationship with heart beat in subjects with type 2 diabetes was identified (Corella, et al., 2014).

**2.8 FAIM2 protein**

*FAIM2* gene is made up of 37322 bases. *FAIM2* reading frame encodes for an evolutionary well-conserved protein FAIM2 which is 316-amino acid long. FAIM2 was first discovered during the identification of inhibitory molecule in Fas signaling by using human lung fibroblast cell line (Reimers, et al., 2006; Somia, et al., 1999). FAIM2 was predicted as a seven membrane-spanning protein based on the hydropathy plots. It is evolutionarily well-conserved and was found highly homologous to mouse and rat at the percentage of 91% and 92%, respectively after protein alignment (Reimers, et al., 2006; Somia, et al.,

1999). FAIM2 was suggested to be a membrane associated protein after examination using confocal microscopy that reveal the accumulation of overexpression of FAIM2 in plasma membrane. C-terminal contains a consensus motif from a conserved hydrophobic transmembrane protein, UPF0005 that mediate their localization to membrane. The structure of FAIM2 resembles the BAX inhibitor 1 (BI-1), an anti-apoptotic protein (Reimers, et al., 2006; Somia, et al., 1999).

### 2.8.1 Functions of FAIM2 protein

FAIM2 is an anti-apoptotic protein which acts as an inhibitor of Fas apoptotic signaling pathway. Apoptosis is a programmed cell death that occurs under a normal regulation. As shown in Figure 2.4, Fas-mediated apoptotic signaling pathway is activated when Fas ligand (FasL) bind to the Fas receptor (FasR). Upon activation of the signaling pathway, caspase-8 cleaves Bap31 into p20 fragment and Bid into tBid. The p20 fragment will bind to the endoplasmic reticulum (ER) and induces the efflux of calcium ions ($Ca^{2+}$) to the mitochondria. At the same time, tBid stimulates the permeabilization of mitochondria and release of cytochrome C (Cyt c). Caspase 3 is then activated by Cyt c released and induce apoptosis. FAIM2 is only able to inhibit the ER step. This had been proved by experimental studies. Overexpression of *FAIM2* exert its anti-apoptotic effect by inhibiting the release of $Ca^{2+}$ from ER. In addition, the effect Bcl-$X_L$ on released of $Ca^{2+}$ appeared to be relied on expression of *FAIM2*. Overexpression of *FAIM2* inhibit the release of Cyt c from mitochondria due to the reduction in $Ca^{2+}$ released. *FAIM2*-transduced

cells showed obvious decreased in Cyt c released after Fas stimulation (Elmore, 2007; Planells-Ferrer, et al., 2016; Urresti, Ruiz-Meana, and Coccia, 2016).

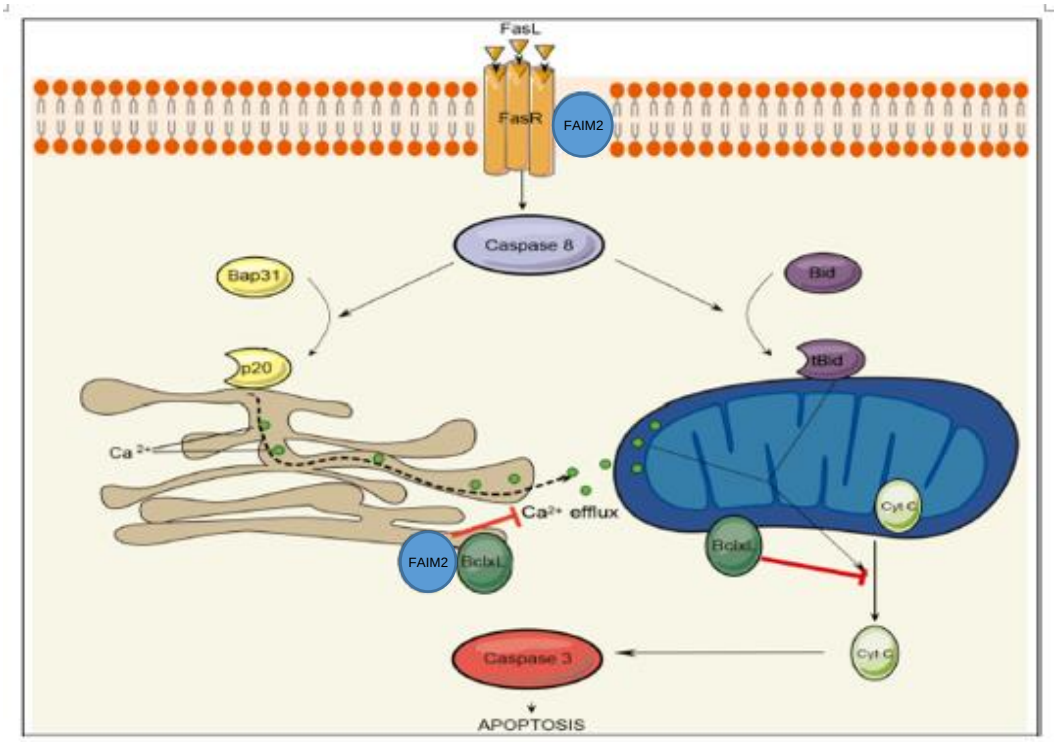


**Figure 2.4**: Schematic diagram of Fas apoptotic signaling pathway and the hypothetic steps suppressed by FAIM2 (Urresti, et al., 2016)

*FAIM2* anti-apoptotic role in nervous system was first established in 2015 that involved silencing of *FAIM2* in rat cerebellar granular neurons. *FAIM2* was silenced by short-interfering RNAs that induce the cleavage of caspase 8 after Fas stimulation and eventually led to cell death. Expression of *FAIM2* in neuron is dependent on the phosphatidylinositol 3 kinase-Akt (PI3K-AKT) pathway (Beier, Wischhusen and Gleichmann, 2005).

Moreover, another possible function of FAIM2 is as a calcium leak channel. FAIM2 is highly homologous to transmembrane Bax inhibitor motif (TMBIM). It is possible to form a pH-sensitive calcium leak channel from the C terminal

region of FAIM2 in endoplasmic reticulum. The important amino acid that accounts for this activity is aspartic at position 183 and 213 (Chang, et al., 2014).

*FAIM2* is highly expressed in hippocampus which may affect the neural development of feeding (Li, et al., 2013). *FAIM*2 may also play an essential role in the development of cerebellum by influencing the size of cerebellum, thickness of internal granular layer and development of Purkinje cells. During postnatal development of brain, transcripts and protein of FAIM2 are intensely upregulated and are highly expressed in Purkinje cells and cerebellar granule neurons Schweitzer, Suter and Taylor, 2002). Proper development and survival of Purkinje cells and cerebellar granule neurons require the presence of FAIM2 that prevents the apoptosis of neurons (Hurtado de Mendoza, et al., 2011).

Furthermore, FAIM2 has been associated with disease including cancers due to its anti-apoptotic role. Role of FAIM2 was studied in breast cancer models and demonstrated the anti-apoptotic role of FAIM2 in cancer progression. FAIM2 prevents tumor cell from Fas-mediated programmed cell death and increases the aggressiveness of cancer cells (Bucan, et al., 2010). Other than breast cancer, its role in neuroblastoma aggressiveness was also discovered. Downregulation of *FAIM2* led to the reduction in cell adhesion, enhanced the growth of cancer cells and promoted migration of cells and metastasis (Planells-Ferrer, et al., 2014).

Currently, extensive efforts have been invested into the development of novel diagnostic marker and potential therapeutic target especially for cancers such as

small-cell lung carcinoma, by utilizing the characteristic of *FAIM2* (Kang, et al., 2016). Identification of role of *FAIM2* in obesity is also an important field, but there is limited studies which provide evidence for the role of *FAIM2* in the lipid and metabolic traits. Thus, overexpression of *FAIM2* in adipocyte and association study between obesity and *FAIM2* rs7138803 among UTAR population was carried out in this study for a better understanding on the gene and its SNP.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Research Methodology

This study was divided into 2 parts which are genotyping of *FAIM2* rs7138803 and overexpression of *FAIM2*. The overview of the methodology of this study is summarized in a flow chart as shown in Figure 3.1.

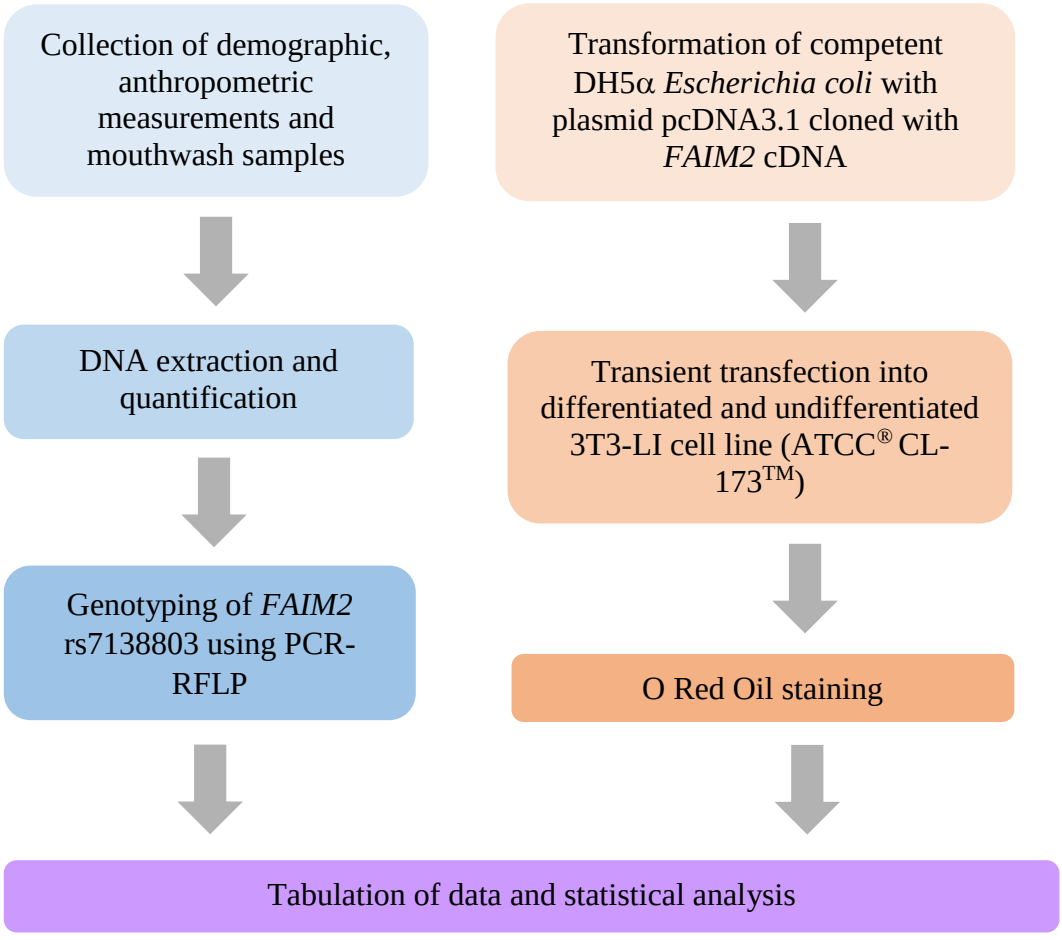


**Figure 3.1:** Overview of experimental flow of this study.

### 3.2 Genotyping of *FAIM2* rs7138803

#### 3.2.1 Specimen and sample population

The DNA specimen used in this study was extracted from buccal cells. Ethical approval (Appendix A) for the study involving materials and data of human subjects was granted by the UTAR Scientific and Ethical Review Committee (SERC) before the commencement of study. In this study, ethical principles was followed as stated in the Declaration of Helsinki.

Convenience sampling was selected as the mode of sampling in this study in order to collect the data of demographic and anthropometric measurement, as well as the mouthwash sample as DNA sample for genotyping of *FAIM2* rs7138803 SNP. It is a non-probability sampling that depends on data collection from subjects who are conveniently available and accessible to take part in this study.

Distribution of consent forms (Appendix B) was done prior to the buccal cells collection using mouth wash method. Explanation of procedures, risks involved, and their privacy and confidentiality rights to the participants was performed prior collection of mouth wash sample. Signature on the consent form upon approval indicated the participant was aware of the risks and agreed to the genomic DNA extraction from the collected buccal cells.

In this research, UTAR students aged between 18 and 30 were selected as the sample population. A total of 502 consenting participants were involved in this

study by completing the distributed questionnaire and donating the buccal cells for extraction of DNA.

### 3.2.2 Demographic and anthropometric measurement

Demographics (Appendix C) including age, gender and ethnicity were used to study the sample population with an approximate of 17000 students. Anthropometric measurement (Appendix C) were performed twice in order to obtain the average readings of each measurements. Upon consent, the blood pressure (mmHg) and pulse rate (bpm) of the participant was measured by using a blood pressure monitor exact model (Omron, Japan). The normal range of pulse rate for adult is ranged between 60 to 100 beats per minute. Well-trained athletes might have lower pulse rate ranged due to better efficiency of heart function (American Heart Association, 2016). A normal healthy adult should have blood pressure lower than 120/80 mmHg (American Heart Association, 2017).

Waist circumference (WC, cm), hip circumference (HC, cm) and height (cm) were measured using a measuring tape. The combination of WC and BMI is better in prediction of variance in health risk than BMI alone. WC was measured at the midpoint between the lower region of last palpable ribs and the uppermost of the iliac crest (World Health Organization, 2008). The cut-off points for abdominal obesity for males and females were 90 cm and 80 cm, respectively (Misra, et al., 2006).

