ASSOCIATION OF *ATP2A1* rs3888190 SINGLE NUCLEOTIDE POLYMORPHISM WITH OBESITY AND ALLERGIC CONDITIONS AMONG UTAR KAMPAR CAMPUS STUDENTS

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ABSTRACT

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JANICE WONG TSE KEI

Obesity and allergic diseases such as asthma, allergic rhinitis and atopic dermatitis are increasing worldwide. Both have attracted the attention of public due to the reduced quality of life and increased expenditure on medication and treatment. *ATP2A1* rs3888190 single nucleotide polymorphism (SNP) was previously associated with obesity through adipogenesis, glucose transport and thermogenesis. Yet, the association with allergy phenotypes is still unknown. However, the SERCA1 protein encoded by this gene is involved in the regulation of intracellular Ca^{2+} concentration which mediates the histamine release from mast cells. Therefore, the main objective of this study was to identify the association of *ATP2A1* rs3888190 SNP with obesity and allergic conditions among UTAR Kampar Campus students. A total of 453 subjects were recruited for the collection of demographics, anthropometric measurements, questionnaire data and mouthwash samples. Skin prick test was only performed on 308 subjects. Genotyping was performed using TaqMan

SNP genotyping assay. Among the 453 subjects, 377 subjects had CC genotype, 74 subjects had CA genotype and only 2 subjects had AA genotype. The overall minor allele frequency was 0.09 (Malays = 0.001; Chinese = 0.08; Indians = 0.004). Only *ATP2A1* rs3888190 genotype of Chinese males showed significant association with the mean value of total body fat (TBF) ($p < 0.01$). Atopic dermatitis was significantly associated with body mass index (BMI) ($p = 0.01$) and waist circumference (WC) ($p = 0.01$). There was also a significant association of asthma + atopic dermatitis with BMI ($p = 0.01$). However, genotype of Chinese females did not show any association with the means of anthropometric measurements. No significant association was also found between *ATP2A1* rs3888190 allele and gender, ethnicity, BMI, WC and TBF. *ATP2A1* rs3888190 allele was not significantly associated with asthma, atopic dermatitis, asthma + allergic rhinitis, asthma + atopic dermatitis, allergic rhinitis + atopic dermatitis and asthma + allergic rhinitis + atopic dermatitis as well. In conclusion, *ATP2A1* rs3888190 SNP increases the risk of acquiring high TBF in Chinese males but not in Chinese females, and it is not associated with allergic conditions among UTAR students.

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DECLARATION

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

…….

JANICE WONG TSE KEI

APPROVAL SHEET

This project report entitled **"ASSOCIATION OF *ATP2A1* rs3888190 SINGLE NUCLEOTIDE POLYMORPHISM WITH OBESITY AND ALLERGIC CONDIIONS AMONG UTAR KAMPAR CAMPUS STUDENTS"** was prepared by **JANICE WONG TSE KEI** and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) in Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by,

……..... Date:……..…….

(Dr. Say Yee How)

Supervisor

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Date: ……

PERMISSION SHEET

It is hereby certified that **JANICE WONG TSE KEI** (ID No: **13ADB06839**) has completed this final year project entitled **"ASSOCIATION OF *ATP2A1* rs3888190 SINGLE NUCLEOTIDE POLYMORPHISM WITH OBESITY AND ALLERGIC CONDITIONS AMONG UTAR KAMPAR CAMPUS STUDENTS"** under the supervision of Dr. Say Yee How from the Department of Biomedical Science, Faculty of Science.

I understand that University will upload softcopy of my final year project in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

…….

(Janice Wong Tse Kei)

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LIST OF ABBREVATIONS

A	Asthma
AD	Atopic Dermatitis
ADRB3	Adrenergic β3 Receptor
AMP	Adenosine Monophosphate
AR	Allergic Rhinitis
ATP	Adenosine Triphosphate
ATP2A1	ATPase sacrcoplasmic/endoplasmic reticulum Ca^{2+} Transporting 1
BAT	Brown Adipose Tissue
BDNF	Brain-Derived Neurotrophic Factor
BMI	Body Mass Index
CA^{2+}	Calcium ion
cAMP	Cyclic Adenosine Monophosphate
CART	Cocaine- and Amphetamine-regulated Transcript
CDKAL1	CDK5 Regulatory Subunit Associated Protein 1-Like 1
CNR1	Endocannabinoid Receptor 1
DBP	Diastolic Blood Pressure
FAM120AOS	Family with Sequence Similarity 120A Opposite Strand
FAS	Fatty Acid Synthase
FTO	Fat Mass and Obesity-associated Gene
GLUT4	Glucose Transporter Type 4

GP2	Glycoprotein 2
GWAS	Genome-Wide Association Studies
HC	Hip Circumference
IP_3	Inositol 1, 4, 5-Triphosphate
JAK2	Janus Kinase 2
KLF9	Kruppel-Like Factor 9
LD	Linkage Disequilibrium
MAF	Minor Allele Frequency
MC4R	Melanocortin 4 Receptor
n	Frequency
NEGR1	Neuronal Growth Regulator 1
NTC	No Template Control
PCR	Polymerase Chain Reaction
PCSK1	Pro-Hormone Convertase 1
POMC	Pro-opiomelanocortin
RM	Resting Metabolism
SBP	Systolic Blood Pressure
SERCA1	Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} ATPase
SF	Subcutaneous Fat
SH2B1	Src-homology 2B Adaptor Protein 1
SM	Skeletal Muscle
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Package for the Social Sciences
SR/ER	Sarcoplasmic/Endoplasmic Reticulum

TBF	Total Body Fat
UTAR	Universiti Tunku Abdul Rahman
VFL	Visceral Fat Level
WC	Waist Circumference
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Based on the WHO definition of obesity, it is defined as abnormal fat accumulation in the body that poses a risk to health. Obesity is measured by BMI, in which the weight in kilograms is divided by the square of height in metres (kg/m^2). An overweight individual has a BMI equal to or more than 25. Nowadays, at least 2.8 million of overweight or obese individuals die each year (World Health Organization, 2016c). Obesity has now become a major public health concern because it leads to many adverse metabolic effects which in turn increase the risk of many common chronic diseases causing early death, such as cardiovascular disease, type 2 diabetes as well as cancer (Cheung and Mao, 2014).

Besides obesity, allergic diseases such as asthma, allergic rhinitis (AR) and atopic dermatitis (AD) are also rising worldwide, especially among the youths. Wheezing and dyspnea are commonly seen in individuals with asthma, while the symptoms for AR are nasal congestion, itch and discharged. Skin caused by AD will normally appear red, itchy, dried and cracked. All these allergic reactions in response to allergen are mediated by IgE (Leynaert, et al., 2000; Kim and Mazza, 2011; Allergy Central Malaysia, n.d.).

In South East Asia, the lowest prevalence was indicated by 14% in both genders for overweight and 3% for obesity (World Health Organization, 2016c). Meanwhile in Malaysia, the prevalence of overweight and obesity was 43.8% in men and 48.6% in women who aged 20 years and above (Ng, et al., 2014). The prevalence of Asian adults with asthma was between 0.7% and 11.9%, while the prevalence of AR was 8.7% (Lim, et al., 2015). For AD, 1% to 3% of grown-ups were affected globally (Lee and Detzel, 2015).

Currently, the interaction of environmental and genetic factors is being emphasized for the determination of body weight and allergy. Environmental factors such as unhealthy dietary habits, sedentary lifestyle with low physical activity and certain medications will increase the risk of obesity (Centers for Disease Control and Prevention, 2015). The severity of effects caused by these changes will increase if the population carries a genotype that predisposes to fatness (World Health Organization, 2000a; Ismail, et al., 2002). As a result, obesity with a monogenic or polygenic origin can affect the intake of food, nutrient turnover and thermogenesis in the body (Marti, et al., 2004). The environmental factors that cause allergic diseases include indoor (dust mites) and outdoor (pollen) allergens, physical activity, tobacco smoke, stress and chemicals in workplace (World Health Organization, n.d.). Also, several SNPs within the obesity-susceptible genes from GWAS were shown to have association with asthma (Melen, et al., 2010).

According to genome-wide association studies (GWAS) and candidate gene studies, the upstream variant rs3888190 located at the upstream of *ATP2A1* gene is in perfect linkage equilibrium (LD) with several SNPs in *SH2B1* gene, which were confirmed to be involved in obesity (Beckers, et al., 2011). According to Ensembl (2016), the lead SNP rs3888190 was also shown to be associated with BMI.

ATP2A1 encodes the SERCA1 protein of SR and ER to regulate the cytoplasmic Ca^{2+} in the muscle cells by removing the Ca^{2+} from the cytosol back to the lumen using ATP hydrolysis (Brini and Carafoli, 2009). SERCA1 also regulates the themogenesis of BAT by releasing heat during ATP hydrolysis via coupled and uncoupled Ca^{2+} transport (De Meis, et al., 2005; Arruda, et al., 2008). In human adipocytes, lipogenesis and lipolysis events are controlled by the intracellular Ca^{2+} (Parikh and Yanovski, 2003). Increase in intracellular Ca^{2+} in adipocytes is shown to enhance glucose transport into the cells as well (Funai, et al., 2013). Besides that, intracellular Ca^{2+} is also involved in allergic reaction, in which the release of histamine from mast cells relies on the increased Ca^{2+} concentration (Takei, et al., 1989).

Previous studies mostly found out that decreased expression of SERCA1 in fast-twitch muscles had led to Brody's myopathy and congenital pseudomyotonia characterized by impaired muscle relaxation and stiffness after exercise (Brini and Carafoli, 2009). Plus, *ATP2A1* rs3888190 was also

confirmed to be associated with BMI and had high LD with a few *SH2B1* SNPs involved in obesity (Speliotes, et al., 2010; Beckers, et al., 2011). Therefore, this research is carried out to find out the association of *ATP2A1* rs3888190 SNP with obesity, asthma, AR and AD among the multi-racial Malaysian subjects as the association is still unknown in the population.

The objectives of this study are:

1. To collect demographic information and anthropometric measurements for the study of obesity prevalence among UTAR subjects.
2. To collect the data of UTAR students through allergy questionnaire and skin prick test to determine the prevalence of asthma, AR and AD.
3. To collect the mouthwash samples for the study of prevalence of *ATP2A1* rs3888190 (C>A) SNP through DNA extraction and TaqMan SNP genotyping assay.
4. To analyze the association of *ATP2A1* rs3888190 (C>A) SNP with obesity, asthma, AR and AD among UTAR students.

CHAPTER 2

LITERATURE REVIEW

2.1 Definition

2.1.1 Definition of Obesity

Overweight and obesity are defined as the excess accumulation of body fat that may affect the health of an individual. In order to classify the overweight and obese adults aged more than 18 years, BMI is usually adopted as it can be calculated easily just by dividing the weight of a person in kilograms with his or her height in meter square (kg/m^2). By using this convenient method, the terms of overweight and obesity can be further defined as having BMI more than or equal to 25 and BMI more than or equal to 30 respectively (World Health Organization, 2016d). The fact that BMI provides a good prediction of fatness is actually shown by many years of research (Harvard T.H. Chan School of Public Health, 2016). However, the association of BMI with the risk of mortality and morbidity may not be the same in different ethnic groups. Therefore, some countries have come up with their own cut-off to assess the risk by using BMI (Caballero, 2007). For example in Asian countries, based on the WHO's proposed classification of weight by BMI, the cut-off point of overweight is 23 kg/m^2 whereas for obesity is 25 kg/m^2 (World Health Organization, 2000b).

Adiposity is the extra fat that can be found around the waist, and it also indicates another important category of obesity which is not mentioned by BMI. Basically, abdominal obesity in Asians is defined as having a WC greater than or equal 80 cm in women, while in men as having a waist size greater than or equal to 90 cm (World Health Organization, 2000b). In addition, the cut-off points of waist-hip ratio for Asians are 0.90 for men and 0.80 for women. When compared to waist-hip ratio, WC is still the preferred choice to measure abdominal obesity (World Health Organization, 2008).

Other methods to measure obesity are through bioelectric impedance analysis, underwater weighing, computed tomography and magnetic resonance imaging (Bell, et al., 2005). Although these techniques are more laborious, they are generally more accurate. But still, BMI, WC, HC and waist-hip ratio are the common measurements for analysis as those laborious methods are not readily available in sample collection (Kettunen, 2010).

2.1.2 Definition of Asthma, Allergic Rhinitis and Atopic Dermatitis

Asthma is the chronic inflammation of smooth muscles in the airways which can be triggered by allergic (allergens) and non-allergic (exercise) stimuli (Kim and Mazza, 2011). In the presence of allergen, IgE bound to the receptors of mast cells and basophils is cross-linked. The mediators are then released to induce bronchoconstriction and inflammation (Ishmael, 2011). Asthma is mainly characterized by wheezing, shortness of breath, coughing and chest

tightness (Kim and Mazza, 2011). Individuals with asthma have limitations and impacts in their physical and daily activities, emotions and social life (Leynaert, et al., 2000).

AR is the inflammation of nasal mucosa soft tissue with many inflammatory cells penetrating the nasal lining after being stimulated by an allergen (Small and Kim, 2011). The inflammation in response to allergen is also IgE-mediated characterized by the release of chemokines and cytokines from inflammatory cells (Pawankar, et al., 2011). The nasal symptoms of AR are nasal blockage, nasal itch, rhinorrhea and sneezing accompanied by headache, poor concentration, sleep disturbance and thirst (Leynaert, et al., 2000).

AD is an itchy, chronic inflammation of skin caused by skin barrier defects, immune abnormalities, allergens and irritant chemicals (Watson and Kapur, 2011). Allergen stimulates the degranulation of mast cells, causing the damage of skin epithelial cells which in turn releases thymic stromal lymphopoietin to up-regulate skin inflammation. Cytokines and chemokines are also secreted by keratinocytes and local lymphoid cells to attract more immune cells (Peng and Novak, 2015). Diagnosis can be made based on skin appearance and history. Classic signs of AD are itchy, redness, cracking and scaling on the face, arms and trunk. The rash may spread to other skin area if scratching is applied (Allergy Central Malaysia, n.d.).

2.2 Prevalence

2.2.1 Prevalence of Obesity

The prevalence of overweight and obesity has risen dramatically over the past 3 decades (Cheung and Mao, 2014). Over 600 million adults (13%) from the 1.9 billion overweight adults (39%) who aged 18 years and above were obese in 2014 (World Health Organization, 2016d). Based on the British medical journal The Lancet, obesity will be increased to 18% of men and 21% of women by 2025 globally (Berlinger, 2016). Over a quarter of extremely obese individuals in the world are from the highly developed countries such as United States, United Kingdom, Canada, Ireland, Australia and New Zealand, followed by the two second most countries Middle East and North Africa that account for 13.9% of the obese population (Berlinger, 2016).

Again according to The Lancet, Malaysia was rated as the highest prevalence for obesity among the Asian countries, with 44% and 49% of obese men and women in 2014 (Corporate 21 Media Group, 2014). A prevalence rate with standardized age of overweight and obesity had been computed for the 20 years old and above population and for ages below 20 years with the standard population distribution. It was done by referring to the World Population Prospect 2012 revision which consisted of the average country-level population distribution by age (UN Department of Economic and Social Affairs, 2011; Ng, et al., 2014). Based on the findings, the prevalence of overweight and obesity in Malaysian boys and girls aged less than 20 years was 22.5% and 19.1% respectively, while the prevalence of obese showed 8.8% in boys and 7.2% in

girls. On the other hand, the prevalence of overweight and obesity in Malaysian men and women aged 20 years and older was 43.8% and 48.6% respectively, while the prevalence of obese showed 11.4% in men and 16.7% in women (Ng, et al., 2014).