Weight (kg), body mass index (BMI, kg/m$^2$), total body fat (TBF, %), subcutaneous fat (SF, %), visceral fat level (VFL, %), skeleton muscle (SM, %) and resting metabolism (RM, kcal) were measured using Karada Scan body composition monitor (Omron, Japan). By comparing weight and BMI, BMI was more reliable to be used as it involved adjustment of weight with height. BMI was calculated by dividing weight of subject (in kg) with the square of height (in m$^2$). Asian population with BMI in between 23 kg/m$^2$ and 24.9 kg/m$^2$ is classified as overweight, whereas individual with BMI more than 25 kg/m$^2$ is considered obese (Aziz, Kallur and Nirmalan, 2014).

BMI might not be the most suitable parameter to indicate overweight and obesity. Some individuals especially body builders and athletes might have BMI within overweight range, but they have high muscle mass instead of body fat. Measurement of TBF was more reliable in determining overweight and obesity (Blaak, 2014). The healthy percentage of TBF for young men and women are 12% - 15% and 25% - 28%, respectively. The cut-off points of TBF for obesity are 20% and 30% for men and women, respectively (Omron, n.d.).

### 3.2.3 Questionnaire: environmental exposure

Questionnaire (Appendix C) regarding the environmental factors related to overweight and obesity was completed by the consenting participants. Data including frequency of vigorous activity per week, the duration spend in front of computer or television, frequency of alcohol consumption and smoking status of the subjects were obtained through the questionnaire.

### 3.2.4 Collection of buccal cells

Consenting participants were advised to rinse their mouth with tap water briefly before the collection of mouth wash sample to eliminate contaminants such as food particles that may affect the purity of DNA. The participants were requested to rinse their mouth vigorously with 5 mL of sterile 3% sucrose solution (PROCHEM, USA) and rubbed their tongue on the oral mucosa while rinsing. After gargling for at least 1 min, the wash was spitted into the centrifuge tube. After that, 3 mL of TNE buffer containing 17 mM Tris/HCl (pH8.0) (Bio Basic Inc., Canada), 50 mM sodium chloride (HmbG Chemicals, Malaysia) and 7 mM EDTA (SYSTERM ChemAR, Malaysia) that diluted in 66% ethanol (Copens Scientific (M) Sdn. Bhd., Malaysia) was added into the centrifuge tube (Aidar and Line, 2007). The sample was stored at -20°C freezer before the subsequent genomic DNA extraction.

### 3.2.5 Genomic DNA extraction and quantification

Extraction of genomic DNA from the collected buccal cells was performed following manufacturer's protocol by using Wizard® Genomic DNA Purification Kit (Promega, USA). After extraction, the concentration and purity of the extracted DNA was determined using the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, USA) at the wavelength of 260 nm and 280 nm as nuclei acid absorbs maximally at these wavelengths. One microliter of the extracted genomic DNA was transferred to the measurement window and the window was covered with the 1 mm path length cover. The sterile deionized water was used as blank. The ratio of $A_{260}$ and $A_{280}$ is commonly used to check

for the purity of DNA. Indication of a pure DNA is the ratio of $A_{260}$/A280 within the range of 1.8 to 2.0 (GE Healthcare Life Sciences, 2016).

### 3.2.6 Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP)

A pair of primers were used for the Polymerase Chain Reaction (PCR) and the sequence of primers are as shown in Table 3.1. The reaction mix for the PCR containing PCR buffer (BioTeke Corporation, China), magnesium ion ($Mg^{2+}$) (BioTeke Corporation, China), deoxynucleotides (dNTPs) (BioTeke Corporation, China), forward and reverse primers (Integrated DNA Technologies, Inc., USA), *taq* polymerase (BioTeke Corporation, China), genomic DNA and appropriate amount of sterile deionized water. The volume and concentration of each components in reaction mix are Table 3.2.

**Table 3.1:** Sequences of forward and reverse primers used for Polymerase Chain Reaction.

| Primer | Primer sequence | Desired product size |
|---|---|---|
| Forward | 5’ -GCATCCTGTTTCCCTTCT-3’ | 278 bp |
| Reverse | 5’-CCATTCACTACCTGCCTTC-3’ | |

(Huang, et al., 2012)

**Table 3.2:** Components of the reagent mix for PCR.

| PCR components | Stock concentration | Final concentration | Volume (μL) |
|---|---|---|---|
| PCR Buffer | 10 × | 1 × | 2.0 |
| Magnesium ions ($Mg^{2+}$) | 25 mM | 1.25 mM | 1.0 |
| Deoxynucleotides (dNTPs) | 10 μM | 0.20 μM | 0.4 |
| Forward primer | 10 μM | 0.25 μM | 0.5 |
| Reverse primer | 10 μM | 0.25 μM | 0.5 |
| Taq polymerase | 5 U/μL | 0.50 U/μL | 0.2 |
| Genomic DNA | 50 ng/μL | 2.50 ng/μL | 1.0 |
| Sterile deionized water | | | 14.4 |
| **Total** | | | 20.0 |

Optimization of the annealing temperature to be used in the PCR reaction was performed prior to the screening of samples. Various annealing temperatures ranged from 50°C to 70°C were used during the optimization process. After determination of optimum primer annealing temperature, PCR reaction was carried out to amplify the target of interest by using thermos cycler (Biorad, California) based on the condition showed in Table 3.3. The entire process took two hours to complete. The PCR products were subjected to gel electrophoresis.

**Table 3.3:** Condition used in each PCR reaction after optimizing.

| Steps | Temperature (°C) | Duration (sec) | Cycles |
|---|---|---|---|
| Initial denaturation | 95 | 300 | 1 |
| Denaturation | 95 | 30 | 40 |
| Annealing | 68 | 30 | 40 |
| Extension | 72 | 60 | 40 |
| Final extension | 72 | 600 | 1 |
| End | 12 | Until electrophoresis | 1 |

Consecutively, the PCR amplicons were digested with *PspE*I from Vivantis, USA. The recognition site of the *PspE*I is shown in Figure 3.2. Each reaction had the total volume of eight microliter which consists of 7 μL of PCR product, 0.9 μL of *PspE*I buffer and 0.1 μL of *PspE*I. The samples were incubated in a water bath with 37°C for overnight. The digested samples were then subjected to gel electrophoresis.

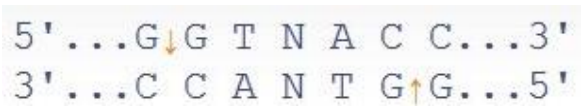


**Figure 3.2**: Recognition site of restriction enzyme *PspE*I (Thermo Fisher Scientific, 2017).

### 3.2.7 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to separate and qualify the presence of desired amplified PCR products and the size of RFLP products after restriction digestion. Approximately 5 μL of PCR or RFLP product was mixed with one microliter of 6× loading dye and was loaded onto a 2% agarose gel (Hydragene, China) and electrophoresed at 90V for 50 min using electrophoresis set from Major Science, USA. First well of the gel was loaded with one microliter of GeneRuler 100 bp DNA Ladder (Thermo Scientific, USA). SB buffer which made up of boric acid (EMSURE®, USA) and sodium hydroxide (Sigma-Aldrich, US) was used as the electrophoresis buffer. The gel was then stained with GelRed solution (Midori Green, Portsmouth) for 10 min. The presence of bands were detected by exposing the gel to ultraviolet light by using gel imager (Bio-Rad, ChemiDoc™ XRS+ System, US).

The ideal band size of the PCR amplicons is 278 bp. The presence of the desired PCR amplicons was identified through eye estimation by comparing to the DNA ladder. Genotype of subjects was determined based on the gel electrophoresis of RFLP products. The wildtype (GG) genotype showed two bands: 150 bp and 128 bp. Three bands: 278 bp, 150 bp and 128 bp were visualized for the heterozygous (GA) Genotype while the homozygous mutant (AA) showed only one band which is 278 bp. All the bands were separated properly on the agarose gel. The cutting site of the restriction enzyme in the PCR amplicon is illustrated in Figure 3.2.

| |
|---|
| Wildtype sequence (150 bp, 128 bp)<br><br>5' –<br>*GCATCCTGTTTCCCTTCT*TAACTCACCATGGATTTACTTCTGTGAAT<br>CTTTCTCAATGTATTTCGAGCCTACTTATGTTTTCGCACATGCCAC<br>TGCTTGGGCATAATGAATTCCACAAG↓GTCACCCTGCTGTGCAAA<br>GTATTTCATAGAATCATAGAATCTTACATTTTTATTTATCATTGTT<br>CAAACCCAAAGGATTCCCTGCACTCAAGAATTATACAAGGTGTG<br>TTCAGGGCAGTGAGGCTGAGGAAAGGTCTGT*GAAGGCAGGTAGT*<br>*GAATGG* – 3' |
| Mutant sequence (278 bp)<br><br>5' –<br>*GCATCCTGTTTCCCTTCT*TAACTCACCATGGATTTACTTCTGTGAAT<br>CTTTCTCAATGTATTTCGAGCCTACTTATGTTTTCGCACATGCCAC<br>TGCTTGGGCATAATGAATTCCACAAGATCACCCTGCTGTGCAAA<br>GTATTTCATAGAATCATAGAATCTTACATTTTTATTTATCATTGTT<br>CAAACCCAAAGGATTCCCTGCACTCAAGAATTATACAAGGTGTG<br>TTCAGGGCAGTGAGGCTGAGGAAAGGTCTGT*GAAGGCAGGTAGT*<br>*GAATGG* – 3' |

**Figure 3.3**: Cutting site of the *PspE*I and the expected fragment sizes. The green downward arrow "↓" indicate the recognition site of the *PspE*I. The letter highlighted in yellow represents the SNP of the sequence. Primer sequences appear in red italicized form.

### 3.2.8 Statistical analysis

The data obtained during the study was analyzed by using IBM Statistical Package for the Social Sciences 20. Demographic data (gender and ethnicity), genotype and alleles were classified as the categorical variables. The anthropometric measurements data were categorized under the continuous variables. Continuous data with normal distribution were tested using Kolmogorov-Smirnov test. Meanwhile, log-transformation of variables that are

not normally distributed were performed before testing. Moreover, Pearson's Chi-Square test was employed to determine the significance of association of the genotypes and polymorphism with obesity. The association between rs7138803 allele distribution and classes of demographics and anthropometric was shown. The allelic and genotypic frequencies were calculated. Genotypes frequency was calculated in order to determine the Hardy-Weinberg equilibrium and Chi-Square test was performed on the expected and observed values. In addition, the significant difference of the mean values was calculated by using univariate analysis of variance which involve the usage of age and ethnicity (as the covariance) between the outcome variables. The adjusted means of anthropometric measurements for rs7138803 genotype allele was tested for its statistical significance. When performing stratified analysis of mean, non-parametric Kruskal Wallis and parametric One-Way ANOVA tests were used to analyze the means ± standard deviation of means and *p*-values that obtained the anthropometric measurements. The *p*-value of statistical significance was set at less than 0.05.

### 3.3 Overexpression of *FAIM2*

#### 3.3.1 *FAIM2* cDNA construct propagation

The *FAIM2* cDNA construct was purchased from Genscript Biotech Corporation, USA. *FAIM2* cDNA (NCBI Reference Sequence: XM_005268730.2) is cloned into multiple cloning site of pcDNA 3.1$^{+}$/C-(K)-DYK plasmid. The pcDNA 3.1$^{+}$/C-(K)-DYK vector map is shown in Figure 3.4. The size of the pcDNA 3.1$^{+}$/C-(K)-DYK cloned with *FAIM2* cDNA is 6269 bp as the size of *FAIM2* cDNA cloned into the plasmid is 825 bp.

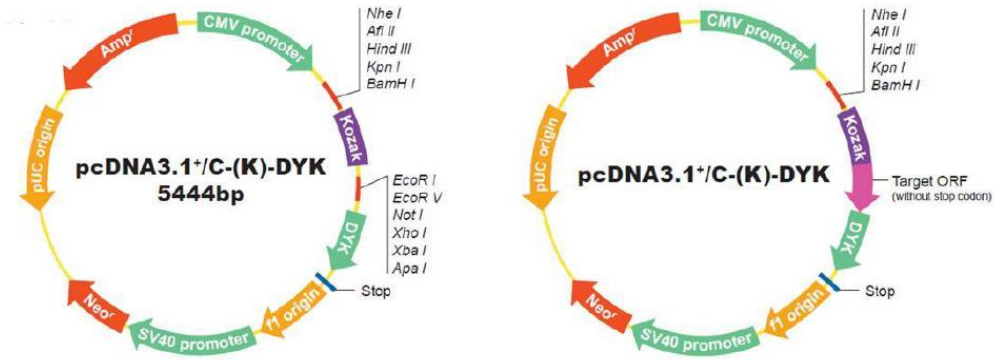


**Figure 3.4**: pcDNA 3.1$^{+}$/C-(K)-DYK vector map (GenScript, 2016).

Before use, the vial containing the pcDNA 3.1$^{+}$/C-(K)-DYK cloned with *FAIM2* cDNA was centrifuged at 6000 ×g for 60 sec at 4°C. After that, 10 µg of lyophilized plasmid DNA was dissolved in 100 µL of sterile deionized water and stored at -20°C

### 3.3.2 Transformation of DH5α *E. coli* with plasmid DNA containing *FAIM2* cDNA

DH5α *E. coli* was allowed to thaw on ice for 15 min. The tube was tapped gently multiple times to obtain uniform suspension. One microliter of *FAIM2* cDNA plasmid DNA was added directly to approximately 50 μL of DH5α *E. coli* in microcentrifuge tube. The mixture was mixed gentle by tapping. The tube was then incubated on ice for half an hour. The tube was then incubated in 42°C water bath (Memmert, Germany) for 45 sec, without shaking. The tube was transferred on ice for 5 min. Five hundred microliter of LB broth (Fisher Scientific, USA) was then added into the microcentrifuge tube and incubated at 37°C, 250 rpm for 1 hr. After incubation, the tube was centrifuged at 3000 ×g for 5 min. Approximately 400 μL of the supernatant was discarded and the pellet was resuspended with the remaining supernatant. One hundred microliter of the cell suspension was transferred onto the LB agar (Merck, Germany) containing 100 ng/mL ampicillin (Bio Basic Inc., Canada) and was spread using sterile spreader. A negative control plate (with ampicillin) and a positive control plate (without ampicillin) was prepared using DH5α *E. coli*. The LB agar plates were incubated overnight at 37°C. After overnight incubation, a colony was picked and inoculated into 5 mL LB broth supplemented with 100 ng/mL of ampicillin. The tube was then incubated in a shaking incubator at 37°C, 250 rpm.