Based on the National Health and Morbidity Survey 2015 in Malaysia, two guidelines known as the Malaysian Clinical Practice Guidelines of Obesity (2004) and WHO (2008) were used to classify BMI. According to the Malaysian Clinical Practice Guidelines of Obesity (2004), the national prevalence overweight and obesity was 33.4% and 30.6%. The prevalence of overweight in males (35.8%) was significantly higher than females (30.9%). However, higher prevalence of obesity was observed in females (33.6%) compared to males (27.8%). When WHO (2008) classification was used, the national prevalence of overweight and obesity had dropped to 30.0% and 17.7%. The prevalence of overweight was still the highest in males (31.6%). However, the prevalence of obesity was significantly lower in males (15%) compared to females (20.6%) (Institute for Public Health, 2015).

2.2.2 Prevalence of Asthma, Allergic Rhinitis and Atopic Dermatitis

Asthma is a major non-transmissible disease among the children. A number of 235 million people was estimated to have asthma (World Health Organization, 2013). Based on the most recent data from Centers for Disease Control and Prevention (2016), the prevalence of current asthma was 7.4% among adults

who aged 18 and above in 2014. The prevalence in male and female adults was 5.1% and 9.6% respectively. In Malaysia, the prevalence of asthma diagnosed by doctors was 9.6% (Lim, et al., 2015).

According to World Allergy Organization (2016b), the prevalence of AR was between 5.9% and 29%. In middle and low-income Asia countries, Lim, et al. (2015) also stated that the prevalence could reach 45% in adolescents. Besides that, the prevalence of AR in adults shown in Asia-Pacific surveys was 8.7% (Bjorkdten, et al., 2008; Katelaris, et al., 2011; Lim, et al., 2015). In Malaysia, 40% of the population was affected (Allergy Centre Malaysia, n.d.).

The prevalence of AD in children and young adults was 5% and 10% respectively (World Allergy Organization, 2016a). In the United States, there were 31.6 million of people with AD. The prevalence was 10.7% in children while in adults it could reach 10.2% (National Eczema Association, n.d.). Based on the ISAAC Phase III study, the prevalence of eczema was between 1.8% and 23.4% in children and between 0.9% and 21.1% in adolescents. In Malaysia, the prevalence in adolescents was 9% (Odhiambo, et al., 2009; Lee and Detzel, 2015).

2.3 Environmental and Genetic Factors

2.3.1 Behavioral and Community Environmental Factors of Obesity

Obesity can be resulted from behavior factors such as long-term unhealthy dietary habits, reduced physical activity, sedentary lifestyle and medication use. Other factors that contribute to obesity may include the environment for food and physical activity, food promotion and marketing as well as education (Centers for Disease Control and Prevention, 2015).

In recent decades, the number of fast food outlets is increasing in Malaysia in pace with the global westernization of eating habits (Ismail, 2002; Ismail, et al., 2002). This has led to excessive dietary consumption of fast energy-dense food and high chances to eat the whole day, resulting in a higher obesity prevalence (Drewnowski and Specter, 2004; Caballero, 2007). An increased sweetened beverage intake has also replaced fruits and vegetables, in which the intake is lower than the recommended level. Also, such reduced consumption is influenced by the unhealthy food advertisements and the increased intake of junk food (Schmidt, Affenito and Striegel-Moore, 2005; Caballero, 2007).

Screen-based sedentary lifestyles are now gradually emerging (Kautiainen, et al., 2005; Mitchell, et al., 2013; 23 Pey, et al., 2014). Television, computer, video game and internet are currently the choices of life, thus decreasing the physical activity of many people (Pratt, et al., 2008). The significant association of reduced television-watching and computer use with lower BMI

was successfully shown by Epstein, et al. (2008). Based on Pey, et al. (2014), individuals with less than one hour of moderate to vigorous physical activity daily are at four times the risk of becoming obese.

Furthermore, the built environmental factors have significantly affected the BMI of population (Frank, Andresen and Schmid, 2004; Caballero, 2007). Based on the Center for Disease Control and Prevention (2015), decisions are made by the people in accordance to the environment and community, for examples, car use promoted by urban planning and unsafe public spaces for walking and cycling to work. Besides that, the daily behaviors can also be affected by the infrastructures of home, school, workplace and healthcare. As a result, these environments should be created in a way that allows the physical activity and healthy diet to be engaged easily.

2.3.2 Environmental Factors of Asthma, Allergic Rhinitis and Atopic Dermatitis

Since 1990s, environmental factors associated with allergy have intensively become the current interest among the public (Strachan, 2000). Allergic diseases are often initiated by the inhaled particles that provoke the airways, including indoor (dust mite, storage mite, cockroach, carpet and pet dander) outdoor allergens (pollen and mould spores) (World Health Organization, n.d.). Based on Malaysian study, the most common house dust mite is *Blomia tropicalis*, followed by *Dermatophagoides pteronyssinus* and *Malayoglyphus*

intermedius (Lim, et al., 2015). Besides that, second-hand smoke is a certain risk for asthma (Pearce and Strachan, 2014). Other factors such as exercise (endurance), cold air, medications (aspirin, β-blockers and non-steroid anti-inflammatory drugs) and excessive emotional stimulation can induce asthma as well (World Health Organization, n.d.). Also, irritants in the workplace (metals, reactive dyes, isocyanates and acid acid anhydrides) and air pollution (CO, NO_2 and SO_2) have the potential to stimulate respiratory and skin allergies (World Allergy Organization, 2013). Furthermore, bacterial or viral infections are found to worsen the allergic diseases especially the endotoxin of gram-negative bacteria, which induces inflammation in asthma and AR. Differences in lifestyle such as nutrient intake, food, personal hygiene standard and stress can enhance the symptoms and allergic sensitization (Wang, 2005).

2.3.3 Genetic Factors of Obesity

Besides environmental factors, genetic predisposition is also a contributor to obesity. Different reactions of different people to the same environmental conditions indicate that genetic predisposition plays an important role in the development of obesity (Via and Hawthorne, 2005; Cheung and Mao, 2014).

According to the evolutionary standpoint, individuals carrying the thrifty genes are not greatly influenced by malnutrition, indicating the reason why the diverse populations are prone to obesity. Based on the family studies, the heritability of BMI was approximately 25% to 50% (Marti, et al., 2004).

Therefore, children whose parents are obese will experience eight times the risk for severe obesity (Bouchard, 2001). Twin studies had also revealed the contribution of genetic factor for obesity was 70% (Marti, et al., 2004).

On the other hand, input and output signals as well as central mechanisms used in body weight regulation may be determined by the genes. Multiple peptides and monoamines regulate the appetite, nutrient utilisation, physical activity response and adipocyte metabolism. As mentioned in Figure 2.1, the susceptible genes are shown to be involved in these processes (Loktionov, 2003; Palou, Pico and Bonet, 2003; Marti, et al., 2004). Besides that, genes responsible for the thermogenesis, energy expenditure and hormone profile can also influence the balance of energy (Rosenbaum, Leibel and Hirst, 1997; Barsh, Farooqi and O'Rahilly, 2000).

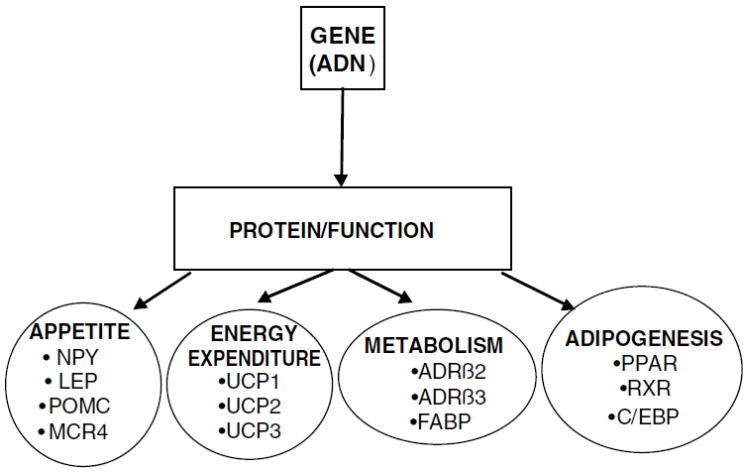

Figure 2.1: Classification of genes involved in body weight homeostasis by processes (Adopted from Marti, et al., 2004).

2.3.3.1 Monogenic Obesity

Obesity is contributed by mutations, deletions and SNPs. Most cases are polygenic obesity, yet there are still others caused by monogenic obesity (Puiu, Emandi and Arghirescu, 2013). Monogene is defined as a gene that shows extreme phenotype caused by a single gene variant without influenced by the environment (Hinney, Vogel and Hebebrand, 2010). Individuals with monogenic obesity may develop severe phenotypes such as early onset of childhood obesity with serious metabolic disorders (Zhao, et al., 2014).

The genetic factor of monogenic obesity is connected to the leptin-melanocortin pathway (Burrage and Mc Candless, 2007). Leptin in adipose tissue activates hypothalamic neurons to secrete peptides to reduce food intake and increase energy expenditure (Zhao, et al., 2014). Patients with congenital leptin deficiency are characterised by elevated energy intake, hyperphagia, hyperinsulinemia, dyslipidemia and liver steatosis (Funcke, et al., 2014). Peptides derived from *POMC* gene are mediated via melanocortin receptors to reduce feeding and increase energy utilization. Early-onset obesity and hyperphagia are often displayed in POMC-deficient individuals (Schwartz, et al., 2000; Pritchard, Turnbull and White, 2002).

The most common form of monogenic obesity is caused by MC4R deficiency. Yet, severe obesity shows incomplete penetration in some individuals who carry heterozygous mutations (Puiu, Emandi and Arghirescu, 2013). MC4R is

specifically involved in appetite regulation (Pritchard, Turnbull and White, 2002). According to Spradley, Palei and Granger (2015) and Burrage and Mc Candless (2007), MC4R deficiency promotes obesity, hyperphagia and increased fat mass and lean mass without the presence of hypentension.

2.3.3.2 Polygenic Obesity

Polygenic obesity is the most common obesity whereby the genetic makeup is susceptible to the environmental risk factors which encourages more energy intake than energy expenditure (Hinney, Vogel and Hebebrand, 2010). Each allele itself contributes a small effect on the obesity phenotype, only a complex interaction between multiple predisposing variants and environment will exert a fairly large effect on body weight (Alshafai, 2015).

Many candidate gene approaches have been used to search obesity and related phenotypes. This hypothesis-driven approach can determine the relationship between a variant of the candidate gene and an obesity trait, besides being able to be performed in unrelated subjects. It must be done on huge size for the detection of small effects caused by the variants in usual traits. For instance, children with mutation (Leu34Phe) in *CART* gene were found to be in relation to obesity and decreased energy utilization, whilst adolescents carrying this mutation would experience extreme obesity at early age (Miraglia, et al., 2001; Tabor, Risch and Myers, 2002; Bell, Walley and Froguel, 2005; Miraglia, et al., 2006; Cheung and Mao, 2014). Candidate gene association studies had also

discovered over 127 candidate genes including the genetic variants in *MC4R*, *ADRB3*, *PCSK1*, *BDNF* and *CNR1* which were the strongly replicated genes being identified (Alshafai, 2015).

GWAS is commonly used to identify the novel SNPs associated with the traits of interest. Besides being characterized as hypothesis-free, the entire genome is also screened at high degree of resolution by GWAS (Boezen, 2009). Currently, the association between SNP and BMI indicated by the Genetic Investigation of Anthropometric Traits (GIANT) Consortium using GWAS was absent from significant gender difference. A new downstream variant of *FAM120AOS* gene was found to be associated with BMI (Warrington, et al., 2015). Besides, GWAS also identified additional four novel loci (*CDKAL1, KLF9, PCSK1 and GP2*) related to BMI in East Asian population (Wang, et al., 2016). However until now, variants in *FTO* gene still have the greatest effect on obesity-related traits among the other loci as shown in Table 2.1 (Barroso and Fawcett, 2010). GWAS also showed that obesity caused by *SH2B1*, *BDNF* and *NEGR1* was due to impaired hypothalamus (McCarthy, 2010).

Table 2.1 Identification of the major BMI- and obesity-susceptibility loci via candidate gene and genome-wide association studies.

Chromosomal Location	**Gene**
1p31.1	*NEGR1*
1q41	*LYPLA1*

Table 2.1 (continued).

Chromosomal Location	Gene
1q25.2	*SEC16B, RASAL2*
2p25.3	*TMEM18*
2q14.1	*INSIG2*
3q27	*ETV5*
4p13	*GNPDA2*
5q13.3	*CART*
5q15-q21	*PCSK1*
6p12	*TFAP2B*
6p22.2-p21.3	*PRL*
6q14-q15	*CNR1*
8p12-p11.2	*ADRB3*
8p23.1	*MSRA*
10p12	*PTER*
11p11.2	*MTCH2*
11p13	*BDNF*
12q13	*BCDIN3D, FAIM2*
14q31	*NRXN3*
16p11.2	*SH2B1, ATP2A1*
16q12.2	*FTO*
16q22-q23	*MAF*
18q11-q12	*NPC1*
18q22	*MC4R*
19q13.11	*KCTD15*

Table 2.1 (continued).

Chromosomal Location	Gene
19q31	*CHST8*
Xq23-24	*SLC6A14, CUL4B*

(Cheung and Mao, 2014)

2.4. Gene Structures of *SH2B1* and *ATP2A1* Genes

2.4.1 *SH2B1* Gene

The protein coding *SH2B1* gene as shown in Figure 2.2 is located at chromosome 16p11.2 and has an exon count of 14. The protein produced regulates the kinase activation and signals the cytokine and growth factor receptors. It also mediates cellular transformation (NCBI, 2016d)

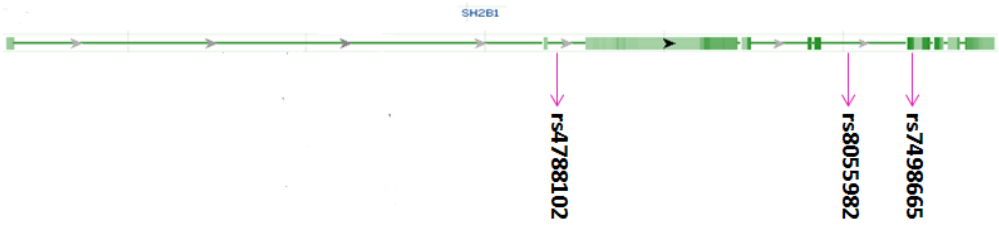

Figure 2.2 *SH2B1* gene (Adopted from NCBI, 2016d).

2.4.2 *ATP2A1* Gene

As shown in Figure 2.3, *ATP2A1* is a protein coding gene with cytogenetic location at 16p12.1 (NCBI, 2016c). It is also known as ATPase, Ca^{2+}

transporting, cardiac muscle or fast twitch 1 (OMIM, 2013). *ATP2A1* gene instructs the production of SERCA1 which is a member of the ATPase enzyme family (U.S. National Library of Medicine, 2016).