### 3.3.3 Extraction of recombinant plasmid DNA through alkaline lysis

The overnight culture was centrifuged at 900 rpm for 10 min and the supernatant was discarded. The pellet was resuspended with 200 μL of Solution I [50 mM Tris (pH 8.0) (R&M Chemical, UK), 10 mM EDTA, 100 μg/mL RNase A].

Then, 200 μL of Solution II [200 mM sodium hydroxide, 1% SDS (Bendosen, Malaysia)] was added and mixed by gently inverting. Approximately 200 μL of Solution III (3.0 M potassium acetate, pH 5.5) was added and mixed by gently inverting. Formation of white precipitate was observed. The tube was centrifuged at 14000 rpm for ten min then the supernatant was transferred to a sterile microcentrifuge tube. Next, 900 μL of 100% ethanol was added to the supernatant and inverted for several times to mix well. The tube was centrifuged at 14000 rpm for 20 min. The supernatant was discarded and the DNA pellet was air-dried for fifteen min. Lastly, the pellet was resuspended in 50 μL of TE buffer [10 mM Tris (pH8), 1 mM EDTA]. The concentration and the purity of the extracted plasmid DNA was measured using NanoDrop 2000 UV-Vis Spectrophotometer. The plasmid DNA was then stored in -20°C freezer.

### 3.3.4 Linearization of extracted plasmid DNA

The extracted plasmid DNA was linearized through restriction enzyme digestion by *BamH*I (Thermo Fisher Scientific, USA). A total volume of ten microliter of the reaction mixture contained 1 mg of plasmid DNA, 0.1 μL of *BamH*I and 0.9 μL of *BamH*I Buffer. The tube containing the restriction enzyme reaction mixture was incubated in 37°C water bath for overnight. Heat inactivation of the restriction enzyme activity was performed by incubating the tube in 80°C water bath for 20 min. After that, 5 μL of the mixture (containing loading dye) was loaded onto 1% agarose gel and was electrophoresed at 90 V for 45 min. One microliter of GeneRuler 1 kb DNA Ladder was loaded onto the first well of the gel. TAE buffer was used as the electrophoresis buffer. The gel was then stained with GelRed solution for 10 min. The presence of bands were

detected by exposing the gel to ultraviolet light by using gel imager. The linearized plasmid DNA containing *FAIM2* cDNA showed only one band: 6269 bp.

### 3.3.5 Cell line

3T3-LI Cell line (ATCC® CL-173™) which is the fibroblast cells from mouse (*Mus musculus*) was used in this study. The cryopreserved cells that were cultured in the appropriate culture medium was observed using an inverted phase contrast microscope to assure the condition of the cultured cells. Maintenance, and sub-culturing of 3T3-L1 cell line was performed according to the instruction from ATCC.

### 3.3.6 Cell culture medium and differentiation

Different medium were used at different cell culturing stage. The basic medium used in culturing 3T3-L1 cells was Dulbelcco's Modified Eagle's Medium (DMEM). DMEM with the volume of 900 mL was composed of 13.4 g DMEM powder (Corning, USA), 3.7 g sodium bicarbonate (Sigma-Aldrich, US) and 1% of Penicillin-Streptomycin mixed solution (Nacalai Tesque, Japan). The pH of the medium was adjusted to 7.4. There are three types of cell culture medium including, Pre-adipocyte Expansion Medium (PEM), Differentiation Medium (DM) and Adipocyte Maintenance Medium (AMM) were used in culturing 3T3-L1 cells. The formulation of each medium was showed in Table 3.4.

**Table 3.4:** Formulation of cell culture medium used.

| Medium | Composition |
|---|---|
| Pre-adipocyte Expansion (PEM) | 90% DMEM, 10% Fetal Bovine Serum (FBS) (Thermo Scientific, USA) |
| Differentiation (DM) | 90% DMEM, 10% FBS, 1 μg/mL insulin (Nacalai Tesque, Japan), 0.25 μM dexamethasone (Calbiochem, Germany), 2 μM rosiglitazone (TCL, Japan), 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX) (Calbiochem, U.S. and Canada) |
| Adipocyte Maintenance (AMM) | 90% DMEM, 10% FBS, 1 μg/mL insulin |

The differentiation of 3T3-L1 cells took 12 days to complete. PEM was used to maintain the seeded cells for 2 days. At Day 2, PEM was discarded and an equal amount of the DM was added. The cells were then incubated in a carbon dioxide incubator at 37°C, 5% (v/v) $CO_2$ for 2 days. At Day 4, an equal amount of AMM was replaced DM. Two days later, the medium was change to DM to induce differentiation again. After 48 hr, DM was discarded and equal amount of AMM was added. At Day 12, the cells were completely differentiated under the induction.

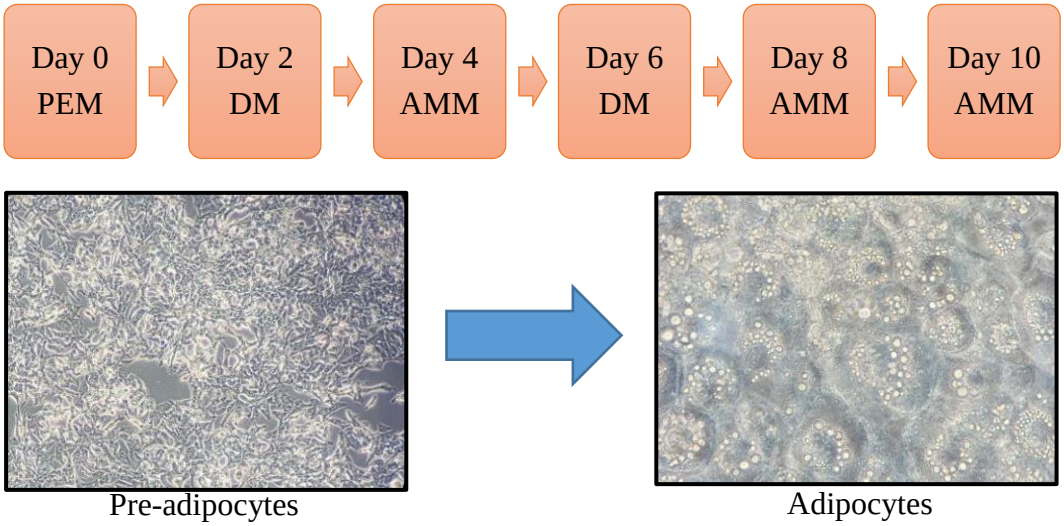


**Figure 3.5:** Protocol of differentiation of 3T3-L1 cells.

### 3.3.7 Thawing and maintenance of 3T3-L1 cells

Cryopreserved 3T3-L1 cells were thawed directly in 37°C water bath for about 2 min. One microliter of the thawed cells was transferred to a centrifuge tube containing 8 mL of DMEM and 1 mL of FBS and were gently resuspended. The cells were then centrifuged at 500 ×g for 10 min and supernatant was discarded. The cell pellet was then resuspended in 1 mL of DMEM. The resuspended cells were then added into a 25 $cm^2$ cell culture flask containing 2.6 mL of DMEM and 400 µL of FBS. The flask was then incubated in a carbon dioxide incubator (C150, BINDER, Germany) at 37°C, 5% (v/v) $CO_2$. An inverted phase contrast microscope (Olympus, Japan) was used to observe the cells regularly. The cell culture medium was replaced every two days to maintain the healthy level of the cell.

### 3.3.8 Sub-culturing of 3T3-L1 cells

When the cells reached 70% confluency, the cells were sub-cultured. First, the older medium in the flask was removed and was rinsed with Phosphate Buffer Saline (PBS) (Takara, Canada) to remove the FBS in the flask that will affect the trypsinization of the cells. One milliliter of 0.25% (w/v) trypsin-EDTA (Biology Industries, USA) was added into the flask and the flask was incubated in the carbon dioxide incubator at at 37°C, 5% (v/v) $CO_2$ for 10 min. After 10 min, detachment of cells was observed under phase contrast inverted light microscope. Approximately 100 μL of FBS was added into the flask to inhibit the reaction of trypsin once the cells were completely detached. The mixture in the flask was transferred to a centrifuge tube and was centrifuged at 500 ×g for 5 min. The supernatant was discarded and the cells were resuspended in 1 mL of DMEM. The resuspended cells were then added into a 75 $cm^2$ cell culture flask containing 6.2 mL of DMEM and 800 μL of FBS. The flask was then incubated in a carbon dioxide incubator at 37°C, 5% (v/v) $CO_2$.

### 3.3.9 Cryopreservation of 3T3-L1 cells

The older medium in the flask was removed and was washed with PBS to remove the FBS in the flask that will inhibit the activity of trypsin. One milliliter of 0.25% (w/v) trypsin-EDTA was added into the flask and the flask was incubated in the carbon dioxide incubator at 37°C, 5% (v/v) $CO_2$ for 10 min. After 10 min, detachment of cells was observed under phase contrast inverted light microscope. Approximately 100 μL of FBS was added into the flask to inhibit the reaction of trypsin once the cells were completely detached. The mixture in the flask was transferred to a centrifuge tube and was centrifuged at

500 ×g for 5 min. The supernatant was removed and the cell pellet was resuspended in 500 μL of DMEM. The resuspended cells were then added into a cryogenic vial containing of 350 μL, 100 μL of FBS and 50 μL of 100% filtered dimethyl sulphoxide (DMSO) (Merck. Germany). The cryogenic vial was then stored in -80°C for 1 days. The next day, the cryogenic vial containing the cells was cryopreserved.

### 3.3.10 Cell counting

Hemocytometer (BLAUBRAND® Neubauer improved, Germany) was used for the cell counting of 3T3-L1 cells. Ten microliters of the cell suspension was mixed with 20 μL of DMEM and 10 μL of tryphan blue (Life Technologies, USA) which was used to stain the viable cells. After that, 10 μL of the diluted cell suspension was transferred to the hemocytometer through the capillary action. Cell counting was performed under 10 × magnification using phase contrast inverted light microscope. The average number of cells in total four grid and the concentration of cell suspension was calculated using the formula as follows:

Average number of cells per mL=

$$\frac{\text{Total number of cells in 4 grids} \times \text{Dilution factor}}{4} \times 10^4$$

Volume of cell suspension needed in each well=$\frac{\text{Number of cells needed per well}}{\text{Average number of cells per mL}}$

### 3.3.11 Transient transfection with *FAIM2* cDNA plasmid DNA

Transient transfection was performed at Day 8 of differentiation. The 3T3-L1 cells were seeded in 12-well plate at cell density of $2.3 \times 10^5$ cells/ well and the standard induction of differentiation was followed as showed in Figure 3.5. Firstly, three milligram of plasmid DNA containing *FAIM2* cDNA was added into 150 μL of antibiotic free DMEM (without penicillin and streptomycin). This tube was labeled as D1. Six microliter of TurboFect transfection reagent (Thermo Scientific, USA) was added into 150 μL of antibiotic free DMEM. This tube was labeled as T1. In addition, six microliter of TurboFect transfection reagent was added into 300 μL of antibiotic free DMEM. This tube was labeled as T2. All the tubes were incubated at room temperature for 5 min. Next, solution in D1 and T1 were mixed together. The tube was incubated at room temperature for 20 min to allow the formation of DNA-transfection reagent complex. After incubation, 100 μL of the complex was transferred to another adipocytes in triplicate. One hundred microliter of the solution in T2 was also transferred to the adipocytes in triplicate and 100 μL of antibiotic free DMEM was transferred to pre-adipocytes in triplicate as well. The plate was then incubated in a carbon dioxide incubator at 37°C, 5% (v/v) $CO_2$.

### 3.3.12 Oil Red O staining

After 48 hr of transient transfection, Oil Red O (ORO) staining was performed to determine the lipid accumulation in the cells. The cells were washed with 1 mL of PBS for two times. After washing, the cells were fixed with one milliliter of 10% formalin at room temperature for 10 min. After fixation, the cells were washed with 1 mL of PBS twice. Diluted ORO solution (R&M Chemical, UK)

was prepared by mixing 3 parts of 0.5% ORO in isopropanol and 2 parts of sterile distilled water. Next, one milliliter of the freshly prepared ORO solution was added into each well. The cells were allowed to stain for 15 min. The cells were washed with PBS again after staining. The cells were visualized under phase contrast inverted light microscope and images of the cells were captured. Then, one milliliter of isopropanol (QRëC™, Singapore) was added into each well and incubate for 10 min to dissolve the ORO for quantitative analysis. One hundred microliter of the solution of each well were transferred to a microplate. Isopropanol was used as the blank. Absorbance of each well was measured at the wavelength of 540 nm using multichannel microplate reader (M200, Tecan, USA). The results obtained were tabulated and a bar chart was drawn.

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 Genotyping of *FAIM2* rs7138803

### 4.1.1 Optimization of annealing temperature

Prior to the genotyping, the optimum annealing temperature was chosen based on the gel image of amplicon. Various annealing temperatures were used during the optimization process. The PCR products were subjected to gel electrophoresis on a 2% agarose gel. Based on the gel image (Figure 4.1), the annealing temperature chosen was 68°C due to the absence of smearing and non-specific band. A sufficient number of PCR amplicons were produced at annealing temperature of 68°C which was determined based on the high intensity of band. All annealing temperatures below 68°C produced smearing or multiples non-specific bands, or both; whereas annealing temperature above 68°C showed no PCR amplicon, indicating unsuccessful amplification of the target sequence. Presence of non-specific amplicons at variable length is due to low PCR stringency (Lorenz, 2012).

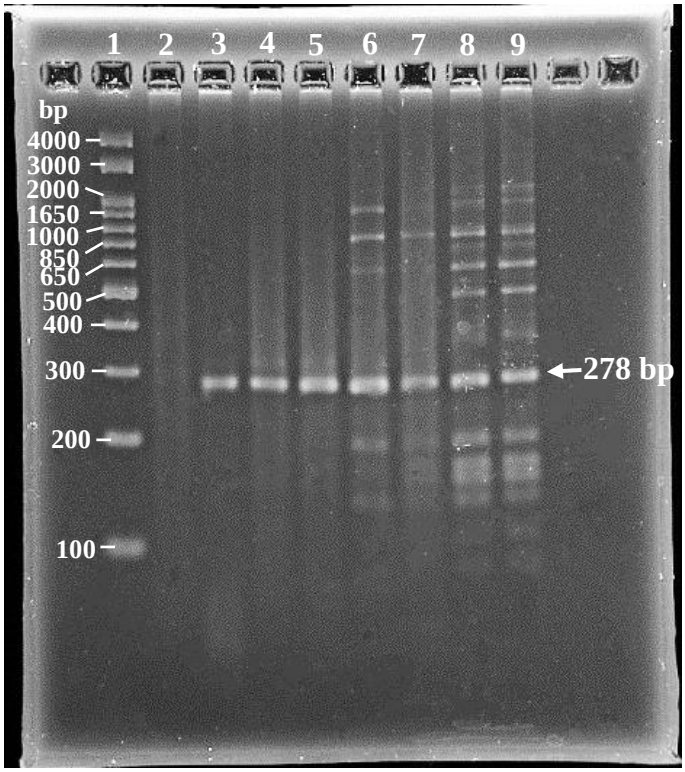


**Figure 4.1:** Optimization of annealing temperature to be used for subsequent PCR process. PCR product in 2% agarose gel. The 100 bp DNA ladder was loaded onto lane 1. DNA amplicons produced were loaded from lane 2 to lane 9 in the decreasing manner of annealing temperatures, which was 70°C, 68°C, 66°C, 62°C, 58°C, 54°C, 51°C and 50°C, respectively.

### 4.1.2 Genotyping by PCR-RFLP

The PCR amplicons were restriction digested by restriction enzyme *PspE*I. The digested samples were subjected to gel electrophoresis through a 2% agarose gel. Based on the gel image (Figure 4.2), the genotype of the samples were determined. For GG genotype, the amplicon was cleaved into two fragments with the sizes of 150 bp and 128 bp; whereas amplicon of AG genotype showed three fragments (278 bp, 150 bp and 128 bp) as only the G allele was cleaved. Amplicon of AA genotype could not be cleaved due to the absence of restriction enzyme site, thus it maintained at 278 bp. The genotypes of the 502 samples are shown in Appendix D.

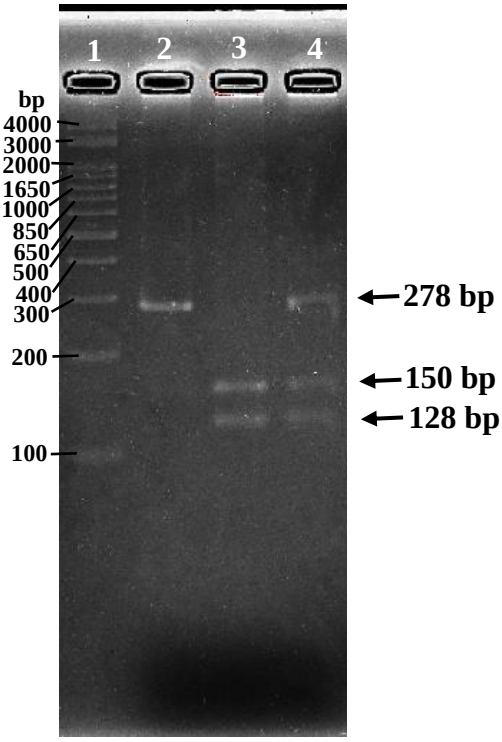


**Figure 4.2:** RFLP products of *FAIM2* rs7138803 genotypes in 2% agarose gel. The 100 bp DNA ladder was loaded onto lane 1. The genotype of the RFLP product loaded onto lane 2, 3 and 4 are AA, GG and GA, respectively.

### 4.1.3 Demographic, anthropometric characteristics and environmental exposures of subjects

A total of 502 students from UTAR Kampar campus participated in this study. Among the 502 subjects, there were 223 (44.42%) males and 279 females (55.58%). Table 4.1 illustrates the demographic and anthropometric characteristics of the subjects based on gender. Of this, Chinese was the prevailing ethnic group, making up of 95.22% of the population. Indian was another ethnic group participated in the study which made up 4.78% of the population only. The prevalence of obesity among studied population was higher in males (21.50%) than in females (18.30%). There was no significant association between gender with ethnicity, BMI, WC and TBF among UTAR students.

**Table 4.1:** Demographics and anthropometric characteristics of the subjects according to gender.

| Variables | Males (*n* = 223) | Females (*n* = 279) |
|---|---|---|
| **Ethnicity** | | |
| Chinese | 216 (96.90) | 262 (93.90) |
| Indian | 7 (3.10) | 17 (6.10) |
| $\chi^2$; *p* | 2.38; 0.12 | |
| **BMI Class** | | |
| Non-obese | 175 (78.50) | 228 (81.70) |
| Obese | 48 (21.50) | 51 (18.30) |
| $\chi^2$; *p* | 0.82; 0.36 | |
| **WC Class** | | |
| Normal | 182 (81.60) | 224 (80.30) |
| High | 41 (18.40) | 55 (19.70) |
| $\chi^2$; *p* | 0.14; 0.71 | |
| **TBF Class** | | |
| Normal | 152 (68.20) | 191 (68.50) |
| High | 71 (31.80) | 88 (31.50) |
| $\chi^2$; *p* | 0.05; 0.94 | |

Parentheses indicate percentage within the same gender.

Rampal and team (2007) showed that women (13.8%) had higher prevalence of obesity as compared to men (9.6%). According to National Health and Morbidity Survey 2015, the prevalence of obesity was significantly lower in males (27.8%) compared to females (33.6%). The overall prevalence of obesity among UTAR students was lower than the prevalence proposed in National Health and Morbidity Survey 2015 (Institute of Public Health, 2015). The

differences between these two proposed prevalence could be explained by the ages of the target populations. National Health and Morbidity Survey 2015 studied the overall prevalence between genders of all age group but in the study of UTAR population, most of the subjects were students who aged below 30 years old. According to a study which involved medical students in Malaysia as subjects, the prevalence of pre-obese was higher in males (18.3%) as compared to females (13.8%). The prevalence of obese subjects was also higher in males (9.2%) than females (1.9%) (Gopalakrishnan, et al., 2012). Education level was suggested to be one of the factors for obesity among students (Rampal, et al., 2007).

Other than BMI, waist circumference (WC) is parameter to determine central obesity. WC is correlated with accumulation of abdominal fat. High WC was observed in 18.40% of males and 19.70% of females. The prevalence of getting high WC was much higher in women (26.0%) as compared to in men (7.2%) (Norafidah, Azmawati and Norfazilah, 2013). According to National Health and Morbidity Survey 2015, prevalence of central obesity was significantly higher in females (60.2%) than males (38.2%) (Institute of Public Health, 2015). UTAR females had slightly higher prevalence of abdominal obesity than males. Other than WC, TBF was also another anthropometric measurement studied. Slightly greater number of males (31.80%) had high TBF as compared to females (31.50%) as different individual has different rate of energy metabolism.

Environmental exposures are responsible for the development of obesity. Out of the 502 subjects, the study of environmental factors including physical

activity, time spending for computer/TV, alcohol consumption and smoking status were performed based on the questionnaires of 486 subjects. Data from 16 subjects was excluded due to the missing data which were needed for the analysis. Table 4.2 illustrates the environmental exposures of the subjects according to gender.

Physical activity is essential to maintain balance between intake and expenditure of energy. Based on the study, physical activity was significantly associated with genders. Females (80.20%) tended to have less physical activity compared to males (68.50%) as females never or only occasionally performed vigorous physical activity. Involvement of males in physical activity could be used to explain the lower percentage of males with high WC as compared to females. There was consistent evidence showed that physical activity was negatively associated with adiposity (Jimenez-Pavon, Kelly and Reilly, 2010). Vigorous physical activity was significantly inversely associated with WC. An hour vigorous physical activity was associated with 0.38 cm decrease in WC. It is suggested that abdominal obesity can be reduced by physical activity by utilizing less fat from gluteal region, but more fat from the intraabdominal region, which eventually lead to the redistribution of adipose tissue (Koh-Banerjee, et al., 2003).

There was no significant differences among males and females on the time spending for computer/TV, alcohol consumption and smoking status. Most of the students spent at least one hour on computer/TV, only 10.30% of males and 8.40% of females spent less than one hours on computer/TV. For the alcohol

consumption, 44.22% of subjects were occasional drinkers; whereas 51.39% were non-drinkers. Majority of the subjects were non-smokers, making of 201 males and 271 females among 502 subjects. No significant differences among these three environmental exposures making them not applicable for explaining the differences in BMI, WC and TBF among male and female subjects.

**Table 4.2:** Environmental exposures of the subjects according to gender.

| **Variables** | **Males (*n* = 213)** | **Females (*n* = 273)** |
|---|---|---|
| **Physical activity** | | |
| Never or only occasionally | 146 (68.50) | 219 (80.20) |
| 1 – 2 times/week | 44 (20.70) | 49 (17.90) |
| ≥ 3 times/week | 23 (10.80) | 5 (1.80) |
| $\chi^2$; *p* | 19.33; **<0.001*** | |
| **Time spending for computer/TV** | | |
| < 1 hour | 22 (10.30) | 23 (8.40) |
| 1 – 3 hours | 79 (37.10) | 106 (38.80) |
| > 3 – 5 hours | 60 (28.20) | 80 (29.30) |
| > 5 hours | 52 (24.40) | 64 (23.40) |
| $\chi^2$; *p* | 0.66; 0.88 | |

**Table 4.2** (continued).

| Variables | Males ($n$ = 213) | Females ($n$ = 273) |
|---|---|---|
| **Alcohol consumption** | | |
| Frequent | 3 (1.40) | 3 (1.10) |
| Occasional | 109 (51.20) | 113 (41.40) |
| Non-drinker | 101 (47.40) | 157 (57.50) |
| $\chi^2$; $p$ | NP | |
| **Smoking status** | | |
| Smoker | 4 (1.90) | 0 (0.00) |
| Ex-smoker | 8 (3.80) | 2 (0.70) |
| Non-smoker | 201 (94.40) | 271 (99.30) |
| $\chi^2$; $p$ | NP | |

Parentheses indicate percentage within the same gender. NP indicated Chi-Square Test not performed due presence of cell having the count of less than 5; *Statistical significance at $p < 0.05$.

### 4.1.4 Demographics and anthropometric characteristics according to *FAIM2* rs7138803 genotypes and alleles

Table 4.3 demonstrates the distribution of genotypes and alleles of *FAIM2* rs7138803 according to the demographic and anthropometric classes, Among the 502 subjects, 244 of them (48.61%) carried homozygous G alleles and 210 of them (41.83%) were heterozygotes. Only 48 of them (9.56%) carried homozygous A alleles. According to study that involved 7161 participants, the prevalence of the genotype for *FAIM2* rs7138803 was: 42.2% homozygous G allele, 44.9% heterozygotes and 12.3% homozygous A alleles (Corella, et al., 2014), which was similar to the prevalence of UTAR population. The minor allele frequency (MAF) of *FAIM2* rs7138803 in the studied population was 0.30.

Based on the database available, the MAF for East Asian was 0.27 which was similar to the MAF of UTAR population (Ensembl, 2017). The MAF for Chinese and Indians in the studied population was 0.30 and 0.46, respectively. According to 1000 Genome Browser, the MAF for population of Han Chinese in Beijing (CHB) and Indian Telegu from UK (ITU) was 0.30 and 0.37 which was similar as compared to the MAF of Chinese and Indian in UTAR population (National Centre for Biotechnology Information, 2017c).