ATP2A1 rs3888190 is an upstream gene variant (TGAGGCCGGGGATGGAAGAGGGCTC[A/C]GGGAAGAACTGGGGGG ATGAGTTTG) at the chromosome location 16: 28878165 (forward strand). The global MAF for allele A is 0.26 (NCBI, 2016b). According to Ensembl (2016), the ancestral allele is A. The phenotypes that are significantly associated with this SNP are BMI (p = 2.27e^{-10}) and Crohns disease (p = 2.10e^{-8}). Its dbSNP HGVS names are NM_001286075.1:c.-2086C>A, NM_001310136.1:c.121-411263G>T, NM_004320.4:c.-507C>A and NM_173201.3:c.-507C>A, while its Ensembl HGVS name is NC_000016.10:g.28878165C>A.

Figure 2.3 *ATP2A1* gene (Adopted from NCBI, 2016a).

2.5 Genome-wide Association Studies of *SH2B1* and *ATP2A1* Genes

SH2B1 was found to be one of those obesity genes associated with severe early onset obesity (Bochukova, et al., 2010). Obesity was observed in *SH2B1*

knockout mice. Besides correcting the metabolic disorders, leptin signalling mediated by JAK2 and orexigenic neuropeptide was improved after restoration of SH2B1β (Maures, Kurzer and Carter-Su, 2007). Neuronal *SH2B1* over-expression was also shown to prevent obesity. The reciprocal duplication of this gene was associated with leanness as well (Volckmar, et al., 2012). Based on GWAS, the obesity-associated SNPs found might actually exert their effects by altering the transcriptional regulation, for example rs7498665 (Voisin, et al., 2015). BMI was also found to increase by almost 0.15 BMI units (kg/m^2) when obesity risk allele was present at *SH2B1* rs7498665 (Volckmar, et al., 2012).

According to Jamshidi, et al. (2007), significant association was shown between rs7498665 and serum leptin, total fat mass, waist circumference and weight. Besides that, five variants (rs4788102, rs8055982, rs7498665, rs7359397 and rs3888190) in the human *SH2B1* gene were also found to be in high LD (Jamshidi, et al., 2007).

Based on another GWAS study on BMI with a total of 339,224 subjects, 97 genetic loci had been identified in association with increased BMI (Volckmar, et al., 2015). The chromosomal region 16p11.2 involved was tagged by one of the lead SNPs rs3888190, which was near *ATP2A1* and *SH2B1* genes (Volckmar, et al., 2015). The SNP rs3888190 was chosen to study as it was in perfect LD with the SNPs in or near *SH2B1*. As shown in Figure 2.4, the black shaded box of rs3888190 indicates 100% LD (Beckers, et al., 2011).

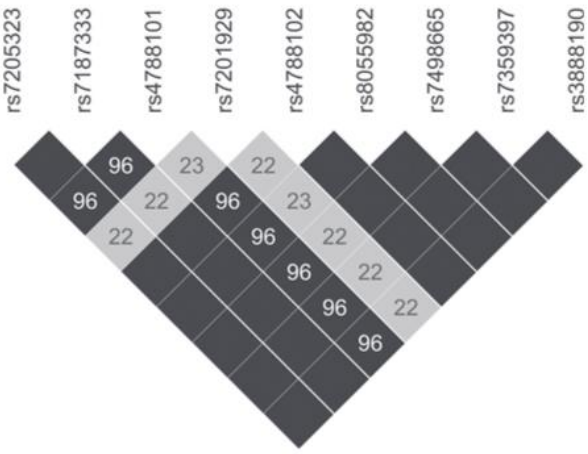

Figure 2.4 Linkage disequilibrium of different SNPs (Adopted from Beckers, et al., 2011).

2.6 *ATP2A1* Proteins and Functions

2.6.1 Isoforms of SERCA1

ATP2A1 gene encodes SERCA1 of SR of skeletal and cardiac muscles and also of ER of all cells. *ATP2A1* gene consists of 23 exons. The transcript as shown in Figure 2.5 is alternatively spliced to produce two isoforms, specifically SERCA1a and SERCA1b. The faster SERCA1a found in the fast-twitch skeletal muscles of adult is expressed together with fast myosin heavy isoforms. Meanwhile, neonatal SERCA1b contains the highly charged eight amino acid sequences rather than the E994 residue found in adult form. Furthermore, two other transcripts resulted from the splicing in the 5' region produce SERCA1T and SERCA1T+4 which are incapable of transporting Ca^{2+} (Chami, et al., 2000; Fajardo, et al., 2013).

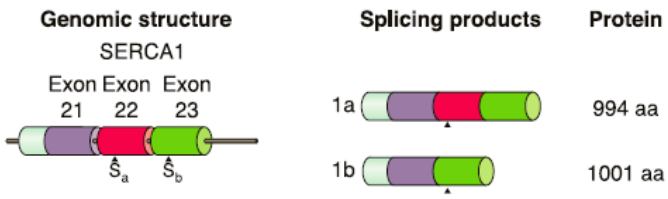

Figure 2.5 Generation of SERCA1 isoforms by alternative splicing of the human *ATP2A1* gene (Adopted from Brini and Carafoli, 2009).

2.6.2 General Properties of SERCA1

Mitochondrial respiration rate and bioenergetics are triggered by the release of Ca^{2+}, causing the increase of ATP production in turn to enhance SERCA1 function (Mekahli, et al., 2011). SERCA1 pump is the major system that regulates the intracellular free Ca^{2+} in the muscle cells by transporting Ca^{2+} from the cytosol back to the lumen after hydrolysis of ATP, thereby restoring the ER Ca^{2+} concentration (Fajardo, et al., 2013).

As shown in Figure 2.6, SERCA1 pump exists in E1 and E2 states. In E1 state, the pump has high affinity for Ca^{2+}, causing the interaction with Ca^{2+} at single side of the membrane. In E2 state, the pump has lower Ca^{2+} affinity, leading to the Ca^{2+} release at the opposite side. The changes of structure involving the transmembrane domains and protruding cytoplasmic region occur after the binding of Ca^{2+}, permiting the γ-phosphate of ATP to phosphorylate the catalytic D-residue. When high Ca^{2+} affinity E1-P pump is transited to the lower affinity E2-P pump, Ca^{2+} will dissociate from the pump. The hydrolysis

then reproduces the Ca^{2+}-free E2 ATPase to establish the catalytic cycle (Brini and Carafoli, 2009).

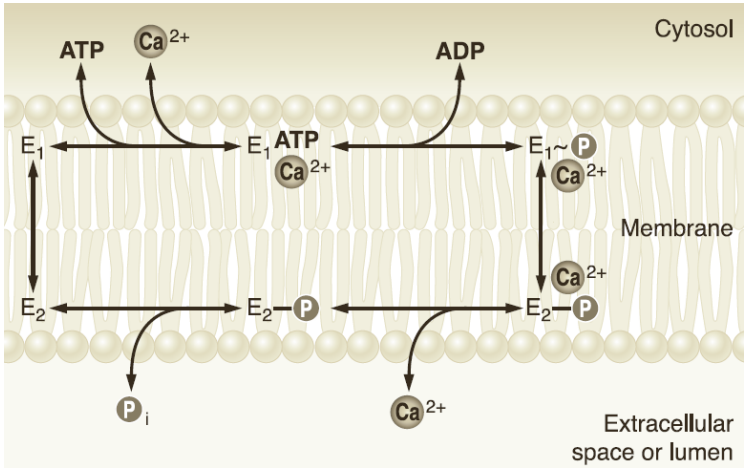

Figure 2.6 A simplified transport cycle of the SERCA1 pump (Adopted from Brini and Carafoli, 2009).

2.6.3 Regulation of Ca^{2+} in Thermogenesis

According to De Meis, et al. (2005), BAT thermogenesis is used to understand the non-shivering heat production and energy wasting mechanisms as it can rapidly convert fat stores to heat. In order to mediate the thermogenic activity, the activation of α_1-adrenoreceptors is coupled to IP_3 production, which is involved in the lipid signalling (De Meis, 2003). When the α_1-adrenoreceptors are activated, Ca^{2+} will also be released into the cytosol from the intracellular stores. Besides increasing Ca^{2+} release, the release of free fatty acids is also promoted by the β_3-adrenergic receptors (Leaver and Pappone, 2002). The storage of free Ca^{2+} in ER helps to determine the intracellular calcium

concentration, which plays a significant role in the metabolic disruption related to obesity (Parikh and Yanovski, 2003).

Besides storing Ca^{2+} inside the ER, SERCA1 plays a role in the thermogenesis (De Meis, et al., 2005). It hydrolyzes ATP via coupled and uncoupled Ca^{2+} transport. In the coupled reaction, part of the energy produced from the ATP hydrolysis is used to transport Ca^{2+} through the membrane, while the remaining part is converted into heat. The uncoupled reaction uses a shortcut where the heat is converted from all the energy released from ATP cleavage (Arruda, et al., 2008). According to a number of studies, thermogenesis has been predicted to contribute to 15% of energy expenditure in mankind (Feldmann, et al., 2009; Lockie, et al., 2014).

2.6.4 Regulation of Ca^{2+} in Glucose Transport

FAS deficiency has the tendency to alter the insulin sensitivity by changing the phospholipid compound and transport efficiency of the SR, thereby giving rise to the intracellular Ca^{2+} in the cytoplasm. AMP-activated protein kinase is then activated by the elevated Ca^{2+}concentration due to the disrupted SERCA1 function, causing an increase in glucose transport regulated by the translocation of GLUT4 glucose transporter to the surface membrane (Funai, et al., 2013). Besides that, glucose tolerance can also be enhanced in adipose tissue when the GLUT4 is over-expressed (Shepherd, et al., 1993). However, up-regulated GLUT4 expression in genetically modified mice was shown to cause increased

glucose influx and adipocyte hyperplasia through increased esterification of fatty acids (Rutkowski, Stern and Scherer, 2015).

2.6.5 Regulation of Ca^{2+} in Adipogenesis

Adipogenesis is the differentiation of adipoctes mediated by the intracellular Ca^{2+}. This ion regulates lipid metabolism of adipocytes via stimulation of lipogenesis and inhibition of lipolysis (Sun and Zemel, 2003). Studies have shown that increase in cytosolic Ca^{2+} concentration caused by the inhibition of SERCA1 can suppress adipocyte differentiation through the inhibition of cAMP level in pre-adipocytes. Furthermore, increased intracellular Ca^{2+} serves to stimulate FAS activity, followed by accumulation of triglycerides and hypertrophy of adipocytes (Shi, et al., 2000).

2.6.6 Regulation of Ca^{2+} in Histamine Release

The intracellular Ca^{2+} concentration is correlated to histamine release in mast cells. According to one study conducted by using tryptase inhibitor, it effectively suppresses the first and second increases of intracellular Ca^{2+} which then lead to the inhibition of histamine release (Takei, et al., 1989).

CHAPTER 3

MATERIALS AND METHODS

3.1 Research Methodology

The overall flow of this study from the data and sample collection to the data analysis is summarized in a flow chart as shown in Figure 3.1.

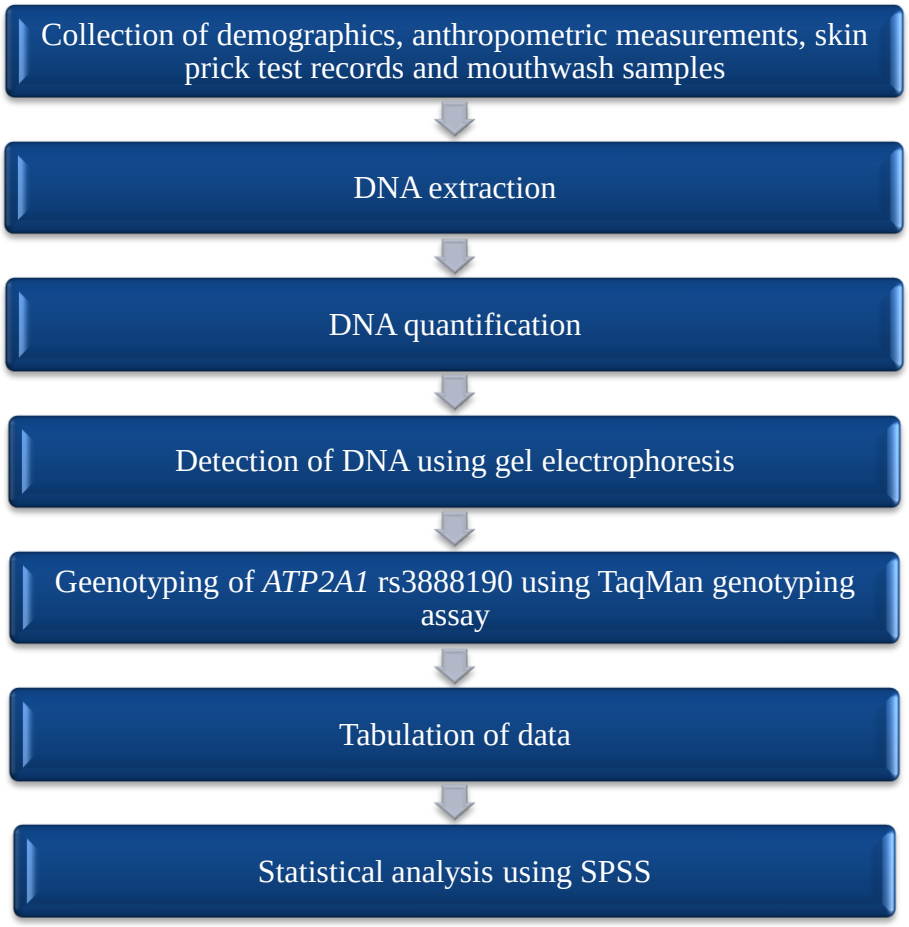

Figure 3.1 Flow chart of study design and methods.

3.2 Respondents

The whole process of this research was carried out in UTAR. The application for ethical approval (Appendix A) of this research involving the data and material of human subjects was approved by the UTAR Scientific and Ethical Review Committee prior to the commencement of study. This study was also conducted by following the ethical principles as stated in the Declaration of Helsinki developed by the World Medical Association (World Medical Association, 2008).

A non-probability sampling method known as convenience sampling was performed in this study to determine the demographics, anthropometric measurements and the genetic factor *ATP2A1* rs3888190 SNP of UTAR students. Convenience sampling was a method of choice as the subjects were easily selected due to their convenient recruitment and accessibility to the researcher (Castillo, 2009).

A cohort of 453 UTAR students between the age of 18 and 26 participated in this study. Majority of the subjects were Chinese, followed by Indians and Malays. An interview was conducted to exclude unhealthy subjects with hypothyroidism and diabetes that would result in weight gain. Subjects who had chronic inflammatory disorders or had taken anti-histamine drugs the day before the skin prick test were also rejected. An informed written consent (Appendix B and D) was obtained from every subject at each demographics

and anthropometric measurements, skin prick test as well as DNA sample collection.

3.3 Questionnaire

3.3.1 Part A: Demographics and Anthropometric Measurements

Demographics shown in Appendix C were used to study the UTAR population with an estimated population of 17,000 students based on three factors such as age (from 18 to 26 years old), gender (male or female) and ethnicity (Malay, Chinese or Indian).

Anthropometric measurements shown in Appendix C were performed twice for the subjects to obtain their average readings. SBP (mmHg), DBP (mmHg) and pulse rate (bpm) were measured by using a blood pressure monitor exact model (Omron, Japan). Regarding the normal resting pulse rate, the range was recorded as 60 to 100 beats per minute for adults. However, a lower resting heart rate might be seen in an athlete with more efficient heart function (Laskowki, 2015).