**Table 4.3**: Association of *FAIM2* rs7138803 genotype and allele distribution with demographic and anthropometric classes.

| Variables | Genotype | | | Allele | |
|---|---|---|---|---|---|
| | **GG** | **GA** | **AA** | **G** | **A** |
| **Gender** | | | | | |
| Male | 107 (43.90) | 97 (46.20) | 19 (39.60) | 310 (44.50) | 136 (44.30) |
| Female | 137 (56.10) | 113 (53.80) | 29 (60.40) | 387 (55.50) | 171 (55.70) |
| $\chi^2$; *p* | | 0.75; 0.69 | | 0.00; 0.96 | |
| **Ethnicity** | | | | | |
| Chinese | 236 (96.70) | 200 (95.20) | 42 (87.50) | 671 (96.30) | 285 (92.80) |
| Indian | 8 (3.30) | 10 (4.80) | 6 (12.50) | 26 (3.70) | 22 (7.20) |
| $\chi^2$; *p* | | 7.49; **0.02*** | | 5.53; **0.02*** | |
| **BMI Class** | | | | | |
| Non-obese | 192 (78.70) | 172 (81.90) | 39 (81.20) | 555 (79.60) | 251 (81.80) |
| Obese | 52 (21.30) | 38 (18.10) | 9 (18.80) | 142 (20.40) | 56 (18.20) |
| $\chi^2$; *p* | | 0.77; 0.68 | | 0.61; 0.43 | |

**Table 4.3** (continued).

| Variables | Genotype | | | Allele | |
|---|---|---|---|---|---|
| | **GG** | **GA** | **AA** | **G** | **A** |
| **WC Class** | | | | | |
| Normal | 197 (80.70) | 174 (82.90) | 35 (72.90) | 567 (81.30) | 245 (79.80) |
| High | 47 (19.30) | 36 (17.10) | 35 (27.10) | 130 (18.70) | 62 (20.20) |
| $\chi^2$; $p$ | | 2.50; 0.29 | | 0.33; 0.57 | |
| **TBF Class** | | | | | |
| Normal | 170 (69.70) | 139 (66.20) | 34 (70.80) | 478 (68.60) | 208 (67.80) |
| High | 74 (30.30) | 71 (33.80) | 14 (29.20) | 219 (31.40) | 99 (32.20) |
| $\chi^2$; $p$ | | 0.79; 0.68 | | 0.07; 0.80 | |

Parentheses indicate percentage within the same genotypes or alleles; NP indicated Chi-Square Test not performed due presence of cell having the count of less than 5; *p*-values by Pearson's Chi-Square Test; *Statistical significance at $p < 0.05$.

Chi-square test (Table 4.4) was performed to determine whether the distribution of genotypes of rs7138803 is following Hardy-Weinberg equilibrium (HWE). The null hypothesis ($H_0$) was set as the observed genotype frequency is in HWE whereas the alternative hypothesis ($H_1$) was set as observed genotype frequency is not in HWE.

**Table 4.4**: Chi-square test for determination of rs7138803 genotype distribution.

| Genotype | Observed (O) | Expected (E) | (O-E) | $(O-E)^2/E$ |
|---|---|---|---|---|
| **GG** | 244 | 246 | -2 | 0.016 |
| **GA** | 210 | 211 | -1 | 0.004 |
| **AA** | 48 | 45 | 3 | 0.200 |
| **Total** | 502 | 502 | $\chi^2 = 0.22$ | |

In this study, one degree of freedom with 5% of significance level was 3.84. The $\chi^2$ value calculated was less than 3.84, indicating that the null hypothesis was not rejected. Thus, the genotype distribution of *FAIM2* rs7138803 was in HWE.

Gender did not show any significant association with both genotypes and alleles. Ethnicity showed significant association with both genotypes and alleles with the *p*-value of 0.02. The differences among Chinese and Indian could be explained by the migration of them from other countries. Malaysian population are mainly composed of Malay, Chinese and Indian. Mainly of the Chinese community are the descendants of Han Chinese from southern provinces of China including Guangdong and Fujian. Indian migrants were brought from the southeastern and north Indian during the British colonization of Malaysia (Teo, et al., 2009). Migration, a process of micro-evolutionary that affects genetic variations, results in the changes in phenotype of a population. The genetic traits along by the individuals when move from one population to another start to change due to the evolutionary force including drift and admixture (Kampuansai, et al., 2016; Nature Education, 2014). Thus, migration could cause the significant differences in alleles for different ethnicities.

In addition, there was no significant association of BMI with genotype and allele as both of the *p*-values were more than 0.05. The *p*-value obtained in the study showed similarity with few recent studies. According to a GWAS study that involved 2030 Chinese children who came from Adolescent Lipids, Insulin Resistance and Candidate Genes (ALIR) and Comprehensive Prevention Project for Overweight and Obese Adolescents (CPOOA) studies, *FAIM2* rs7138803 was not in association with obesity ($p = 0.542$). Moreover, a study involved a total of 11568 Chinese Han population which were from WD-CAD and Beijing stroke cohort (BJSC) showed no significant effect of rs7138803 on obesity with the *p*-value of 0.51. However, the association between rs7138803 and obesity are still in debate especially in Chinese population as there were some evidences reported the significant association between the SNP and obesity. For instance, Longitudinal Replication Studies of GWAS showed significant association with childhood obesity. The effect of risk allele (A allele) significantly decreased with rising age ($p = 0.004$). The result suggested that the longitudinal BMI changes could be regulated by age-dependent genetic pathways of cell proliferation and apoptosis (Mei, et al., 2011). Thus, UTAR population that consists of all adult subjects showed no association with obesity might because rs7138803 is an age-dependent SNP.

BMI alone is not sufficient for the classification of obesity. BMI provides the overall indication about obesity based on the weight and height only, which might lead to the misclassification (Rothman, 2008). Thus, TBF and WC are required in accessing obesity. TBF measures the overall adiposity. rs7138803 did not exert any significant effect on TBF for both genotype and allele. WC

measures the fat accumulation happen at the intra-abdominal region. WC showed no significant association with the genotype and allele in UTAR population. This indicates that rs7138803 did not exert significant effect in central obesity in UTAR population. A recent GWAS study that involved 3502 Chinese children from BCAMS (Beijing Child and Adolescent Metabolic Syndrome) study suggested that there was no significant association of rs7138803 with WC (Xi, et al., 2013). In the PREDIMED study done by Corella and team (2014), WC was not in association with obesity in both additive and recessive model ($p > 0.05$).

### 4.1.5 Adjusted means of anthropometric measurements for *FAIM2* rs7138803 genotype and allele

Table 4.5 illustrates the adjusted mean of anthropometric measurements for the genotype and allele of *FAIM2* rs7138803. There was no association between most of the anthropometric measurements with rs7138803 genotype and allele. The normal range of SBP and DBP is lower than 120 mmHg and 80 mmHg, respectively (Mungreiphy, Kapoor and Sinha, 2011). A population-based cohort association study carried out by Li and team (2013) also showed no association between blood pressure and rs7138803. PR was also not associated with the SNP among UTAR students.

Mean values for WC fell within the normal range as the cut-off point for males and females are <90 cm and <80 cm, respectively (Misra, et al., 2006). Even there was no significant association, genotype AA showed slightly higher WC as compared to heterozygotes and homozygous G allele, indicated that carrying

both risk alleles might slightly increase WC as rs7138803 was a WC-related SNP (Zhang, et al., 2014). The cut-off points of TBF for males and females are 20% and 30%, respectively. Among UTAR students, the mean of adjusted TBF was approximately 23% which was higher than the cut-off point for males, indicating males in UTAR population tend to have higher total body fat than the normal range due to the differences in fat metabolism (Blaak, 2014). SF, SM, VFL, RM and BMI showed no significant association with genotype and allele of rs7138803 as the anthropometric measurements were similar with each other within the genotype and allele groups. Asian populations have different cut-off points for overweight and obesity compared to Caucasian population, which are 23 – 24.9 kg/m2 and >25 kg/m2, respectively (Aziz, et al., 2014). The adjusted mean of BMI for genotype and allele was less than the both cut-off points of overweight and obesity. This indicates that rs7138803 did not exert any effect on BMI of UTAR population.

**Table 4.5:** Adjusted means of anthropometric measurements for rs7138803 genotype and allele.

| Variables | Genotype | | | Allele | |
|---|---|---|---|---|---|
| | **GG** | **GA** | **AA** | **G** | **A** |
| SBP (mmHg) | 113.76 ± 0.73 | 113.97 ± 0.79 | 113.40 ± 1.65 | 113.82 ± 0.43 | 113.79 ± 0.65 |
| *p* | | 0.91 | | 1.00 | |
| DBP (mmHg) | 69.02 ± 0.55 | 69.31 ± 0.56 | 68.73 ± 1.48 | 69.12 ± 0.32 | 69.11 ± 0.50 |
| *P* | | 0.61 | | 0.64 | |
| PR (bpm) | 82.49 ± 0.77 | 81.84 ± 0.83 | 81.60 ± 1.73 | 82.28 ± 0.45 | 81.79 ± 0.68 |
| *p* | | 0.85 | | 0.60 | |
| WC (cm) | 77.11 ± 0.63 | 76.11 ± 0.68 | 77.52 ± 1.43 | 76.81 ± 0.37 | 76.56 ± 0.57 |
| *p* | | 0.10 | | 0.81 | |

**Table 4.5** (continue).

| Variables | Genotype | | | Allele | |
|---|---|---|---|---|---|
| | **GG** | **GA** | **AA** | **G** | **A** |
| TBF (%) | 23.34 ± 0.36 | 23.19 ± 0.38 | 22.51 ± 0.81 | 23.30 ± 0.21 | 22.99 ± 0.32 |
| *p* | | 0.65 | | 0.67 | |
| SF (%) | 19.49 ± 0.38 | 18.82 ± 0.41 | 18.28 ± 0.87 | 19.27 ± 0.23 | 18.65 ± 0.34 |
| *p* | | 0.84 | | 0.71 | |
| SM (%) | 30.79 ± 0.15 | 30.64 ± 0.16 | 30.95 ± 0.34 | 30.75 ± 0.09 | 30.74 ± 0.13 |
| *p* | | 0.68 | | 0.88 | |
| VFL | 4.92 ± 0.23 | 4.51 ± 0.25 | 4.17 ± 0.52 | 4.80 ± 0.14 | 4.41 ± 0.21 |
| *p* | | 0.52 | | 0.28 | |
| RM (kcal) | 1404.59 ± 11.21 | 1373.79 ± 12.09 | 1396.93 ± 25.29 | 1395.21 ± 6.64 | 1381.36 ± 10.00 |
| *p* | | 0.20 | | 0.28 | |

**Table 4.5** (continue).

| Variables | Genotype | | | Allele | |
|---|---|---|---|---|---|
| | **GG** | **GA** | **AA** | **G** | **A** |
| BMI ($kg/m^2$) | 22.74 ± 0.29 | 22.30 ± 0.25 | 22.13 ± 0.55 | 22.61 ± 0.16 | 22.24 ± 0.21 |
| *p* | | 0.69 | | 0.43 | |
| WHR | 0.81 ± 0.01 | 0.81 ± 0.01 | 0.83 ± 0.01 | 0.81 ± 0.00 | 0.81 ± 0.00 |
| *p* | | 0.06 | | 0.148 | |
| WHRadjBMI | 0.80 ± 0.01 | 0.81 ± 0.01 | 0.83 ± 0.01 | 0.81 ± 0.00 | 0.81 ± 0.00 |
| *p* | | **0.02*** | | **0.03*** | |

Values that are not normally distributed were log transformed before analysis by univariate analysis of variance (General Linear Model). All values were adjusted for gender and ethnicity except SBP, PR, VFL, RM, WHR and WHRadjBMI which only adjusted for gender. DBP and BMI was not adjusted with any co-variant. Values are presented as adjusted mean ± SEM (estimated marginal means ± standard error of the mean); *Statistical significance at $p < 0.05$.

WHR with the adjusted mean of 0.81 (for GG and GA genotype) and 0.83 (for AA genotype) also showed no significant association with rs7138803. The cut-off points of WHR for Asians are 0.90 for males and 0.80 for females. The adjusted mean for WHR of UTAR population was higher than the cut-off point of females for obesity. AA genotypes had slightly higher WHR value compared to GG and GA genotype. The risk allele (A allele) of rs7138803 was associated with higher WHR (Zhang, et al., 2014). Higher WHR indicates more deposition of intra-abdominal fat, which is similar to high WC (Shungin, et al., 2015). WHRadjBMI is a parameter used recently to identify new genetic loci that were linked to central fat accumulation (Strawbridge, et al., 2016). In studied population, WHRadjBMI was significantly associated with genotype ($p$ = 0.02) and allele ($p$ = 0.03). This result indicates that the central accumulation of fat that was independent of total body weight in individuals who carried the risk allele (Strawbridge, et al., 2016).

### 4.1.6 Stratified analysis of WHR according to gender

Stratified analysis of WHR according to gender was performed and the results are shown in Table 4.6. Unadjusted WHR was not associated with both genotype and allele in males and females of UTAR students. WHR only showed significant association with genotype in males after adjusted for BMI. Males with AA genotype had higher WHRadjBMI compared to heterozygotes and homozygous G, indicative risk allele associated with higher WHR (Zhang, et al., 2014). There was no significant association between WHR (adjusted and non-adjusted) with genotypes and alleles in female. However, females with AA genotype had the WHR

mean of 0.80 after adjusted for BMI compared to GG genotype and GA genotype, where the cut-off points of WHR for obesity is 0.8 in female. This again proved that risk allele was associated with higher WHR.

Overall, males had higher mean of WHR as compared to females for both genotypes and alleles. This could be explained by the gender difference in fat metabolism, especially in the regional fatty acid storage. Distribution of body fat is different in males and females. Males tend to have higher WHR as there is greater storage of fat in the abdominal region. Larger visceral adipocytes in male increase the basal lipolysis in visceral adipocytes, thus increase the hydrolysis of lipid and increase the release of free fatty acid in the body (Blaak, 2014). Males also showed greater rate of lipogenesis in visceral adipose tissue to take up more fatty acid as compared to females, thus lead to increase fat accumulation. Thus, greater storage of fat in abdominal region increase WHR. Visceral fat might play an essential role in insulin resistance in liver (Power and Schulkin, 2008). Combination of *FAIM2* rs7138803 risk allele and high visceral fat in male contributed to obesity, especially in central obesity.