WC (cm), HC (cm) and height (cm) were measured by using a measuring tape. In population-based studies, WC was commonly used as the waist for given BMI was associated with the co-morbidity and mortality risks. It indicated the deep adipose tissue and correlated with fat mass (Adem, 2015). The best WC

was assessed by objective measurement where the tape measure was placed at the midpoint between the lowest palpable rib and the upper part of iliac crest. Meanwhile, HC was measured around the widest part of the hips. The accuracy of waist and hip measurements could be increased by snugging the tape around the body without pulling tightly until it constricted (World Health Organization, 2008). The cut-off points for abdominal obesity were 90 cm for Asian men and 80 cm for Asian women (World Health Organization, 2000a).

Weight (kg), BMI (kg/m^2), TBF (%), SF (%), VFL (%), SM (%) and RM (kcal) were measured by using Karada Scan body composition monitor (Omron, Japan). As an indicator of overweight and obesity, it was more reliable to use weight adjusted with height rather than weight alone as weight was greatly correlated with body fat as well as height, which was less associated with body fat (Power, Lake and Cole, 1997; Adem, 2015). BMI was calculated by using the body weight in kilograms (kg) divided by the square of body height in meters (m^2) (World Health Organization, 2016d). The standards for BMI classification in Asian adults were based on the WHO's proposed classification of weight by BMI as shown in Appendix E (World Health Organization, 2000b).

People of different heights might show similar fat masses but different TBF proportions. Obesity signified body fat in excess; therefore the most relevant method was to measure the body fat percentage. Besides that, study also

suggested that girls had more body fats than mature boys at similar BMI (Dietz and Bellizzi, 1999). For young men, the TBF percentage was between 12% and 15% while for young women, it was between 25% and 28% (Jeukendrup and Gleeson, 2010). The cut-off points for obesity based on TBF were 20% for males and 30% for females (Omron, n.d.).

3.3.2 Part B: Core Questionnaire for Asthma

In Part B as shown in Appendix C, the questions were set to find out the history, the frequency and the impacts of asthma. For examples, the subjects were asked when their first onset of asthma was and whether they had wheezing at any time in the past 12 months especially during or right after the exercise and at night. They were also asked whether wheezing would cause them sleep disturbance and speech limitation. The disease classification for asthma is stated in Appendix F.

3.3.3 Part C: Asthma Questionnaire (Optional)

Only the subjects who had asthma in the past 12 months were compulsory to answer Part C. The frequency, the severity and the effects of asthma on daily activities in the past 12 months were made known in this particular part. For examples, the subjects were asked about their frequency of asthma attacks in the daytime and nightime and also the number of days of school or work missed by them. Besides that, their number of visits to the clinic and hospital was also recorded.

3.3.4 Part D: Core Questionnaire for Allergic Rhinitis

In Part D as shown in Appendix C, the questions were prepared mainly on the severity, the frequency and the impacts of allergic rhinitis on daily life. The subjects were asked to select the symptoms of allergic rhinitis, including itchy-watery eyes, itchy nose, sneezing, runny nose, nose blockage, snore and nose bleed. They were also asked to state the frequency and severity of each symptom, as well as whether these symptoms would lead to sleep disturbance, impairment of daily activities and impairment of school or work. The subjects were allowed to state that they had no idea about allergic rhinitis as there was a "don't know" option included. In the end, the "don't know" answers were classified as "no" as the subjects should select "yes" instead of "don't know" if the symptoms really occurred (Poe, et al., 1988; Walonick, 1993). The disease classification for allergic rhinitis is stated in Appendix F.

3.3.5 Part E: Core Questionnaire for Eczema

The questions in Part E as shown in Appendix C were used to record the history, the severity and the frequency of eczema. For examples, the subjects were asked regarding their first onset of itchy rash and whether the rash was coming and going or not cleared completely at a given time. They were also asked whether the itchy rash would affect some specific body parts. The frequency of awaking at night by the itchy rash had also been noted. The disease classification for eczema is stated in Appendix F.

3.3.6 Part F: Environmental Exposure

The questions in Part F as shown in Appendix C were all about the environment factors the subjects exposed. For examples, the number of times of vigorous activities per week to breathe hard, the number of hours spent in front of the TV or computer each day, the frequency of alcohol intake, the smoking status of subjects and their family members, the presence of pets and painted house interior in the past 12 months as well as the use of carpets in the house were part of the environmental exposure that might have caused allergy.

3.4 Skin Prick Test

As shown in Appendix D, skin prick test was performed for 308 subjects in the diagnosis of allergy to four different allergens such as *Blomia tropicalis* (dust mite), *Dermatophagoides pteronyssinus* (dust mite), *Elaeis guineensis* (oil palm pollen) and *Curvularia* spp. (fungus). Histamine as positive control and saline as negative control were used for control testing. Six areas with 1.5 cm apart from each other were marked on the inner forearm. A drop of selected allergen was placed beside the marked area. The skin was then gently pricked by a sterile lancet through the allergen drops. The diameter of wheal and erythema flare on the forearm was examined and measured after 10 minutes. Diameter of at least 3 mm for wheal and 10 mm for flare was categorized as positive result (Asha'ari, et al., 2011). The disease classification for allergy is stated in Appendix F.

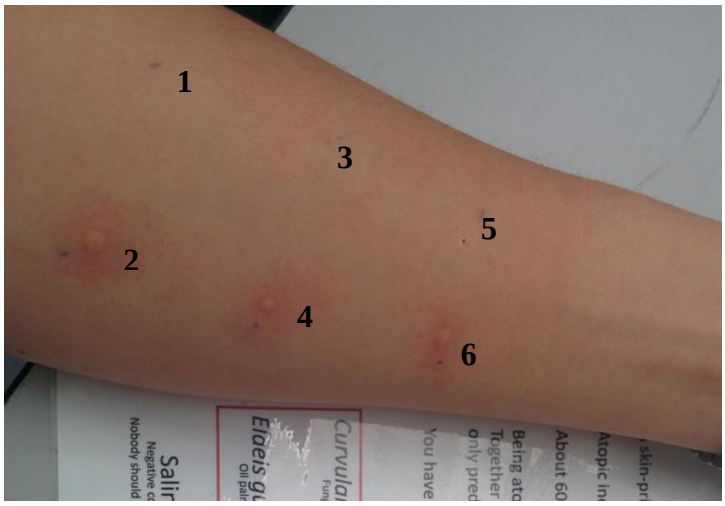

Figure 3.2 Stimulation of wheal and erythema flare towards different allergens used in skin prick test. 1: Saline (negative control); 2: histamine (positive control); 3: *Elaeis guineensis* (oil palm pollen); 4: *Blomia tropicalis* (dust mite); 5: *Curvularia lunata* (fungus); 6: *Dermatophagoides pteronyssinus* (dust mite).

3.5 Collection of Mouthwash Samples

After consenting, the subjects were asked to rinse their mouths with tap water to wash away the food residues. They were then asked to vigorously rinse their mouths again with 5 mL of 3% sucrose solution (PROCHEM, USA) for at least 1 minute. The subjects were also oriented to rub their tongue on the oral mucosa. Each mouthwash was collected in a 15 mL Falcon tube and stored on ice (Aidar and Line, 2007).

TNE solution containing 17 mM Tris/HCl (pH 8.0) (Bio Basic Inc., Canada), 50 mM NaCl (HmbG® Chemicals, Malaysia) and 7 mM EDTA (SYSTERM ChemAR®, Malaysia) was prepared. Tris/HCl is able to maintain pH, interact with lipopolysaccharides and permeabilize membrane. NaCl is used to separate

proteins from DNA. At the same time, DNA is protected from DNases degradation as the complexed Mg^{2+} is chelated by EDTA (Madhad and Sentheil, 2014). Three millilitres of TNE solution diluted in 66% ethanol was then added to each tube. The tubes were then stored in $-20℃$ refrigerator (Aidar and Line, 2007).

3.6 DNA Extraction

The tubes containing the buccal cells were centrifuged at 3000 rpm for 10 minutes at room temperature to obtain the pellet. The supernatant was discarded immediately to avoid pellet slippage. One millilitre of TNE was added to resuspend the cells for second washing. After vigorously vortexing the tubes for 5 seconds, the mixture was transferred to a 1.5 mL microcentrifuge tube. The tubes were centrifuged at 4,000 $\times g$ for 1 minute at room temperature. The supernatant was then poured off immediately (Aidar and Line, 2007).

A volume of 1 mL of detergent-based lysis solution prepared from 10 mM of Tris (pH 8.0), 0.5% of SDS (Bendosen, Malaysia) and 5 mM of EDTA was added to lyse the cells. SDS broke open the cell and nuclear membranes as well as denatured the proteins (Madhad and Sentheil, 2014). After that, 10 µL of 20 mg/mL proteinase K (Novagen, Germany) was added to each tube to digest the proteins. The peptide bonds adjacent to the carboxylic group of aliphatic and aromatic amino acids were cleaved by proteinase K (Promega, 2013). The

tubes were vortexed at medium speed for 5 seconds and sealed with parafilm. The mixture was then incubated overnight in a water bath (Memmert, USA) at 55℃ (Aidar and Line, 2007).

After incubation, 500 µL of solution prepared from 8 M of ammonium acetate (SYSTERM ChemAR®, Malaysia) and 1 M of EDTA was added to remove the unwanted proteins and contaminants, followed by vortexing at high speed for 5 seconds. Positive charged ions, low pH and high salt molarity were provided by ammonium acetate to effectively precipitate the DNA (Madhad and Sentheil, 2014). The tubes were then centrifuged at 17,000 $\times g$ for 10 minutes (Aidar and Line, 2007).

A volume of 750 µL of supernatant was transferred to a 1.5 mL microcentrifuge tube, followed by adding 540 µL of 2-isopropanol (QRëC™, Singapore) to precipitate the DNA. The tubes were inverted gently 20 times to mix the solution and then incubated at room temperature for 1 minute. The tubes were centrifuged at 17,000 $\times g$ for 5 minutes. The supernatant was discarded immediately and each tube was inverted to briefly drain on absorbent paper (Aidar and Line, 2007).

After adding 1 mL of 70% pre-cooled ethanol, the tubes were inverted 10 times to wash the DNA pellet. By this, DNA was purified and concentrated from

isopropanol by forming a separate layer on the top of cell homogenate. DNA was also precipitated in the alcohol layer while the proteins were remained dissolved in the homogenate (Madhad and Sentheil, 2014). The tubes were later centrifuged at 17,000 $\times g$ for 1 minute. The supernatant was poured off carefully. The tubes were inverted to air dry completely on absorbent paper for 45 to 60 minutes. The DNA was then re-suspended in 50 µL of deionized water and stored in $-20℃$ refrigerator (Aidar and Line, 2007).

3.7 DNA Quantification

The purity and concentration of DNA extracted was assessed by using Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA). Nucleic acid absorbs maximally at 260 nm while protein absorbs maximally at 280 nm. The purity of DNA was therefore determined by obtaining the A_{260}/A_{280} ratio. A ratio of 1.8 to 2.0 was accepted as pure for DNA. DNA with different high concentration was standardized to the range between 50 ng/µL and 80 ng/µL by diluting with deionized water (Madhad and Sentheil, 2014).

3.8 Gel Electrophoresis

In gel electrophoresis, 1% of agarose gel works well with DNA size ranges from 100 bp to 2 kb. Agarose gel (1×) was prepared from 100× LE grade agarose powder (Choice Care, Malaysia) and 1× SB buffer made up of boric acid (EMSURE®, USA) and sodium hydroxide (eLabProtocols, 2015). The agarose powder was dissolved completely by heating in a microwave for

approximately 3 minutes. The dissolved agarose was poured on the casting tray and the comb was inserted. The gel was allowed to solidify for 30 minutes, followed by placing it on the electrophoresis box containing the SB buffer (1×) (California Lutheran University, 2006).

One microlitre of GeneRuler 1 kb DNA Ladder (Thermo Scientific, USA) was loaded into the first well of gel. Then, 1 µL of DNA suspended with 1 µL of loading dye was added into the subsequent wells. DNA loading dye (6×) containing 30% (v/v) glycerol (Fisher Scientific, UK) and 0.25% (w/v) bromophenol blue (Fisher Scientific, UK) was used to sink DNA at the bottom of the well and to track DNA migration visually during electrophoresis (Thermo Fisher Scientific, 2016a). The gel was electrophoresed at 80 V for 45 minutes or until the loading dye was approximately 3/4 the way across the gel.

The presence of DNA bands was detected by staining the gel with ethidium bromide for 5 minutes, followed by destaining the gel with water for 1 to 5 minutes. Finally, the gel was placed on the UV transilluminator (Major Science, USA) to visualize the DNA bands.

3.9 TaqMan SNP Genotyping Assay by Real-time PCR

3.9.1 Preparation of Reaction Mix

During the preparation of reaction mix, the desired volume of TaqMan® GTXpress™ Master Mix (2×) (AB Applied Biosystem™, USA), TaqMan® SNP genotyping assays (40×) (AB Applied Biosystem™, USA), nuclease-free water and DNA template per reaction was calculated (Life Technologies, 2014).

Table 3.1 Volume of reaction components for one reaction after optimization.

Reaction Component	**Volume (µL)/Reaction in low profile white PCR tube [1×]**
TaqMan® GTXpress™ Master Mix (2×)	5
TaqMan® SNP genotyping assays (1.88 µL; 40×; Assay ID: C_____2885_10; Cat. # 4351379)	0.25
Nuclease-free water	3.75
DNA template	1
Total volume:	10

(Life Technologies, 2014)

3.9.2 Reaction Mix and DNA Loading

After calculating the number of reactions to be performed, 9 µL of reaction mix containing only the TaqMan® GTXpress™ Master Mix (2×), TaqMan®

SNP genotyping assays (40×) and nuclease-free water was transferred to each 0.1 mL low profile white PCR tube (Axygen®, USA). After thawing and re-suspending, 1 µL of DNA was loaded on the wall of adjacent white PCR tubes. One tube containing only the reaction mix was used as the NTC. The tubes were then centrifuged briefly and subjected to real-time PCR genotyping (Life Technologies, 2014).

3.9.3 Real-time PCR Conditions

The conditions listed in Table 3.4 were optimized for use with TaqMan® Genotyping Assays on CFX96™ Real-Time System (Bio-Rad, USA). All conditions were same as the TaqMan® Genotyping Assays User Guide except for the number of cycles that had been changed from 40 cycles to 50 cycles.

Table 3.2 Real-time PCR conditions after optimization.

Steps	**Predesigned SNP**		
	Temperature	**Duration**	**Cycles**
AmpliTaq Gold®, UP, enzyme activation	95℃	10 minutes	HOLD
Denaturation	95℃	15 seconds	50
Annealing/extension	60℃	1 minute	

(Life Technologies, 2014)

3.9.4 Post Real-time PCR Read and Analysis

Real-time PCR instrument software plots R_n values according to the fluorescence signals from each well by using the fluorescence measurements. After PCR amplification, the allelic discrimination plot was referred to verify the allele types of each sample. According to Thermo Scientific (2016b), allele 1 (wild type allele C) is bound by the FAM dye whereas allele 2 (mutant allele A) is bound by the VIC dye. Ideally as shown in Figure 3.2, the expected results should show the variation in clustering. A good allelic discrimination plot would show three clusters located well away from each other and NTC, which was near the origin. Moreover, the dots in each cluster should be closely grouped together (Life Technologies, 2014).

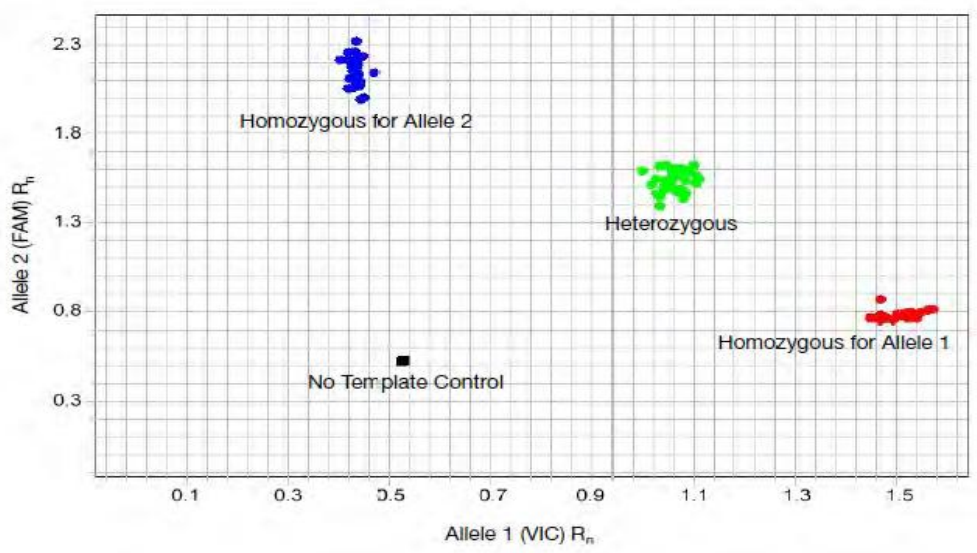

Figure 3.3 Expected result of allelic discrimination plot (Adopted from Life Technologies, 2014).

3.10 Statistical Analysis

IBM Statistical Package for the Social Sciences 20 was used to analyze the data obtained from this research. Gender, ethnicity, genotype, allele and allergy conditions were placed under the categorical variables. Meanwhile, the data of anthropometric measurements were placed under the continuous variables. Kolmogorov-Smirnov test was used to test the normal distribution of continuous variables. The variables were log-transformed if they were not normally distributed. Besides that, Pearson's Chi-Square test was used to obtain the χ^2 and *p*-values. The association of rs3888190 allele distribution with demographics and anthropometric classes was shown. Next, the association of allergy status with gender, allele and anthropometric classes was also determined. Fisher's Exact test was also performed to obtain the *p*-values when the sample size was less than five in the cells. The frequencies of genotypes and alleles were also calculated. Hardy-Weinberg equilibrium was determined by calculating the genotype frequencies and performing Chi-Square test on the observed and expected values. Furthermore, univariate analysis of variance was performed to test the significant difference of the mean values by using the covariance (age and ethnicity) between the outcome variables. The statistical significance of adjusted means of anthropometric measurements for rs3888190 genotype allele was tested. When stratified analysis of means was performed, the means ± standard error of means and *p*-values obtained from the anthropometric measurements were analyzed with the rs3888190 genotype using parametric One-Way ANOVA and non-parametric Kruskal Wallis test. The *p*-values in all tests were statistically significant if less than 0.05.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Genomic DNA Detection

The DNA samples collected were subjected to 1% agarose gel electrophoresis to confirm the presence of DNA band. In Figure 4.1, lanes 1 to 24 show the presence of thick DNA bands of more than 1 kb, except for lane 7 and 15. DNA samples without degradation will appear as very thick DNA bands which migrate not very far out of the wells (Michaelis, Flanders Jr and Wulff, 2011). Only the samples with thick bands were subjected to TaqMan SNP genotyping assay using real-time PCR. On the other hand, the presence of faint smears extending down a short distance from the DNA bands indicated the degradation of large fragments of DNA into many smaller fragments (Michaelis, Flanders Jr and Wulff, 2011). The degradation of DNA might be due to the repeated thawing of DNA samples.

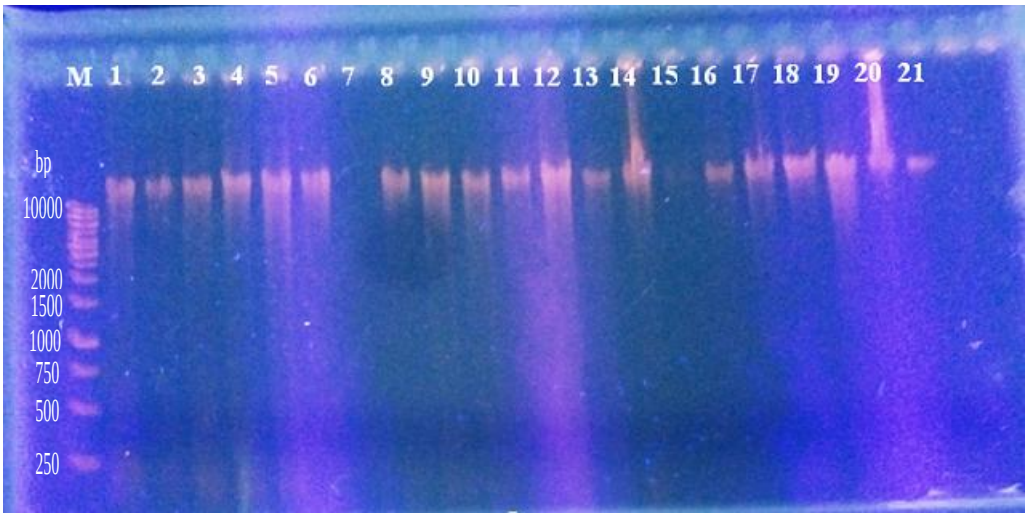

Figure 4.1 Genomic DNAs on 1% agarose gel after staining with ethidium bromide for the confirmation of DNA in the samples. Lane M: 1 kb DNA ladder; Lane 1 to 24: DNA samples.

4.2 TaqMan SNP Genotyping Assay

The allelic discrimination plot as shown in Figure 4.2 ideally shows three clusters and one NTC. NTC is correctly located at the bottom left corner. The orange clusters are slightly trailing at the lower right corner. They represent the wild type homozygous allele C bound by FAM dye. The blue cluster at the upper left corner represents the mutant homozygous allele A bound by VIC dye. Meanwhile, the green clusters located approximately midway between the homozygous allele C and allele A clusters are also trailing. They represent the heterozygous allele A and C. The possible reason to explain the trailing clusters is the unequal quantities of DNA samples due to inaccurate DNA quantitation in which the DNA concentrations are diluted between the range of 50 and 80 µg/µL (Life Technologies, 2014). The genotypes of 453 subjects are listed in Appendix G.

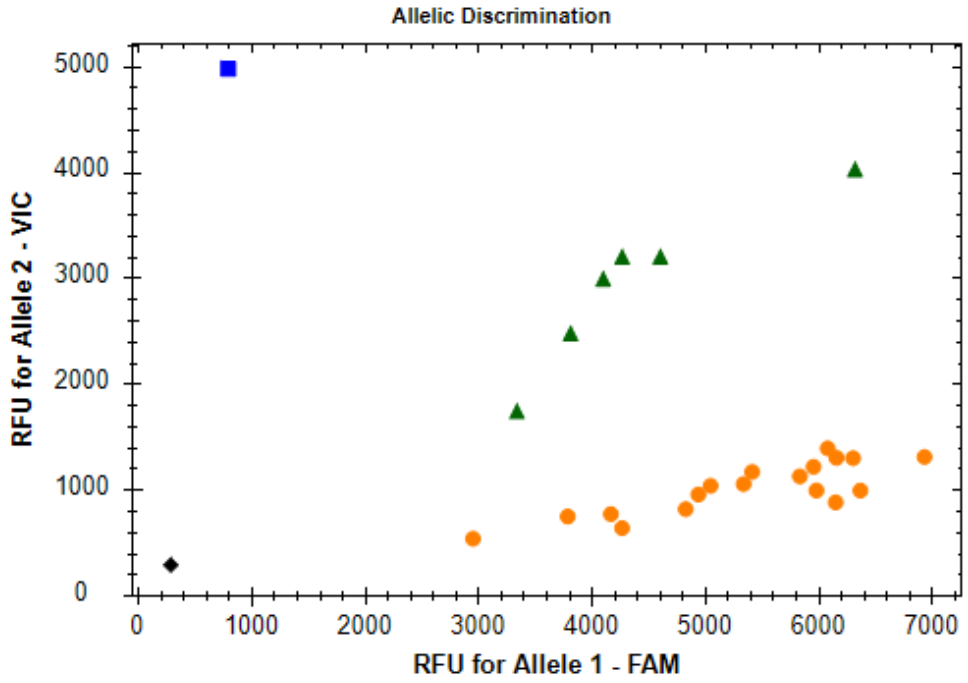

Figure 4.2 Allelic discrimination plot generated after TaqMan SNP genotyping assay using genomic DNAs from different subjects. Orange cluster: homozygous allele C (wild type); Green clusters: Heterozygous allele A and C; Blue clusters: homozygous allele A (mutant); Black dot: NTC.

4.3 Demographics and Anthropometric Characteristics of Subjects

Table 4.1 illustrates the demographics and anthropometric characteristics of UTAR students from Kampar campus. Among the total number of 453 subjects, 214 of them were males while 239 were females. Chinese subjects were the majority ethnic in this population study, followed by Indians and Malays. More than half of the males and females were non-obese and had normal WC and TBF. Based on the National Health and Morbidity Survey 2015, 31.6% of males and 28.3% of females in Malaysia who aged 18 years and above had BMI equal to or more than 25 kg/m^2. The prevalence of obesity in Malays, Chinese and Indians was 35.4%, 21.9% and 43.5%, respectively. For

abdominal obesity, there were 38.2% of males and 60.2% of females who showed WC more than 90 cm and 80 cm, respectively (Institute for Public Health, 2015). As compared to UTAR subjects, only 22.9% of males and 16.7% of females were obese. High WC was seen in 19.2% of males and 20.5% of females. By referring to both studies, males showed a higher prevalence of obesity than the females whereas females showed a higher prevalence of abdominal obesity as compared to males. Greater number of males (33.6%) showed high TBF than the females (28.9%) in this study. The high BMI and TBF in males could be explained by one study which suggested that watching television for 2 hours or longer everyday in childhood might contribute to 17% of early adult overweight. Indeed, males were shown as higher users of screen-based media than females nowadays (Sweeting, 2008).

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

Variables	**Male (*n* = 214)**	**Female (*n* =239)**
Ethnicity		
Malay	0 (0.00)	3 (1.30)
Chinese	208 (97.20)	217 (90.80)
Indian	6 (2.80)	19 (7.90)
BMI Class		
Non-obese	165 (77.10)	199 (83.30)
Obese	49 (22.90)	40 (16.70)

Table 4.1 (continued).

Variables	Male (*n* = 214)	Female (*n* =239)
WC Class		
Normal	173 (80.80)	190 (79.50)
High	41 (19.20)	49 (20.50)
TBF Class		
Normal	142 (66.40)	170 (71.10)
High	72 (33.60)	69 (28.90)

Parentheses indicate percentage within the same gender.

4.4 Demographics and Anthropometric Characteristics According to *ATP2A1* rs3888190 Genotype and Allele

Table 4.2 shows the distribution of *ATP2A1* rs3888190 C/A genotype and allele with demographics and anthropometric characteristics. Among all of the subjects, 377 of them carried the homozygous wild type allele C, while 74 of them carried the heterozygous alleles. Only 2 subjects were found to carry homozygous mutant allele A.

In this study, the MAF of both genders was 0.09. According to Malays, Chinese and Indians, their MAFs were 0.001, 0.08 and 0.004, respectively. Based on Ensembl (2016), the overall MAF of East Asian for allele A was 0.13, which was quite similar to the MAF of both genders. According to Teo, et al. (2009), the Chinese community was mainly made up of Han Chinese in

Malaysia. Besides that, the paternal ancestries tracing had also shown that majority of Indians migrated from southeastern India to Malaysia were consisted of Telugu and Tamils. Therefore for Southern Han Chinese, their MAF was 0.14 whereas for Telugu Indians, their MAF 0.26 (Ensembl, 2016). However, the MAF discovered between both Chinese and Indians in this study and those in China and India did not show similarity. The possible reason might be due to the insufficient number of mutant alleles being detected among the 453 subjects. The observation on Malays could not be done because there was no published data available currently to make any comparison.

Table 4.2 Association of *ATP2A1* rs3888190 genotype and allele distribution with demographic and anthropometric classes.

Variables	**Genotype**			**Allele**	
	CC	**CA**	**AA**	**C**	**A**
Gender					
Male	178 (47.20)	35 (47.30)	1 (50.00)	391 (47.20)	37 (47.40)
Female	199 (52.80)	39 (52.70)	1 (50.00)	437 (52.80)	41 (52.60)
χ^2; *p*		NP		0.001; 0.97	
Ethnicity					
Malay	2 (0.50)	1 (1.40)	0 (0)	5 (0.60)	1 (1.30)
Chinese	354 (93.90)	69 (93.20)	2 (100.00)	777 (93.80)	73 (93.60)
Indian	21 (5.60)	4 (5.40)	0 (0)	46 (5.60)	4 (5.10)
χ^2; *p*		NP		0.52; 0.77	

Table 4.2 (continued).

Variables	Genotype			Allele	
	CC	**CA**	**AA**	**C**	**A**
BMI Class					
Non-obese	301 (79.80)	62 (83.80)	1 (50.00)	664 (80.20)	64 (82.10)
Obese	76 (20.20)	12 (16.20)	1 (50.00)	164 (19.80)	14 (17.90)
χ^2; *p*		NP		0.16; 0.69	
WC Class					
Normal	304 (80.60)	58 (78.40)	1 (50.00)	666 (80.40)	60 (76.90)
High	73 (19.40)	16 (21.60)	1 (50.00)	162 (19.60)	18 (23.10)
χ^2; *p*		NP		0.55; 0.46	
TBF Class					
Normal	261 (69.20)	50 (67.60)	1 (50.00)	572 (69.10)	52 (66.70)
High	116 (30.80)	24 (32.40)	1 (50.00)	256 (30.90)	26 (33.30)
χ^2; *p*		NP		0.19; 0.66	

Parentheses indicate percentage within the same demographic/anthropometric class; NP = Chi-Square Test not performed due presence of cell having the count of less than 5; *p*-values by Pearson's Chi-Square Test; **p*-value significant at < 0.05.

None of the gender and ethnicity successfully showed significant difference in their *ATP2A1* rs3888190 allele distribution. Also, BMI did not show any significant association with *ATP2A1* rs3888190. The *p*-value of BMI obtained in this study was quite similar to two recent studies. According to Ahmad, et al.

(2015), their study involving the BMI associated SNPs in the total of 16,157 Pakistan Risk of Myocardial Infarction Study (PROMIS) cohort showed that *ATP2A1* rs3888190 was not significantly associated with BMI (p = 0.45). Meanwhile according to the Gene-Lifestyle Interactions and Complex Traits Involved in Elevated Disease Risk (GLACIER) study (2016) with a total of 3541 adult participants, BMI-associated loci *ATP2A1* rs3888190 with 10-year change in BMI did not show association with BMI (p = 0.16) as well (Ahmad, et al., 2016). In this study, 5% significance level for 1 degree of freedom was 3.84. The distribution of *ATP2A1* rs3888190 genotype and allele was in Hardy-Weinberg equilibrium as the χ^2 value = 0.77 and p = 0.42, which was more than 0.05. Thus, the null hypothesis stating that the population was in Hardy-Weinberg equilibrium was not rejected.