**Table 4.6:** Stratified analysis of WHR between males and females.

| Variables | Genotype | | | Allele | |
|---|---|---|---|---|---|
| | **GG** | **GA** | **AA** | **G** | **A** |
| **Male** | | | | | |
| Unadjusted | 0.83 ± 0.01 | 0.84 ± 0.01 | 0.88 ± 0.03 | 0.84 ± 0.00 | 0.85 ± 0.01 |
| *p* | | 0.20 | | 0.42 | |
| BMI adjusted | 0.84 ± 0.01 | 0.84 ± 0.01 | 0.89 ± 0.02 | 0.84 ± 0.01 | 0.85 ± 0.01 |
| *p* | | **0.047*** | | 0.11 | |
| **Female** | | | | | |
| Unadjusted | 0.78 ± 0.01 | 0.78 ± 0.01 | 0.79 ± 0.01 | 0.78 ± 0.00 | 0.78 ± 0.00 |
| *p* | | 0.33 | | 0.77 | |

**Table 4.5** (continue).

| Variables | Genotype | | | Allele | |
|---|---|---|---|---|---|
| | **GG** | **GA** | **AA** | **G** | **A** |
| **Female** | | | | | |
| BMI adjusted | 0.78 ± 0.01 | 0.78 ± 0.01 | 0.80 ± 0.01 | 0.78 ± 0.00 | 0.78 ± 0.01 |
| *p* | | 0.29 | | 0.39 | |

Unadjusted mean values and standard error of the mean by One-Way ANOVA; *p*-values of unadjusted WHR by Mann-Whitney test. Values for WHRadjBMI by univariate analysis of variance. Values are presented as adjusted mean ± SEM (estimated marginal means ± standard error of the mean); *Statistical significance at $p < 0.05$.

## 4.2 Overexpression of *FAIM2*

### 4.2.1 Transformation of plasmid (pcDNA 3.1+/C-(K)-DYK) cloned with *FAIM2* cDNA DH5α *Escherichia coli*

Chemical method was used to transform DH5α *Escherichia coli* with plasmid (pcDNA $3.1^{+}$/C-(K)-DYK) cloned with *FAIM2* cDNA. Figure 4.3 shows the growth of DH5α *E. coli*. Positive control was used to access the viability of competent DH5α *E. coli*. Extensive growth of DH5α *E. coli* on the LB agar plate showed that the cells were viable prior to the transformation. Negative control was used to prove that untransformed DH5α *E. coli* was unable to grow on the ampicillin supplemented agar plate as they did not gain the ampicillin resistance gene present on the plasmid. DH5α *E. coli* that were successfully transformed with plasmid (pcDNA $3.1^{+}$/C-(K)-DYK) cloned with *FAIM2* cDNA were able to grow on the ampicillin supplemented plate as the bacterial cells gained the ampicillin resistance from the plasmid. After that, a colony was picked, then inoculated into ampicillin supplemented LB broth and incubated at 37°C, 250 rpm. Alkaline lysis method was performed to isolate the recombinant plasmid DNA from transformed DH5α *E. coli*. The concentration of isolated plasmid DNA was 2140.10 ng/μL. Purity of the extracted plasmid DNA was also accessed. The ratio of absorbance at 260 nm to 280 nm within the range of 1.8 to 2.0, indicative a pure sample. An $A_{260}/A_{230}$ ratio of 2 or greater is indicative of high level of sample purity (GE Healthcare Life Sciences, 2016). The reading of $A_{260}/A_{280}$ and $A_{260}/A_{230}$ of the extracted plasmid DNA was 1.98 and 2.32, respectively, which indicated that a pure plasmid DNA was extracted.

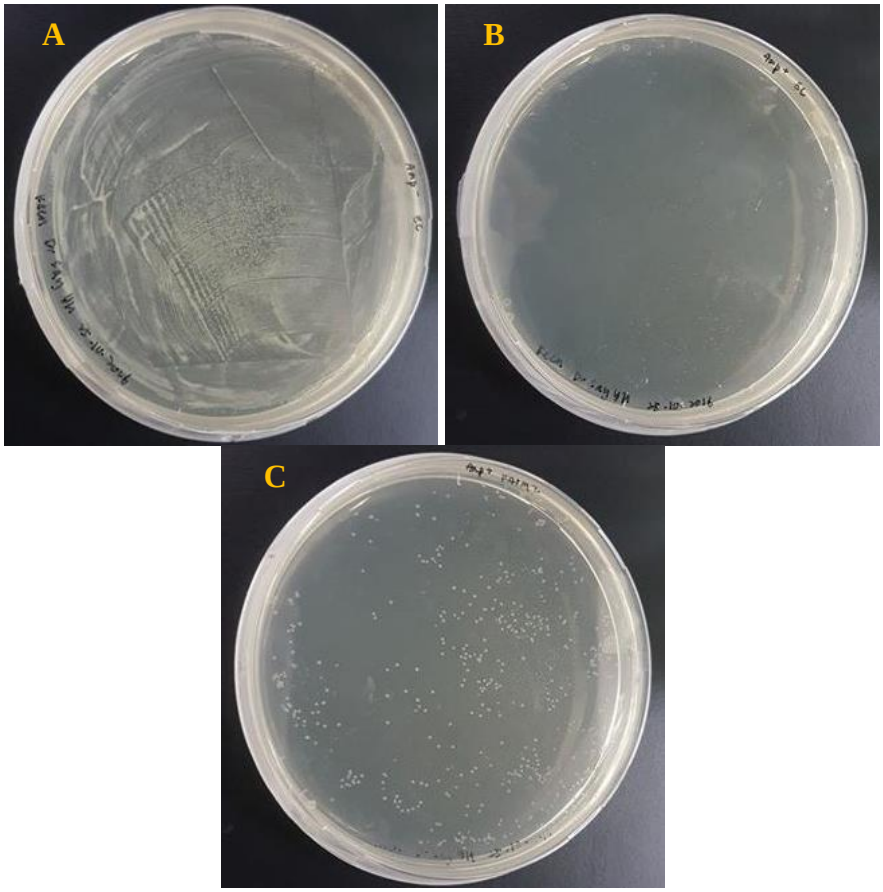


**Figure 4.3**: Growth of DH5α *Escherichia coli*. **(A)** Positive control. Untransformed DH5α *E. coli* was grew on LB agar plate without supplemented with ampicillin. **(B)** Negative control. Untransformed DH5α *E. coli* was unable to grow on LB agar plate supplemented with ampicillin (100 ng/μL). **(C)** DH5α *E. coli* transformed with plasmid (pcDNA $3.1^{+}$/C-(K)-DYK) cloned with *FAIM2* cDNA. Each colony represented the successful transformation.

After accessing the concentration and purity of extracted plasmid DNA, the plasmid DNA was linearized through restriction digestion by restriction enzyme *BamH*I. Extracted plasmid DNA contained only one *BamH*I restriction site. After restriction digestion with *BamH*I, the circular plasmid DNA was linearized and only one band of DNA was showed on the gel (Figure 4.4) after gel electrophoresis. The purity of the extracted plasmid DNA was accessed again based on gel image. The purity of plasmid DNA was high are there was no/limited smear present on the gel. Based on eye estimation, the only band

showed at the position around 6000 bp of the ladder was the extracted plasmid DNA with the size of 6269 bp.

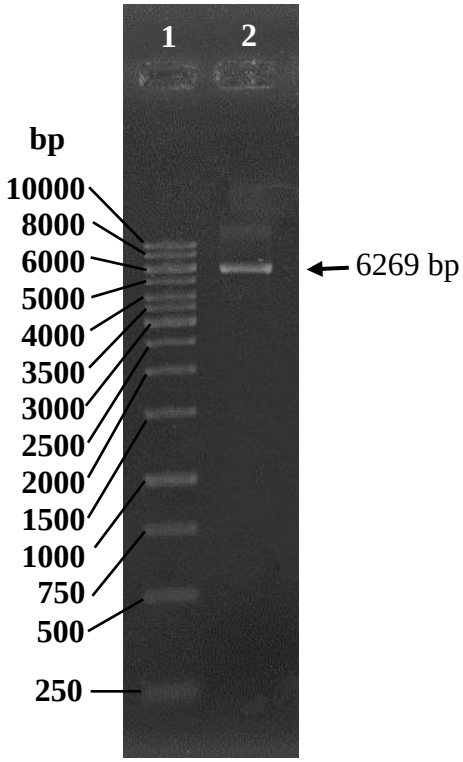


**Figure 4.4:** Linearization of plasmid containing *FAIM2* cDNA with *BamH*I. Lane 1 and 2 was loaded with 1 kb DNA ladder and linearized plasmid with *FAIM2* cDNA, respectively.

### 4.2.2 Intracellular lipid accumulation

ORO staining was performed on pre-adipocytes and adipocytes with or without *FAIM2* cDNA transfection. The intracellular lipid accumulation were as shown in Figure 4.5. Observable differences were identified between pre-adipocytes (undifferentiated cells) and adipocytes (differentiated cells). Pre-adipocytes maintained at its fibroblast-like morphology and no intracellular lipid accumulation was observed; whereas adipocytes was not in fibroblast shape and intracellular lipid accumulation was observed. Adipocytes without transfection with *FAIM2* cDNA was act as the control cells in the study. There was no obvious differences between the intracellular lipid accumulation in control cells and adipocytes transfected with *FAIM2* cDNA based on light microscopy.

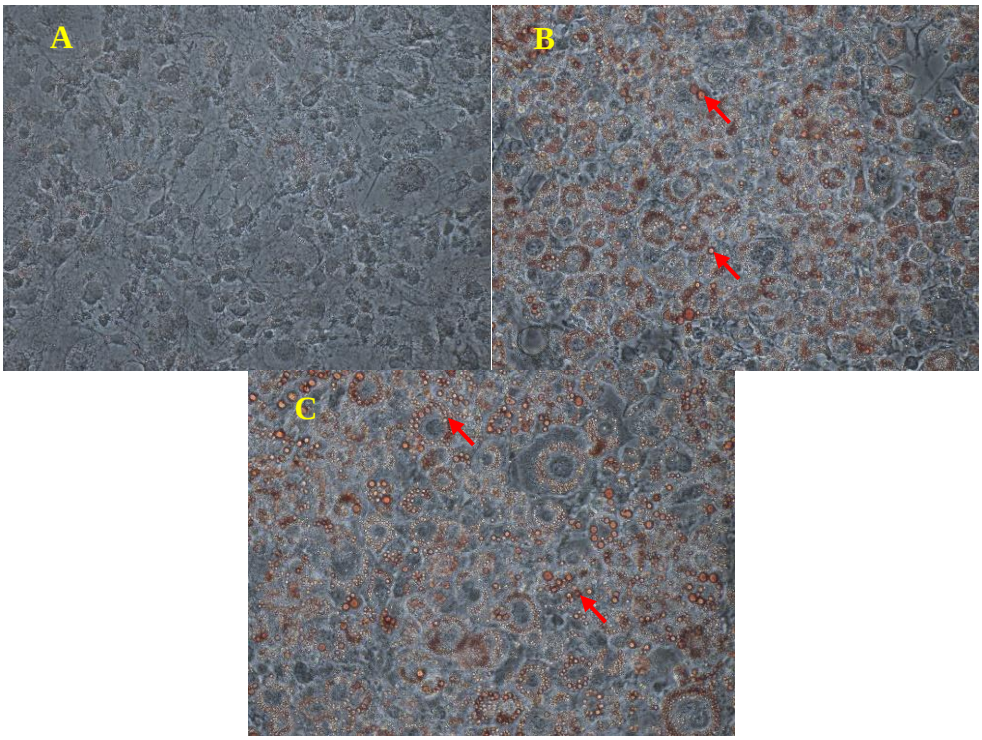


**Figure 4.5:** Microscopic image of 3T3-L1 cells with the total magnification of 2000 ×, captured after Oil Red O staining by using Nikon Eclipse TS100 phase contrast microscope. **(A)** Pre-adipocytes. **(B)** Adipocytes. **(C)** Adipocytes transfected with *FAIM2* cDNA. Lipid droplets were stained with Oil Red O staining and appeared as red in the image which indicated by red arrow "↑".

After observation under light microscope, the ORO stain on the intracellular lipid droplets was dissolved with isopropanol and the absorbance was measured at 540 nm. A bar chart (Figure 4.6) was generated based on the absorbance readings. Control cells and adipocytes transfected with *FAIM2* cDNA showed approximately four fold increase in the intracellular lipid accumulation as compared to the pre-adipocytes with the *p*-value of 0.01 and 0.0003, respectively. The presence of absorbance reading for pre-adipocytes might due to the incomplete removal of ORO stain during the washing process. There was no significant difference between absorbance reading of control and adipocytes transfected with *FAIM2* cDNA ($p$ = 0.51). However, adipocytes transfected

with *FAIM2* cDNA showed slightly decrease in the absorbance reading compared to control cells, indicating adipocytes with *FAIM2* overexpression had lower lipid accumulation in the cells.