By referring only to the BMI, we could not exactly state that there was no association between *ATP2A1* rs3888190 allele and obesity because BMI is just a phenotype of obesity (Say, et al., 2014). In another word, the discrimination between muscle and adipose tissue and the assessment of regional adiposity could not be determined by BMI. Therefore, the *p*-values of abdominal adiposity (WC) and overall adiposity (TBF) were also referred as they were able to assess the adiposity even in the absence of change in BMI (Stevens, McClain and Truesdale, 2008). However, both *p*-values did not show significant association with *ATP2A1* rs3888190 allele.

4.5 Adjusted means of anthropometric measurements for *ATP2A1* rs3888190 genotype and allele

Based on Table 4.3, AA genotype carriers showed slightly higher mean values of WC, BMI, TBF, SF, VFL and RM than other two genotypes although there were no significant associations between anthropometric measurements and *ATP2A1* rs3888190 genotype and allele. The mean value of WC for AA genotype was 79.64 cm, which was lower than the cut-off points for male (< 90 cm) and female (< 80 cm) abdominal obesity for Asian population recommended by WHO (2008). However, the mean value of BMI for AA genotype was higher than the recommended values for overweight (23 kg/m^2) by WHO (2000), while the mean value of TBF was also greater than the cut-off points of male (20%) and female (30%) by Omron (n.d.), therefore both were considered as overweight and obesity, respectively.

According to the Ensembl (2016), rs3888190 is an upstream gene variant, which is 5 kilobase upstream of the most distal transcription start site. As a result, the location of SNP might influence the transcription and translation processes. Yet, the actual effects have to be further confirmed (Cingolani, et al., 2012). Therefore in this study, we hypothesized that the function of SERCA1 pump might be affected by this upstream variant, causing a disruption in intracellular Ca^{2+} balance involved in glucose transport, adipogenesis and thermogenesis (Shi, et al., 2000; Funai, et al., 2003; De Meis, et al., 2005).

Table 4.3 Adjusted means of anthropometric measurements for rs3888190 genotype and allele.

Variables	**Genotype**			**Allele**	
	CC	**CA**	**AA**	**C**	**A**
WC (cm)	77.17 ± 0.51	76.99 ± 1.15	79.46 ± 6.98	77.15 ± 0.34	77.12 ± 1.11
p		0.98		0.98	
BMI (kg/m^2)	22.51 ± 0.21	22.43 ± 0.47	24.69 ± 2.86	22.50 ± 0.14	22.55 ± 0.46
p		0.78		0.94	
TBF (%)	22.76 ± 0.31	22.94 ± 0.69	39.09 ± 4.21	22.78 ± 0.21	23.77 ± 0.68
p		0.35		0.36	
SF (%)	18.45 ± 0.26	18.68 ± 0.58	20.26 ± 3.52	18.47 ± 0.17	18.76 ± 0.56
p		0.88		0.75	
VFL (%)	4.80 ± 0.18	4.62 ± 0.41	7.93 ± 2.49	4.78 ± 0.12	4.79 ± 0.40
p		0.72		0.93	

Table 4.3 (continued).

Variables	**Genotype**			**Allele**	
	CC	**CA**	**AA**	**C**	**A**
SM (%)	31.00 ± 0.12	31.09 ± 0.28	29.12 ± 1.69	31.01 ± 0.08	30.99 ± 0.27
p		0.63		0.96	
RM (kcal)	1397.82 ± 8.87	1406.56 ± 20.02	1439.45 ± 121.79	1398.6 ± 5.97	1408.25 ± 19.45
p		0.82		0.54	

All values were log transformed before analysis by univariate analysis of variance (General Linear Model), adjusted for co-variates: gender (all) and ethnicity (for BMI, SF and SM only); Values are presented as adjusted mean ± SEM (estimated marginal means ± standard error of the mean); **p*-value significant at < 0.05.

SERCA1 is well-known in the regulation of intracellular Ca^{2+} concentration. The production of energy is more efficient when there is a correct SERCA1 expression in the heart that leads to the shifting of fat to carbohydrate utilization (Waller, et al., 2015). In human adipocytes, intracellular Ca^{2+} controls the lipogenesis and lipolysis. High levels of intracellular Ca^{2+} play a role in triggering the fatty acid synthase involved in de novo lipogenesis. As a consequence, non-obese tends to have less intracellular Ca^{2+} (Parikh and Yanovski, 2003). In thermogenesis, the heat release is reduced when Ca^{2+} gradient is absent across the ER membrane (De Meis, et al., 2005). These conditions might explain why the mean values of WC, BMI, TBF, SF and VFL for AA genotype were higher than the others, causing reduced ATP consumption and fat oxidation. Although SM showed no association with *ATP2A1* rs3888190 genotype and allele, the mean value of SM for AA genotype was slightly lower than the others. According to Mazala, et al. (2015), impaired Ca^{2+} homeostasis and SERCA1 might demonstrate dystrophic phenotype, which is a form of muscle wasting. A decreased marker of muscle damage was also seen in a SERCA1 over-expressed mouse.

4.6 Stratified analysis of means of anthropometric measurements between Chinese male and female subjects

When stratified analysis of means was performed as shown in Table 4.4, AA genotype male carriers showed significantly higher mean values of WC, BMI, TBF, SF and VFL than CC and CA genotypes, whereas AA genotype female carriers showed all normal mean values. According to Blaak (2001), males

stored higher visceral adipose tissue than females caused by the gender-related differences in the regulation of regional fatty acid metabolism. The mean value of WC for AA male carriers was 95 cm, which was greater than the Asian cut-off point (< 90 cm) for abdominal obesity by WHO (2008). Besides that, the mean value of BMI and TBF for AA genotype had also exceeded the cut-off points of BMI (25 kg/m^2) and TBF (20%) for obesity by WHO (2000) and Omron (n.d.).

Although there was no association between BMI and obesity in this study, *ATP2A1* rs3888190 polymorphism was possible to have effects on other phenotypes. Surprisingly, only genotype of male subjects showed significant association with TBF ($p = 0.00$). According to Choi, Pai and Kim (2002), TBF and serum lipid concentrations were more closely associated with males than females during adolescence. The association with TBF instead of BMI might lead to the contradiction of low BMI and high TBF among Asians when compared to Caucasians (Say, et al., 2014). Therefore, body fat mass should be used as an obesity index due to the fact that adipose tissue is an important site for total energy and fat storage (Bray, 1989). The increased mean value of TBF could be resulted from the increased intracellular Ca^{2+} causing the trigger of lipogenic gene expression and an increase in lipid deposition (Zemel, 2002). However, there was no significant association between the means of anthropometric measurements and the genotype of Chinese females.

Table 4.4 Stratified analysis of means of anthropometric measurements between Chinese male and female subjects.

Variables	**Male**			**Female**		
	CC	**CA**	**AA**	**CC**	**CA**	**AA**
WC (cm)	80.13 ± 0.69	79.41 ± 1.81	95.00	73.83 ± 0.76	74.93 ± 1.36	64.25
p		0.28			0.21	
BMI (kg/m^2)	22.80 ± 0.27	22.55 ± 0.75	30.15	22.09 ± 0.32	22.42 ± 0.58	19.20
p		0.26			0.37	
TBF (%)	16.93 ± 0.49	17.03 ± 1.13	55.10	27.44 ± 0.36	27.87 ± 0.77	22.50
p		0.00*			0.37	
SF (%)	11.90 ± 0.36	11.90 ± 0.85	20.10	23.93 ± 0.38	24.50 ± 0.76	19.50
p		0.23			0.31	
VFL (%)	6.08 ± 0.27	5.91 ± 0.70	14.00	3.50 ± 0.26	3.56 ± 0.43	2.00
p		0.23			0.57	

Table 4.4 (continued).

Variables	Male			Female		
	CC	**CA**	**AA**	**CC**	**CA**	**AA**
SM (%)	35.65 ± 0.20	35.78 ± 0.52	30.50	27.04 ± 0.14	27.03 ± 0.28	28.35
p		0.17			0.62	
RM (%)	1588 ± 13.31	1585 ± 32.94	1812	1224 ± 12.31	1253 ± 22.22	1086
p		0.43			0.13	

Mean values ± standard error of the mean by One-Way ANOVA; *p*-values of TBF, SF and SM (male) by One-Way ANOVA; *p*-values of WC, BMI, VFL and RM (male) and *p*-values of WC, BMI, TBF, SF, VFL, SM and RM (female) by Kruskal-wallis test; **p*-value significant at < 0.05.

4.7 Overall prevalence of controls, intermediates and cases of different allergy status

A Venn-diagram in Figure 4.3 illustrates the number of intermediates and cases of asthma, AR and AD. The number of subjects having AR was the highest, followed by asthma and AD. Based on Figure 4.4, case AR + AD showed the highest prevalence (80%) among the other allergy status, followed by intermediate A + AD (66.67%) and asthma (62.41%). The lowest prevalence was belonged to the case A + AD (6.25%). The prevalence of controls of all the allergy status was lower when compared to either intermediates or cases due to the small number of controls among the 235 subjects.

According to Lim, et al. (2015), the prevalence of asthma in Asian adults ranged from 0.7% to 11.9%. In this study, the prevalence of case of asthma (13.48%) was slightly higher as compared to previous study. On the other hand, the prevalence of allergic rhinitis was 74.5% in older Malaysian children (Yadav and Naidu, 2015). In contrast to our findings, the prevalence of allergic rhinitis was only 54.48%. But still, allergic rhinitis was found more frequent than asthma, supported by Burney, et al. (1996) and Casale and Lazarus (1999). According to Allergy Centre Malaysia (n.d.), the prevalence of atopic dermatitis was 20% in Malaysia, which was very similar to the prevalence (21.48%) found in this study. The prevalence varied between previous studies and our study might be due to the differences in sample size, age groups and number of ethnic groups involved.

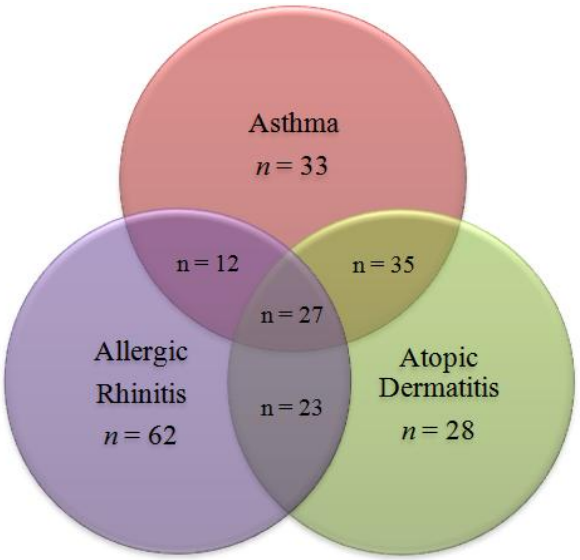

Figure 4.3 Venn diagram showing number of intermediates and cases of asthma, allergic rhinitis and atopic dermatitis (Total number of intermediates and cases = 220).

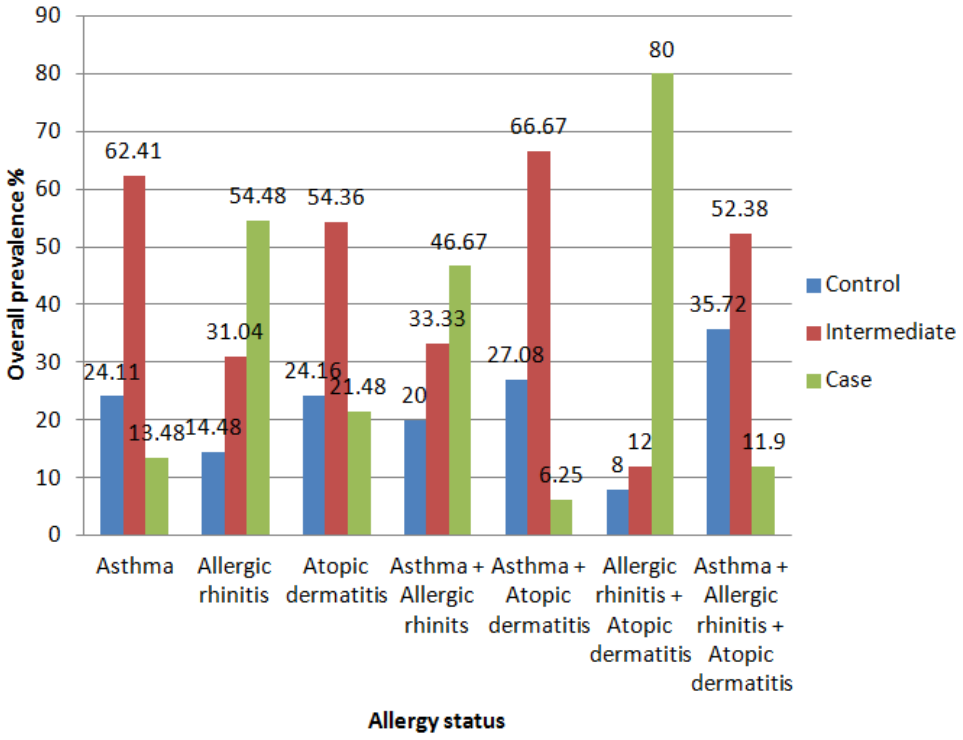

Figure 4.4 Bar chart of overall prevalence of controls, intermediates and cases against different allergy status.

Table 4.5 Association of allergy status with *ATP2A1* rs3888190 genotype and allele distribution and demographics and anthropometric classes.

Variables	**Asthma Status**			**AR Status**			**AD Status**		
	Cont	**Int**	**Case**	**Cont**	**Int**	**Case**	**Cont**	**Int**	**Case**
Gender									
Male	11 (17.70)	39 (62.90)	12 (19.40)	7 (12.30)	19 (33.30)	31 (54.40)	14 (22.20)	39 (61.90)	10 (15.90)
Female	23 (29.10)	49 (62.00)	7 (8.90)	14 (15.90)	26 (29.50)	48 (54.50)	22 (25.60)	42 (48.80)	22 (25.60)
χ^2; p		4.71; 0.1			0.48; 0.79			2.91; 0.23	
Genotype									
CC	28 (24.30)	73 (63.50)	14 (12.20)	18 (15.30)	36 (30.50)	64 (54.20)	29 (23.40)	70 (56.50)	25 (20.20)
CA	5 (20.00)	15 (60.00)	5 (20.00)	3 (11.50)	8 (30.80)	15 (57.70)	6 (25.00)	11 (45.80)	7 (29.20)
AA	1 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (100.00)	0 (0.00)	1 (100.00)	0 (0.00)	0 (0.00)
χ^2; p		NP			NP			NP	

Table 4.5 (continued).

Variables	Asthma Status			AR Status			AD Status		
	Cont	**Int**	**Case**	**Cont**	**Int**	**Case**	**Cont**	**Int**	**Case**
Allele									
C	61 (23.90)	161 (63.10)	33 (12.90)	39 (14.90)	80 (30.50)	143 (54.60)	64 (23.50)	151 (55.50)	57 (21.00)
A	7 (25.90)	15 (55.60)	5 (18.50)	3 (10.70)	10 (35.70)	15 (53.60)	8 (30.80)	11 (42.30)	7 (26.90)
χ^2; *p*		0.83; 0.66			NP			1.67; 0.43	
BMI Class									
Non- obese	26 (22.40)	76 (65.50)	14 (12.10)	19 (15.60)	35 (28.70)	68 (55.70)	29 (23.20)	74 (59.20)	22 (17.60)
Obese	8 (32.00)	12 (48.00)	5 (20.00)	2 (8.70)	10 (43.50)	11 (47.80)	7 (29.20)	7 (29.20)	10 (41.70)
χ^2; *p*		2.76; 0.25			NP			9.07; 0.01*	

Table 4.5 (continued).

Variables	Asthma Status			AR Status			AD Status		
	Cont	**Int**	**Case**	**Cont**	**Int**	**Case**	**Cont**	**Int**	**Case**
WC Class									
Normal	26 (23.40)	70 (63.10)	15 (13.50)	17 (14.70)	35 (30.20)	64 (55.20)	28 (23.50)	71 (59.70)	20 (16.80)
High	8 (26.70)	18 (60.00)	4 (13.30)	4 (13.80)	10 (34.50)	15 (51.70)	8 (26.70)	10 (33.30)	12 (40.00)
χ^2; p		0.14; 0.93			0.20; 0.90			9.16; 0.01*	
TBF Class									
Normal	24 (22.60)	69 (65.10)	13 (12.30)	16 (14.50)	34 (30.90)	60 (54.50)	26 (23.00)	65 (57.50)	22 (19.50)
High	10 (28.60)	19 (54.30)	6 (17.10)	5 (14.30)	11 (31.40)	19 (54.30)	10 (27.80)	16 (44.40)	10 (27.80)
χ^2; p		1.34; 0.51			0.004; 1.00			1.99; 0.37	

Cont: Control; Inter: Intermediate. Parentheses indicate percentage within the same demographic/anthropometric class; NP = Chi-Square Test not performed due presence of cell having the count of less than 5; *p*-values by Pearson's Chi-Square Test; **p*-value significant at < 0.05.

4.8 Association of allergy status with *ATP2A1* rs3888190 genotype and allele distribution and demographics and anthropometric classes

Only 235 subjects who had correctly completed their core questionnaire for allergies and skin prick test were selected to identify the association of allergy status with *ATP2A1* rs3888190 genotype and allele distribution and demographics and anthropometric classes. As shown in Table 4.5, subjects without asthma was classified as controls but they still had the possibility of having AR or AD. Meanwhile, asthma subjects with less severe symptoms were categorized as intermediates while the severe ones were known as cases. Same classification of disease was applied to all other allergy status in accordance to the criteria as stated in the Appendix F. Analysis with control versus combined intermediate and case was performed in Table 4.6.

In Table 4.5, the subjects carrying allele A showed higher prevalence of asthma (cases) and AD than those carrying allele C. AR subjects (cases) with allele A indicated similar prevalence with those with allele C. Meanwhile in Table 4.6, the subjects (cases) carrying allele A also showed higher prevalence of A + AD and A + AR + AD than allele C. AR + AD subjects (cases) with allele A showed same prevalence with those carrying allele C. These results could be explained by several studies involving the intracellular Ca^{2+}. In asthma, the inflammatory response is regulated by mast cells placed adjacent to blood vessels and nerves. Histamine is released and additional cytokines are stimulated after being activated by cross-linking of IgE receptor. Chemotactic factor IL-16 is induced by histamine to attract CD4+ cells (Hart, 2011). In AR,

allergen triggers the release of histamine from degranulated mast cells into the nasal mucosa (Taylor-Clark, 2010). Enhanced histamine release can also be observed in AD, leading to inhibition of terminal differentiation of keratinocytes and impairment of skin barrier (De Benedetto, et al., 2015). According to Takei, et al. (1989), they stated that increased intracellular Ca^{2+} of mast cells is promoted by the antigen. Beaven, et al. (1983) also discovered the degranulation of mast cells can be stimulated by the release of Ca^{2+} from the intracellular store. Furthermore, migration of leukocytes is induced by the chemokines via increased intracellular Ca^{2+}. The vascular permeability of blood vessels is also enhanced by intracellular Ca^{2+} during leukocyte evasion. Based on Valverde, et al. (2011), cytosolic Ca^{2+} regulates the production of cytokines and proliferation of T cells. The effects of cytokines are manifested through changes in intracellular Ca^{2+} as well (Mooren and Kinne, 1998).

In addition to that, the prevalence of obese groups with allergies (cases) was almost all higher than the non-obese groups in terms of BMI, WC and TBF. Based on previous studies, subjects with asthma would experience limitations in their physical activities like sports. Subjects with AR have impairments in their daily activities and always feel exhausted (Juniper, 1997; Leynaert, et al., 2000). Besides that, Lonne-Rahm, et al. (2014) stated that the itch of AD usually gets worse after sweating, thereby causing a decrease in physical exercise. According to Shore (2008), obesity and asthma might share common genetics. Thus with a combination of reduced physical activities and disruption

of intracellular Ca^{2+} concentration in both adipogenesis and histamine release, the subjects were possible to develop obesity and allergy at the same time.

Based on the findings conducted by Lokaj-Berisha, et al. (2015), the differences in study designs and age groups as well as small sample size might explain why the association between allergies and obesity was sometimes supported by some studies while sometimes did not. As shown in Table 4.5, asthma and AR showed no significant association with demographics and anthropometric classes. According to Leung, et al. (2009), gender did not cause consistent impact on the association between obesity and the presence of allergen-specific IgE. There was also no association found between obesity and asthma or AR in Chinese children. However, AD was significantly associated with BMI and WC. These results were supported by Koutroulis, et al. (2015) stating that high BMI was significantly associated with severity of AD for children aged more than 2 years. According to Lee, et al. (2016), significant association was found between high BMI and WC in young women adults and AD. Lokaj-Berisha, et al. (2015) stated that the association between BMI and AD could be due to the production of pro-inflammatory cytokines in the adipose tissue. Other possible reasons might include sedentary lifestyle, limited physical activity or increased energy intake. Moreover, there was also no significant association found between A + AR, AR + AD or A + AR + AD and demographics and anthropometric classes as stated in Table 4.6, except for A + AD which was associated with BMI. The results of this study supported the findings of Koo, et al. (2014) stating that BMI was positively associated with

asthma and AD in male adolescents. According to Ho, et al. (2011), obesity can facilitate airway hyper-responsiveness via low-grade systemic inflammation. Also, obese individuals might develop restrict airway mechanics and decreased tidal volume. The non-significant association between *ATP2A1* rs3888190 allele and all the allergy status could not be compared as there were still no studies being conducted.

Table 4.6 Association of combined allergy status with *ATP2A1* rs3888190 genotype and allele distribution and demographics and anthropometric classes.

Variables	**A + AR Status**			**A + AD Status**			**AR + AD Status**			**A + AR + AD Status**		
	Cont	**Inter**	**Case**	**Cont**	**Inter**	**Case**	**Cont**	**Inter**	**Case**	**Cont**	**Inter**	**Case**
Gender												
Male	0 (0.00)	2 (28.60)	5 (71.40)	4 (18.20)	18 (81.80)	0 (0.00)	1 (12.50)	1 (12.50)	6 (75.00)	6 (31.60)	10 (52.60)	3 (15.80)
Female	3 (37.50)	3 (37.50)	2 (25.00)	9 (34.60)	14 (53.80)	3 (11.50)	1 (5.90)	2 (11.80)	14 (82.40)	9 (39.10)	12 (52.20)	2 (8.70)
χ^2; p*		3.28; 0.70			1.63; 0.20			0.32; 0.57			0.26; 0.61	
Genotype												
CC	2 (20.00)	3 (30.00)	5 (50.00)	9 (22.50)	29 (72.50)	2 (5.00)	1 (5.00)	3 (15.00)	16 (80.00)	14 (37.80)	19 (51.40)	4 (10.80)
CA	1 (20.00)	2 (40.00)	2 (40.00)	3 (42.90)	3 (42.90)	1 (14.30)	1 (20.00)	0 (0.00)	4 (80.00)	1 (20.00)	3 (60.00)	1 (20.00)
AA	0 (0.00)	0 (0.00)	0 (0.00)	1 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
χ^2; p*		NP			NP			NP			NP	

Table 4.6 (continued).

Variables	A + AR Status			A + AD Status			AR + AD Status			A + AR + AD Status		
	Cont	**Inter**	**Case**	**Cont**	**Inter**	**Case**	**Cont**	**Inter**	**Case**	**Cont**	**Inter**	**Case**
Allele												
C	5 (20.00)	8 (32.00)	12 (48.00)	21 (24.10)	61 (70.10)	5 (5.70)	3 (6.70)	6 (13.30)	36 (80.00)	29 (36.70)	41 (51.90)	9 (11.40)
A	1 (20.00)	2 (40.00)	2 (40.00)	5 (55.60)	3 (33.30)	1 (11.1)	1 (20.00)	0 (0.00)	4 (80.00)	1 (20.00)	3 (60.00)	1 (20.00)
p		1.00			0.06			0.35			0.65	
BMI Class												
Non-obese	3 (21.40)	5 (35.70)	6 (42.90)	8 (19.5)	32 (78.00)	1 (2.4)	2 (10.50)	3 (15.80)	14 (73.70)	13 (37.10)	18 (51.40)	4 (11.40)
Obese	0 (0.00)	0 (0.00)	1 (100.00)	5 (71.4)	0 (0.00)	2 (28.60)	0 (0.00)	0 (0.00)	6 (100.00)	2 (28.60)	4 (57.10)	1 (14.30)
p		1.00			0.01*			1.00			1.00	

Table 4.6 (continued).

Variables	A + AR Status			A + AD Status			AR + AD Status			A + AR + AD Status		
	Cont	**Inter**	**Case**	**Cont**	**Inter**	**Case**	**Cont**	**Inter**	**Case**	**Cont**	**Inter**	**Case**
WC Class												
Normal	3 (23.10)	4 (30.80)	6 (46.20)	9 (22.50)	29 (72.50)	2 (5.00)	2 (11.80)	3 (17.60)	12 (70.60)	11 (33.30)	18 (54.50)	4 (12.10)
High	0 (0.00)	1 (50.00)	1 (50.00)	4 (50.00)	3 (37.50)	1 (12.50)	0 (0.00)	0 (0.00)	8 (100.00)	4 (44.40)	4 (44.40)	1 (11.10)
p		1.00			0.19			1.00			0.70	
TBF Class												
Normal	2 (18.20)	5 (45.50)	4 (36.40)	8 (21.10)	28 (73.70)	2 (5.30)	2 (11.10)	3 (16.70)	13 (72.20)	11 (34.40)	17 (53.10)	4 (12.50)
High	1 (25.00)	0 (0.00)	3 (75.00)	5 (50.00)	4 (40.00)	1 (10.00)	0 (0.00)	0 (0.00)	7 (100.00)	4 (40.00)	5 (50.00)	1 (10.00)
p		1.00			0.11			1.00			1.00	

Cont: Control; Inter: Intermediate. Parentheses indicate percentage within the same demographic/anthropometric class; NP = Chi-Square Test not performed due presence of cell having the count of less than 5; *p**-value by Pearson's Chi-Square Test; *p*-values by Fisher's Exact Test; **p*-value significant at < 0.05.

4.9 Limitations of Study

There were a few limitations that could be brought up in this study. First of all, the small sample size as well as insufficient number of Malay and Indian subjects had made this study difficult to represent the whole Malaysian population. Other minority ethnic groups from Sabah and Sarawak were also lack in this study. Hence, further study should be conducted in the future by recruiting more subjects, not only the Malay and Indian subjects but also the other ethnic groups from East Malaysia as Malaysia was made up of different ethnicity. Besides that, this population-based study was to show the association between *ATP2A1* rs3888190 and obesity. Therefore, larger group of obese subjects should be involved as if rs3888190 mutant allele were shown to associate with BMI. In accordance to this, the particular SNP should have been carried by high number of obese subjects. By this, the statistical power could be improved.

On the other hand, the impacts of environmental and lifestyle factors should be included in this study to identify the interaction between the environment and gene so that an accurate and constant finding could be achieved. Lastly, the validity of results could also be improved by gene sequencing of the three possible genotypes to double confirm the SNP carried by the subjects. Another alternative way might involve the examination of altered mRNA and protein functions caused by this specific SNP in the adipose tissues and skeletal muscles by comparing with the normal subject.

CHAPTER 5

CONCLUSION

In conclusion, the prevalence of obesity in male (22.9%) was higher than female (16.7%), so as the prevalence of high TBF content which was higher in males (33.6%) than females (28.9%). However, the prevalence of abdominal obesity in females (20.5%) was higher than males (19.2%). The MAF of mutant allele A was found to be 0.001 in Malays, 0.08 in Chinese and 0.004 in Indians. Besides that, the prevalence of *ATP2A1* rs3888190 (C>A) was 8.61% among both genders. There was no significant association between *ATP2A1* rs3888190 allele distribution and gender, ethnicity, BMI and WC in this study. Besides that, the mean values of all anthropometric measurements of AA genotype among all genders were higher than the CC and CA genotypes, except for SM which showed decrease in mean value. As a result, individuals with mutant AA genotype were at risk of becoming obese. However, there was no significant association between these variables with *ATP2A1* rs3888190 genotype and allele. Only the genotype of Chinese males was significantly associated with the mean value of TBF (p = 0.00), whereas the genotype of females showed no association at all with the mean values of anthropometric measurements.

The prevalence of case AR + AD (80%) was the highest among the other allergy status, followed by intermediate A + AD (66.67%) and asthma (62.41%). Subjects with mutant allele A showed higher prevalence of case asthma, AD, A + AD and A + AR + AD than those with allele C. Therefore, subjects with mutant allele A were also at risk of showing allergy phenotypes. AD status showed significant association with BMI (p = 0.01) and WC (p = 0.01) classes, whereas asthma and AR did not show association with any outcome variables. None of the combined allergy status (A + AR, AR + AD and A + AR + AD) showed significant association with *ATP2A1* rs3888190 allele and demographics and anthropometric classes, except for A + AD which was only significantly associated with BMI (p = 0.01).

Overall, *ATP2A1* rs3888190 SNP was only significantly associated with TBF of Chinese males but not with allergic conditions among UTAR Kampar Campus students.

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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) 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 (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

UNIVERSITI TUNKU ABDUL RAHMAN

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.