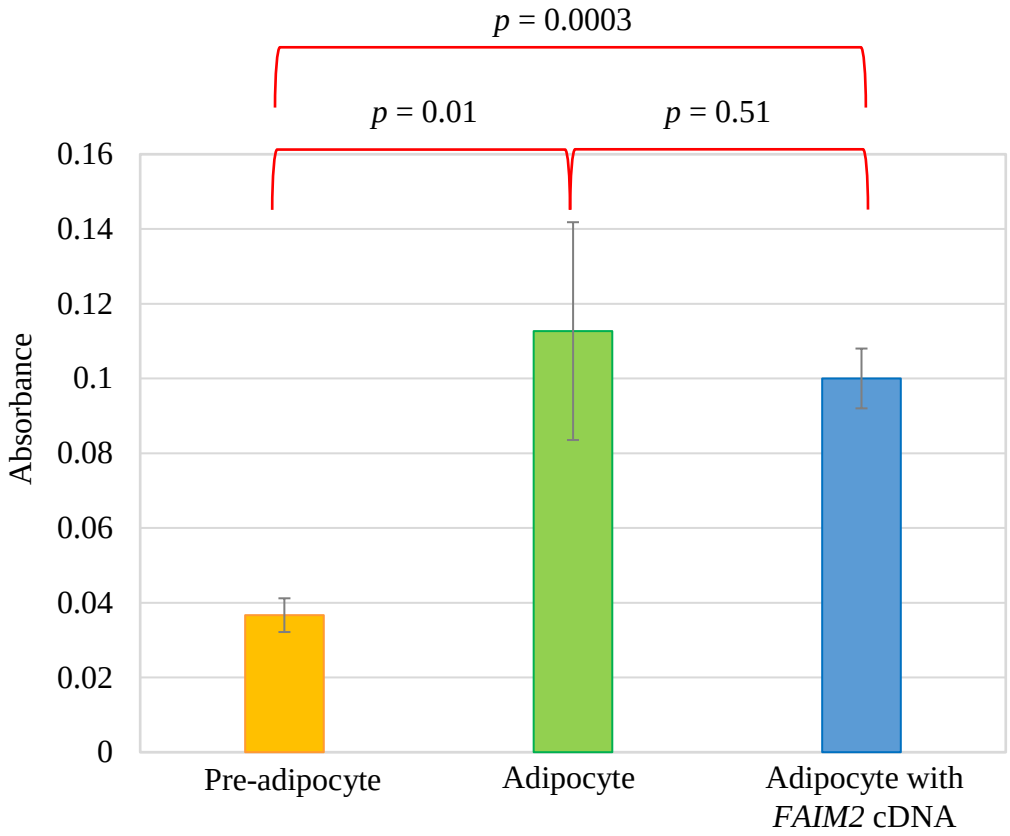


**Figure 4.6:** Bar chart representing absorbance reading of Oil Red O stain (dissolved by isopropanol) of pre-adipocyte, adipocyte (control) and adipocyte transfected with *FAIM2* cDNA.

### 4.3 Proposed mechanism of the action of FAIM2 in obesity

Apoptosis is a programmed cell death that is important for immune defense, development and tissue homeostasis. Imbalance in rate of apoptosis is a threat to body health, as it might causes cancer, autoimmune diseases and neurodegenerative disorder (Elmore, 2007; Czabotar, et al., 2014). Induction of apoptosis causes accumulation of droplets in cells due to *de novo* synthesis of natural lipid. Increased intracellular lipid accumulation is related to the increased lipogenesis (Al-Saffar, et al., 2002). In addition, apoptosis increases the membrane potential of mitochondrial then lead to the intramitochondrial production of reactive oxygen species (ROS). ROS inhibits the β-oxidation of fat and upregulate lipid synthesis, thus leading to more intracellular lipid droplets (Boren and Brindle, 2012).

FAIM2 is an anti-apoptotic protein which inhibits apoptosis by binding to the endoplasmic reticulum. Transfection of *FAIM2* cDNA into the adipocytes during the study led to the overexpression of *FAIM2* in the differentiated cells. Overexpression of *FAIM2* inhibit calcium ions released from endoplasmic reticulum then eventually lead to the inhibition of release of cytochrome C from mitochondria (Planells-Ferrer, et al., 2016; Urresti, et al., 2016). Thus, *FAIM2* overexpression prevents the occurrence of apoptosis in adipocytes, reduces the *de novo* synthesis of lipid, maintains the mitochondrial membrane potential at normal level and prevents the production of ROS that inhibit the β-oxidation of fat.

Mutation of *FAIM2* influences the Fas apoptotic signalling pathway that confers risk for obesity (Tang, et al., 2014). Risk allele of rs7138803 was suggested to reduce the expression or activity of *FAIM2.* Individual carrying the risk allele has less protection against apoptosis and rate of cell apoptosis (Corella, et al., 2014). Decreased in *FAIM2* expression induces more apoptotic cells (Kang, et al., 2016).

Apoptosis of adipocytes promotes recruitment, accumulation and persistence of macrophages in white adipose tissue. Macrophage infiltration and increase in production of pro-inflammatory cytokine such as tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) lead to the low-grade chronic inflammation. TNF-α and IL-6 are crucial in the development of insulin resistance. TNF-α is the major stimulant for apoptosis and main regulator of IL-6 production in adipocytes. TNF-α directly influences the signalling cascade of insulin and impairs insulin-dependent glucose transport. It is also an essential regulator in fat cell function especially in expansion of adipose tissue (Alkhouri, et al., 2010; Tinahones, et al., 2013).

### 4.4 Limitations and future studies

There were several limitations in this study. Chinese was the prevailing ethnic group who made up 95.22% of the studied population. Less Indian subjects and no Malay subjects were included in the study. The subjects was restricted to UTAR students which majority of their age were within the range of 18 to 30 years old. The number of subjects from different ethnics and their age has made this study difficult to represent the Malaysian population. Further studies are

required to recruit more Indian and Malay subjects instead of Chinese subjects in order the increase the reliability and accuracy of the analysis. Other than that, missing data of the subjects might reduce the accuracy of the result. Thus, the researchers are requested to make sure the completion of questionnaire by the subjects before the subject leaves. A greater depth of molecular studies might be included in order to understand better regarding the science behind obesity. It is recommended to perform gene expression study by using quantitative PCR to measure the gene expression of each genotype among the subjects.

In addition, 3T3-L1 cell line used in the study was from mouse (*Mus Musculus*) may not give the similar result when test on the human cell lines. Thus, it is suggested to use multiple cell lines such as human cell line in study of the effect of *FAIM2* overexpression. Brain cell lines might also be included as the *FAIM2* is highly expressed in hypothalamus. Furthermore, protein expression analysis was suggested to be included in the study. Current study only measured the lipid accumulation in the cells despite of the different viability of cells in the culture. Protein expression can be used to normalize the result obtained from ORO assay in order to improve the accuracy of the result for intracellular lipid accumulation. Other assays can also be included to study the effect of *FAIM2* overexpression in cells, including lipolysis assay to detect the lipolytic activity in the cells, MTT assay to measure the cell viability and flow cytometry to determine the number of apoptotic cells.

# CHAPTER 5

# CONCLUSION

Among 502 UTAR students involved in the present study, there were more obese males (21.50%) as compared to obese females (18.30%), albeit without statistical significance ($p$ = 0.36). The percentage of males with high TBF (31.80%) was slightly higher than in females (31.50%). In contrast, the prevalence of getting high WC was much lower in men (18.40%) but higher in women (19.70%), indicating that females had slightly higher prevalence in developing central obesity. Based on the study of environmental exposure, physical activity was significantly associated with genders ($p$ = 0.00). Majority of females (80.20%) had less physical activity compared to males (68.50%). The time spending for computer/TV, alcohol consumption and smoking status showed no significant differences between males and females.

The MAF of *FAIM2* rs7138803 in the studied population was 0.30. The MAF for Chinese and Indian among UTAR students was 0.30 and 0.46, respectively. The distribution of rs7138803 genotypes followed Hardy-Weinberg equilibrium (HWE). Ethnicity showed significant association with both genotypes and alleles ($p$ = 0.02). However, gender, BMI, WC and TBF did not show any significant association with both genotypes and alleles of rs7138803. Majority of the anthropometric measurements showed no association with rs7138803 genotypes and alleles. Most of the mean of anthropometric measurements were similar with each other within the genotype and allele groups. WHRadjBMI is

the only parameter that showed significant association with genotype ($p = 0.02$) and allele ($p = 0.03$), indicating central fat deposition was independent of total body weight in risk-allele carriers. Stratified analysis of WHR showed that WHR only showed significant association with genotype in males after adjusted for BMI ($p = 0.047$). AA genotype males had higher WHRadjBMI mean compared to another two genotypes.

Lipid accumulation in 3T3-L1 cell line was determined by ORO assay. There was no significant difference between level of lipid accumulation of control and adipocytes with *FAIM2* overexpression ($p = 0.51$). However, adipocytes with *FAIM2* overexpression showed slight decrease in the lipid accumulation compared to control cells. Thus, it is suggested that overexpression of *FAIM2* reduce the lipid accumulation by preventing of apoptosis in adipocytes, reducing the *de novo* lipid synthesis, maintaining normal level of mitochondrial membrane potential at normal level and prevent the production of ROS. It is also suggested that risk allele of rs7138803 downregulates the expression or activity of *FAIM2*, leading to increase rate of cell apoptosis and eventually cause obesity.

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# APPENDICES

## APPENDIX A

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

Re: U/SERC/36/2015

7 August 2015

Dr Say Yee How
Department of Biomedical Science
Faculty of Science
Universiti Tunku Abdul Rahman
Jalan Universiti, Bandar Baru Barat,
31900 Kampar
Perak

Dear Dr Say,

**Ethical Approval For Research Project/Protocol**

We refer to your application dated 13 July 2015 for ethical approval of your research project which was circulated on 28 July 2015 for consideration of the UTAR Scientific and Ethical Review Committee (SERC). We are pleased to inform that your application for ethical approval of your research project involving human subjects has been approved by SERC.

The details of your research project are as follows:

| | |
|---|---|
| **Research Title** | Identification of Genetic Variants Associated with Obesity and Allergic Diseases |
| **Investigator(s)** | Dr Say Yee How (PI)<br>Dr Chew Fook Tim (National University of Singapore) |
| **Research Location** | UTAR, Perak Campus |
| **No of Participants** | Between 375 - 500 participants (Age 17 - 50) |
| **Research Costs** | Self-funded |
| **Procedures Involved** | (1) Collection of mouthwash samples<br>(2) Collection of blood samples from finger pricking |
| **Approval Validity** | 2015 - 2016 |

However, you are requested to take into consideration the following suggestions of the Committee in the conduct of this research:

(1) The researcher(s) who will be doing the finger pricking to get blood samples should be trained in the procedure and deemed competent.

(2) University safety procedure should be observed when handling the blood samples.

(3) The samples for DNA testing should not be used for purposes other than that stipulated in the application of this research project.

**Address:** Jalan Sg. Long, Bandar Sg. Long, Cheras, 43000 Kajang, Selangor D.E. **Postal Address:** P.O Box 11384, 50744 Kuala Lumpur, Malaysia
**Tel:** (603) 9086 0288 **Fax:** (603) 9019 8868 **Homepage:** http://www.utar.edu.my

## APPENDIX B


DEPARTMENT OF BIOMEDICAL SCIENCE
FACULTY OF SCIENCE

**INFORMATION FOR PARTICIPANTS**
*for the study of*
**Genetics of obesity and allergy**

- We would like your permission to enroll you as a participant in a research study to identify genes that are involved in obesity and allergy. The prevalence of obesity, asthma and rhinitis are increasing globally, including Malaysia. These complex diseases have diverse genetic and environmental backgrounds. Recent studies have suggested that many genetic variants were associated with these diseases when exposed to certain environmental factors. This study involves a questionnaire on whether you have allergy or not, detection of genetic variants using DNA from your mouthwash and measurement of biochemicals in your blood.
- First, you will be to pour a 5 ml sugar solution into your mouth. Please rinse, rub your cheeks with your tongue for 1 minute and spit it back into a test tube.
- You will then have to answer a series of questions in a questionnaire to assess your allergic conditions and environmental exposures.
- We will then take your body measurements, which include your height, weight, waist and hip circumferences. You will then be asked to step on a scale which will measure your Body Mass Index, Body Fat Percentage, Subcutaneous Fat Percentage, Visceral Fat Percentage, Resting Metabolism Rate and Skeletal Muscle Percentage. We will also take your blood pressure.
- We will also prick your fingertip to collect around 0.5 ml of blood samples. You will feel a little pain (like an ant bite) at first.
- You will receive a small token as an appreciation for your time and effort. Thank you.

**CONSENT FORM**

I, _____

volunteer to participate in the study of

***Genetics of obesity and allergy***

I am willing to give my mouthwash and blood samples. I also understand that I have to answer a series of questions in a questionnaire as honest as possible.

I have also been informed that all the information provided by me and all the results obtained will be kept in strict confidence by the researchers, and all the data and samples from this research project will be destroyed after the end of it.

Hereby, I give my consent to participate in this above study.

| Respondent | Interviewer |
|---|---|
| Signature: _____ | Signature: _____ |
| Date : _____ | Date : _____ |
| Contact No : _____ | Name : _____ |
| E-mail: _____ | |

**Personal Data Protection Statement**

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

**Notice:**

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

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

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

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

**Consent:**

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

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

3. You may access and update your personal data by writing to us at dhr@utar.edu.my.

**Acknowledgment of Notice (Please tick)**

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

☐ I disagree, my personal data will not be processed.

# APPENDIX C

**Respondent No:**

**PART A: Demographics and anthropometrics**
Please complete this part. Fill in the particulars or circle only one most relevant answer. The anthropometric measurements will be performed for you.

1. **Age:** _____ 2. **Gender:** ☐ Male ☐ Female 3. **Ethnicity:** ☐ Malay ☐ Chinese ☐ Indian

4. **Anthropometric measurements**

| Measurement | 1st reading | 2nd reading | Measurement | 1st reading | 2nd reading |
|---|---|---|---|---|---|
| SBP (mmHg) | | | Weight (kg) | | |
| DBP (mmHg) | | | BMI (kg/m²) | | |
| Pulse rate (bpm) | | | TBF (%) | | |
| Waist circumference (cm) | | | SF (%) | | |
| Hip circumference (cm) | | | VFL (%) | | |
| Height (cm) | | | SM (%) | | |
| | | | RM (kcal) | | |

**PART F: Environmental exposure**
Please tick ONE most relevant answer for the questions below.

1. How many times a week do you engage in vigorous physical activity long enough to make you breathe hard?
   ☐ Never or only occasionally
   ☐ 1 – 2 times/week
   ☐ ≥ 3 times/week
2. How many hours do you spend in front of the TV/computer every day?
   ☐ < 1 hour
   ☐ 1 – 3 hours
   ☐ > 3 – 5 hours
   ☐ > 5 hours
3. How often do you consume alcohol?
   ☐ Frequent
   ☐ Occasional
   ☐ Non-drinker
4. What is your smoking status?
   ☐ Smoker
   ☐ Ex-smoker
   ☐ Non-smoker
5. How many people living in your house smoke cigarettes?
   _____ people
6. Does your father (or male guardian) smoke cigarettes?
   ☐ Yes ☐ No
7. Does your mother (or female guardian) smoke cigarettes?
   ☐ Yes ☐ No

 6

## APPENDIX D

| Subject | Genotype | Subject | Genotype | Subject | Genotype |
|---|---|---|---|---|---|
| 1 | GA | 169 | GG | 337 | GG |
| 2 | GA | 170 | GG | 338 | GA |
| 3 | GG | 171 | GG | 339 | GG |
| 4 | GG | 172 | AA | 340 | GG |
| 5 | GG | 173 | GG | 341 | GG |
| 6 | GG | 174 | GA | 342 | GG |
| 7 | AA | 175 | AA | 343 | GG |
| 8 | GA | 176 | GA | 344 | GA |
| 9 | GG | 177 | GG | 345 | GG |
| 10 | GA | 178 | GG | 346 | GA |
| 11 | GA | 179 | GA | 347 | GA |
| 12 | GG | 180 | GG | 348 | GA |
| 13 | GA | 181 | GA | 349 | GG |
| 14 | AA | 182 | GA | 350 | GG |
| 15 | GA | 183 | GA | 351 | GA |
| 16 | GA | 184 | GG | 352 | GA |
| 17 | GG | 185 | GG | 353 | GA |
| 18 | GG | 186 | GG | 354 | GG |
| 19 | GA | 187 | GG | 355 | GG |
| 20 | GG | 188 | GG | 356 | GA |
| 21 | GA | 189 | AA | 357 | GG |
| 22 | GA | 190 | GG | 358 | AA |
| 23 | GG | 191 | GA | 359 | GG |
| 24 | GA | 192 | AA | 360 | GG |
| 25 | GA | 193 | GG | 361 | GA |
| 26 | AA | 194 | GG | 362 | GG |
| 27 | GG | 195 | GG | 363 | GG |
| 28 | GG | 196 | AA | 364 | GA |
| 29 | GG | 197 | GA | 365 | GA |
| 30 | GA | 198 | GA | 366 | GG |
| 31 | GA | 199 | GG | 367 | GG |
| 32 | AA | 200 | AA | 368 | AA |
| 33 | GG | 201 | GA | 369 | GA |
| 34 | GA | 202 | GG | 370 | GG |

*GG: Homozygous wildtype; GA: Heterozygous; AA: Homozygous mutant

| Subject | Genotype | Subject | Genotype | Subject | Genotype |
|---|---|---|---|---|---|
| 35 | AA | 203 | GA | 371 | GG |
| 36 | GA | 204 | GG | 372 | GG |
| 37 | AA | 205 | GA | 373 | AA |
| 38 | AA | 206 | GG | 374 | GG |
| 39 | GG | 207 | GG | 375 | GG |
| 40 | GG | 208 | GG | 376 | GG |
| 41 | GG | 209 | GA | 377 | AA |
| 42 | GA | 210 | GA | 378 | GA |
| 43 | GA | 211 | GG | 379 | GG |
| 44 | GA | 212 | GA | 380 | GG |
| 45 | GG | 213 | GG | 381 | AA |
| 46 | GA | 214 | GA | 382 | GG |
| 47 | GA | 215 | GA | 383 | AA |
| 48 | GA | 216 | GG | 384 | GA |
| 49 | GA | 217 | GA | 385 | GA |
| 50 | GG | 218 | GG | 386 | GG |
| 51 | GG | 219 | GG | 387 | GG |
| 52 | GG | 220 | AA | 388 | GG |
| 53 | GG | 221 | GG | 389 | GA |
| 54 | GA | 222 | GA | 390 | GA |
| 55 | GG | 223 | GG | 391 | GA |
| 56 | GG | 224 | GA | 392 | GA |
| 57 | GG | 225 | GG | 393 | GA |
| 58 | GG | 226 | GG | 394 | GG |
| 59 | GA | 227 | GA | 395 | GA |
| 60 | GG | 228 | GA | 396 | GA |
| 61 | GA | 229 | GA | 397 | AA |
| 62 | GA | 230 | GA | 398 | GG |
| 63 | GA | 231 | GG | 399 | GG |
| 64 | GG | 232 | GA | 400 | GA |
| 65 | GG | 233 | GG | 401 | GA |
| 66 | AA | 234 | GG | 402 | GA |
| 67 | GG | 235 | GA | 403 | GG |
| 68 | GA | 236 | GA | 404 | GG |
| 69 | GA | 237 | GG | 405 | AA |
| 70 | GG | 238 | GG | 406 | GG |

*GG: Homozygous wildtype; GA: Heterozygous; AA: Homozygous mutant

| Subject | Genotype | Subject | Genotype | Subject | Genotype |
|---|---|---|---|---|---|
| 71 | GG | 239 | GG | 407 | GA |
| 72 | GG | 240 | GA | 408 | GA |
| 73 | GA | 241 | GG | 409 | GA |
| 74 | AA | 242 | GA | 410 | GA |
| 75 | GA | 243 | GG | 411 | GG |
| 76 | GG | 244 | GA | 412 | GG |
| 77 | GG | 245 | GA | 413 | GG |
| 78 | GA | 246 | GG | 414 | GG |
| 79 | GA | 247 | GG | 415 | GG |
| 80 | GA | 248 | GG | 416 | GA |
| 81 | GG | 249 | GG | 417 | GA |
| 82 | GA | 250 | AA | 418 | GA |
| 83 | GG | 251 | AA | 419 | GA |
| 84 | GA | 252 | GA | 420 | GA |
| 85 | GA | 253 | GG | 421 | GG |
| 86 | GA | 254 | AA | 422 | GA |
| 87 | GG | 255 | GA | 423 | GA |
| 88 | AA | 256 | GA | 424 | AA |
| 89 | GA | 257 | GG | 425 | AA |
| 90 | GA | 258 | GA | 426 | GG |
| 91 | GG | 259 | AA | 427 | GG |
| 92 | GG | 260 | GG | 428 | GA |
| 93 | GA | 261 | GA | 429 | GA |
| 94 | GA | 262 | GA | 430 | GA |
| 95 | GG | 263 | AA | 431 | GA |
| 96 | GG | 264 | GG | 432 | GA |
| 97 | GG | 265 | GG | 433 | GG |
| 98 | AA | 266 | GG | 434 | GG |
| 99 | GG | 267 | GG | 435 | GG |
| 100 | GG | 268 | GA | 436 | GA |
| 101 | GG | 269 | GG | 437 | GG |
| 102 | GG | 270 | GG | 438 | GG |
| 103 | GG | 271 | GG | 439 | GG |
| 104 | GG | 272 | GA | 440 | GG |
| 105 | GG | 273 | GA | 441 | AA |
| 106 | GA | 274 | GA | 442 | AA |

*GG: Homozygous wildtype; GA: Heterozygous; AA: Homozygous mutant

| Subject | Genotype | Subject | Genotype | Subject | Genotype |
|---|---|---|---|---|---|
| 107 | GA | 275 | GG | 443 | GG |
| 108 | GA | 276 | GG | 444 | GG |
| 109 | GA | 277 | GA | 445 | GA |
| 110 | GG | 278 | GA | 446 | GG |
| 111 | GG | 279 | GG | 447 | GA |
| 112 | GA | 280 | GG | 448 | GA |
| 113 | GA | 281 | GG | 449 | GG |
| 114 | GA | 282 | GG | 450 | GA |
| 115 | GA | 283 | GA | 451 | GG |
| 116 | GG | 284 | AA | 452 | GG |
| 117 | GG | 285 | GG | 453 | GA |
| 118 | GA | 286 | AA | 454 | GA |
| 119 | GA | 287 | GG | 455 | GG |
| 120 | GG | 288 | GG | 456 | GG |
| 121 | GG | 289 | GG | 457 | GG |
| 122 | GG | 290 | GA | 458 | AA |
| 123 | AA | 291 | GG | 459 | GA |
| 124 | AA | 292 | GG | 460 | GG |
| 125 | GA | 293 | GG | 461 | GG |
| 126 | GA | 294 | GG | 462 | GA |
| 127 | GG | 295 | GG | 463 | GG |
| 128 | GA | 296 | GG | 464 | GG |
| 129 | AA | 297 | GA | 465 | GG |
| 130 | GG | 298 | GG | 466 | GG |
| 131 | GG | 299 | GG | 467 | GA |
| 132 | GG | 300 | GA | 468 | GA |
| 133 | GA | 301 | GA | 469 | GG |
| 134 | GA | 302 | GG | 470 | GG |
| 135 | GG | 303 | AA | 471 | GG |
| 136 | GA | 304 | GA | 472 | GG |
| 137 | GA | 305 | GA | 473 | GG |
| 138 | GA | 306 | GG | 474 | GG |
| 139 | GA | 307 | GA | 475 | GA |
| 140 | GG | 308 | AA | 476 | GA |
| 141 | GG | 309 | GG | 477 | GA |
| 142 | GA | 310 | GA | 478 | GG |

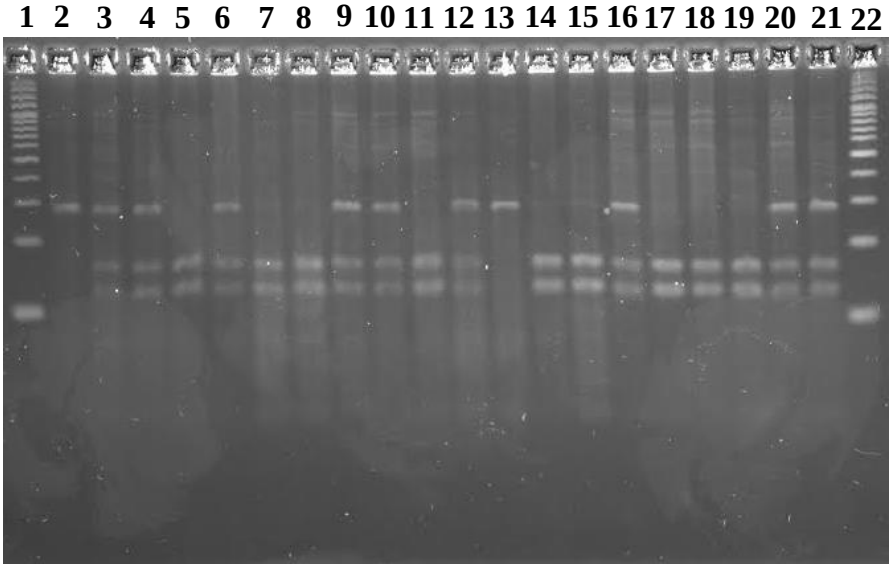
*GG: Homozygous wildtype; GA: Heterozygous; AA: Homozygous mutant

| Subject | Genotype | Subject | Genotype | Subject | Genotype |
|---|---|---|---|---|---|
| 143 | GA | 311 | GA | 479 | GA |
| 144 | GA | 312 | AA | 480 | GA |
| 145 | GG | 313 | GG | 481 | GA |
| 146 | GA | 314 | GA | 482 | GG |
| 147 | GG | 315 | GA | 483 | GA |
| 148 | GG | 316 | GG | 484 | GG |
| 149 | GA | 317 | GA | 485 | GA |
| 150 | AA | 318 | GG | 486 | GA |
| 151 | GA | 319 | GG | 487 | GG |
| 152 | GG | 320 | AA | 488 | GG |
| 153 | GG | 321 | GG | 489 | GA |
| 154 | GG | 322 | GA | 490 | AA |
| 155 | GA | 323 | GG | 491 | GG |
| 156 | GA | 324 | GA | 492 | GA |
| 157 | GA | 325 | GG | 493 | AA |
| 158 | GA | 326 | GA | 494 | GG |
| 159 | GG | 327 | GA | 495 | GG |
| 160 | GA | 328 | GG | 496 | GG |
| 161 | GA | 329 | GG | 497 | GG |
| 162 | GA | 330 | GA | 498 | GG |
| 163 | GA | 331 | GA | 499 | GA |
| 164 | GA | 332 | GA | 500 | GG |
| 165 | GA | 333 | GA | 501 | GG |
| 166 | GG | 334 | GG | 502 | GG |
| 167 | GG | 335 | GG | | |
| 168 | GA | 336 | GA | | |

*GG: Homozygous wildtype; GA: Heterozygous; AA: Homozygous mutant

## APPENDIX E

**Representative gel image for genotyping of rs7138803**




The 100 bp DNA ladder was loaded onto lane 1 and lane 22. The genotypes of each sample is as followed:

| Lane | Genotype | Lane | Genotype |
|---|---|---|---|
| 2 | AA | 12 | AG |
| 3 | AG | 13 | AA |
| 4 | AG | 14 | GG |
| 5 | GG | 15 | GG |
| 6 | AG | 16 | AG |
| 7 | GG | 17 | GG |
| 8 | GG | 18 | GG |
| 9 | AG | 19 | GG |
| 10 | AG | 20 | AG |
| 11 | GG | 21 | AG |

*GG: Homozygous wildtype; GA: Heterozygous; AA: Homozygous mutant