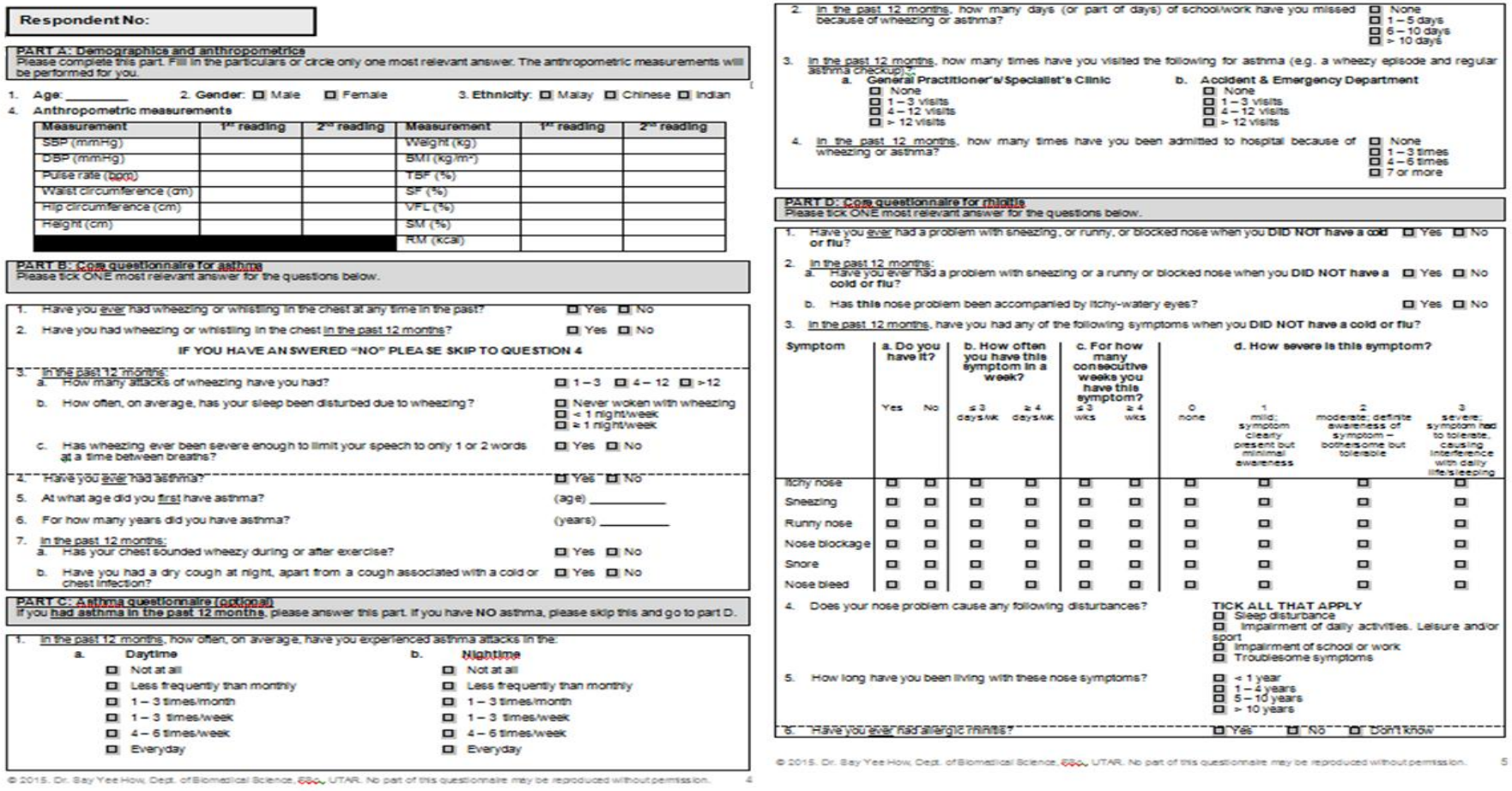
3. You may access and update your personal data by writing to us at dhr@utar.edu.my.

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 B: Core questionnaire for asthma
Please tick ONE most relevant answer for the questions below.

1. Have you ever had wheezing or whistling in the chest at any time in the past? ☐ Yes ☐ No
2. Have you had wheezing or whistling in the chest in the past 12 months? ☐ Yes ☐ No

IF YOU HAVE ANSWERED "NO" PLEASE SKIP TO QUESTION 4

3. In the past 12 months:
   a. How many attacks of wheezing have you had? ☐ 1 – 3 ☐ 4 – 12 ☐ >12
   b. How often, on average, has your sleep been disturbed due to wheezing? ☐ Never woken with wheezing ☐ < 1 night/week ☐ ≥ 1 night/week
   c. Has wheezing ever been severe enough to limit your speech to only 1 or 2 words at a time between breaths? ☐ Yes ☐ No
4. Have you ever had asthma? ☐ Yes ☐ No
5. At what age did you first have asthma? (age) _____
6. For how many years did you have asthma? (years) _____
7. In the past 12 months:
   a. Has your chest sounded wheezy during or after exercise? ☐ Yes ☐ No
   b. Have you had a dry cough at night, apart from a cough associated with a cold or chest infection? ☐ Yes ☐ No

PART C: Asthma questionnaire (optional)
If you **had asthma in the past 12 months**, please answer this part. If you have **NO** asthma, please skip this and go to part D.

1. In the past 12 months, how often, on average, have you experienced asthma attacks in the:

a. Daytime	b. Nighttime
☐ Not at all	☐ Not at all
☐ Less frequently than monthly	☐ Less frequently than monthly
☐ 1 – 3 times/month	☐ 1 – 3 times/month
☐ 1 – 3 times/week	☐ 1 – 3 times/week
☐ 4 – 6 times/week	☐ 4 – 6 times/week
☐ Everyday	☐ Everyday

2. In the past 12 months, how many days (or part of days) of school/work have you missed because of wheezing or asthma? ☐ None ☐ 1 – 5 days ☐ 6 – 10 days ☐ > 10 days
3. In the past 12 months, how many times have you visited the following for asthma (e.g. a wheezy episode and regular asthma check-up)?

a. General Practitioner's/Specialist's Clinic	b. Accident & Emergency Department
☐ None	☐ None
☐ 1 – 3 visits	☐ 1 – 3 visits
☐ 4 – 12 visits	☐ 4 – 12 visits
☐ > 12 visits	☐ > 12 visits

4. In the past 12 months, how many times have you been admitted to hospital because of wheezing or asthma? ☐ None ☐ 1 – 3 times ☐ 4 – 6 times ☐ 7 or more

PART D: Core questionnaire for rhinitis
Please tick ONE most relevant answer for the questions below.

1. Have you ever had a problem with sneezing, or runny, or blocked nose when you **DID NOT have a cold or flu**? ☐ Yes ☐ No
2. In the past 12 months:
   a. Have you ever had a problem with sneezing or a runny or blocked nose when you **DID NOT have a cold or flu**? ☐ Yes ☐ No
   b. Has **this** nose problem been accompanied by itchy-watery eyes? ☐ Yes ☐ No
3. In the past 12 months, have you had any of the following symptoms when you **DID NOT have a cold or flu**?

Symptom	a. Do you have it? Yes	a. Do you have it? No	b. How often you have this symptom in a week? ≤ 3 days/wk	b. How often you have this symptom in a week? ≥ 4 days/wk	c. For how many consecutive weeks you have this symptom? ≤ 3 wks	c. For how many consecutive weeks you have this symptom? ≥ 4 wks	d. How severe is this symptom? 0 none	1 mild: symptom clearly present but minimal awareness	2 moderate: definite awareness of symptom – bothersome but tolerable	3 severe: symptom had to tolerate, causing interference with daily life/sleeping
Itchy nose	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sneezing	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Runny nose	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Nose blockage	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Snore	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Nose bleed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

4. Does your nose problem cause any following disturbances? **TICK ALL THAT APPLY**
   ☐ Sleep disturbance
   ☐ Impairment of daily activities, Leisure and/or sport
   ☐ Impairment of school or work
   ☐ Troublesome symptoms
5. How long have you been living with these nose symptoms?
   ☐ < 1 year
   ☐ 1 – 4 years
   ☐ 5 – 10 years
   ☐ > 10 years
6. Have you ever had allergic rhinitis? ☐ Yes ☐ No ☐ Don't know

PART E: Core questionnaire for eczema
Please tick ONE most relevant answer for the questions below.

1.	Have you ever had an itchy rash which was coming and going for at least 6 months?	☐ Yes ☐ No
2.	Have you had this itchy rash at any time in the past 12 months?	☐ Yes ☐ No
3.	Has this itchy rash at any time affected any of the following places: The folds of elbows, behind knees, in front of ankles, under buttocks, or around neck, cheeks, ears or eyes? **IF YOU HAVE ANSWERED "NO" PLEASE SKIP TO QUESTION 8**	☐ Yes ☐ No
4.	At which age did this itchy rash first occur?	☐ < 2 years ☐ 2 – 4 years ☐ > 5 years ☐ Don't know
5.	Has this rash cleared completely at any time during the last 12 months?	☐ Yes ☐ No
6.	In the past 12 months, how often, on average, have you been kept awake at night by this itchy rash?	☐ Never ☐ < 1 night/week ☐ ≥ 1 night/week
7.	In the past 12 months, have you suffered from dry skin?	☐ Yes ☐ No
8.	Have you ever had eczema?	☐ Yes ☐ No ☐ Don't know

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
8.	In the past 12 months, have you had a cat and/or dog in your house?	☐ Dog ☐ Cat ☐ No
9.	In the past 12 months, has the interior of your house been painted?	☐ Yes ☐ No
10.	Have you ever used carpets in the living room or other bedroom in your house?	☐ Yes ☐ No

- END OF QUESTIONNAIRE. THANK YOU VERY MUCH FOR YOUR ASSISTANCE. -

APPENDIX D

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UNIVERSITI TUNKU ABDUL RAHMAN

DEPARTMENT OF BIOMEDICAL SCIENCE
FACULTY OF SCIENCE

INFORMATION FOR PARTICIPANTS
for the study of
Epidemiology study of allergic conditions

- We would like your permission to enroll you as a participant in a research study to survey the prevalence of allergic conditions like asthma, allergic rhinitis and atopic dermatitis. This study involves a skin prick test (SPT) to determine whether you have allergy or not, and collection of dead skin cells.
- **In order to qualify for SPT, you must NOT be taking any anti-histamines (anti-allergy/anti-flu drugs) for the past 3 days.**
- The SPT is **NOT** painful. This type of testing uses needles (lancets) that barely penetrate the skin's surface. You won't bleed or feel more than mild, momentary discomfort.
- After cleaning the test site with alcohol, we will draw small marks on your skin and apply a drop of allergen extract next to each mark. We will then use a lancet to prick the extracts into the skin's surface. A new lancet is used for each allergen.
- About 15 minutes after the skin pricks, we will observe your skin for signs of allergic reactions. If you are allergic to one of the substances tested, you'll develop a raised, red, itchy bump (wheal) that may look like a mosquito bite. We will then measure the bump's size.
- After recording the results, we will clean your skin with alcohol to remove the marks.
- We will also collect dead skin cells from the flexural portion of your arm. We will use a sticky tape on a device and continually press it for 50 times to get dead skin cells.

CONSENT FORM

I, Pang Li Ying

volunteer to participate in the study of

Epidemiology study of allergic conditions

I am willing to perform the skin prick test and have dead cells taken from my skin.

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]	Signature:	
Date	: 24/2/2016	Date	:
Contact No	: 016-669 1861	Name	:
E-mail:	liyingpang96@gmail.com		

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.

238 3:25pm

Respondent No:

Skin Prick Test Record

No.	Test	Results (mm)	Class
1	Histamine (positive control)	E 12 × 12 W 5 × 5	
2	Saline (negative control)	E — × — W — × —	
3	*Blomia tropicalis* (dust mite)	E 3 × 3 W 3 × 3	
4	*Dermatophagoides pteronyssinus* (dust mite)	E 2 × 2 W 2 × 2	
5	*Elaeis guineensis* (oil palm pollen)	E — × — W — × —	
6	*Cunnilaria* spp. (fungus)	E — × — W — × —	

E: Erythema flare diameter W: Wheal diameter

Class	Description
0	No wheal. Erythema absent of < 1 mm diameter or reaction < control
1+	Wheal absent or slight. Erythema present and < 3 mm diameter
2+	Wheal absent or slight with associated erythema > 3 mm diameter
3+	Wheal ≥ 3 mm and with erythema
4+	Wheal ≥ 3 mm with pseudopodia and erythema

- END OF TEST. THANK YOU VERY MUCH FOR YOUR ASSISTANCE. -

APPENDIX E

BMI CLASSIFICATION FOR OBESITY

Category	BMI (kg/m^2)
Underweight	< 18.50
Normal weight	18.50-22.90
Overweight	≥ 23.00
At risk	23.00-24.90
Obesity class I	25.00-29.90
Obesity class II	≥ 30.00

(World Health Organization, 2000)

APPENDIX F

DISEASE CLASSIFICATION FOR ALLEGIES

1. Asthma

Case acc to survey: If the person answers

- 'Yes' for 'Have you ever had asthma?'

No Asthma: If the person answers

- 'No' to 'Ever has wheezing?' AND
- 'No' to 'Wheezing in the past twelve months?' AND
- 'Don't know' to 'How many attacks of wheezing in the past twelve months?'

No Asthma: If the person answers

- 'Never woken with wheezing' or 'Don't know' for the question 'In the past twelve months, on an average, has your sleep been disturbed?' AND
- 'No' or 'Don't know' to 'In the last 12 months, has wheezing ever been severe enough to limit your speech to only one or two words at a time between breaths?' AND
- 'No' or 'Don't know' to 'In the past 12 months, has your chest sounded wheezy during or after exercise?' AND
- 'No' or 'Don't know' to 'In the past 12 months, have you had a dry cough at night, apart from a cough associated with a cold or chest infection?'

2. Allergic Rhinitis

Case acc to survey: If the person answers

- 'Yes' to 'Have you ever had a problem with sneezing, or runny, or blocked nose when you DID NOT have a cold or flu?' AND

- 'Yes' for more than 1 of the 6 symptoms (Itchy nose, sneezing, runny nose, snore, nose blockage, nose bleed.

No Allergic rhinitis: If the person answers

- 'No' to 'Have you ever had a problem with sneezing, or runny, or blocked nose when you DID NOT have a cold or flu?' AND
- 'No' to 'Have you ever had allergic rhinitis?'

No Allergic rhinitis: If the person answers

- 'No' to 'Have you ever had a problem with sneezing, or runny, or blocked nose when you DID NOT have a cold or flu?' AND
- 'Yes' for less than 2 of the 6 symptoms (Itchy nose, sneezing, runny nose, snore, nose blockage, nose bleed. AND
- 'No' or 'Don't know' to 'Have you ever had allergic rhinitis?'

3. ATOPIC DERMATITIS

Case according to survey: If the person answers

- 'Yes' to 'Have you ever had an itchy rash which was coming and going for at least six months?' AND
- 'Yes' to 'Has this itchy rash at any time affected any of the following places: The folds of the elbows, behind the knees, in front of the ankles, under the buttocks, or around the neck, cheeks, ears or eyes?'

No Atopic Dermatitis: If the person answers

- 'No' to 'Have you ever had an itchy rash which was coming and going for at least six months?' AND
- 'No' to 'Has this itchy rash at any time affected any of the following places: The folds of the elbows, behind the knees, in front of the ankles, under the buttocks, or around the neck, cheeks, ears or eyes?' AND
- 'No' to 'Have you ever had eczema?'

4. COMMON FOR ALL DISEASES

- Disease according to survey AND Skin prick test >=3 + 'Yes' = Case
- No Disease according to survey AND Skin prick test >=3 + 'No' = Control
- No Disease according to survey AND Skin prick test >=3 + 'Yes' = Intermediate

(Note: When AND is used, all the conditions are to be satisfied)

APPENDIX G

Subject	Genotype	Subject	Genotype	Subject	Genotype
1	CC	46	CC	91	CC
2	CC	47	CC	92	CC
3	CA	48	CA	93	CC
4	CC	49	CC	94	CC
5	CA	50	CC	95	CC
6	CC	51	CA	96	CC
7	CC	52	CC	97	CC
8	CC	53	CA	98	CC
9	CC	54	CC	99	CC
10	CC	55	CC	100	CA
11	CC	56	CC	101	CC
12	CC	57	CC	102	CC
13	CC	58	CC	103	CC
14	CA	59	CC	104	CC
15	CC	60	CC	105	CC
16	CC	61	CC	106	CC
17	CA	62	CC	107	CA
18	CC	63	CC	108	CC
19	CA	64	CC	109	CC
20	CC	65	CC	110	CC
21	CC	66	CA	111	CC
22	CC	67	CC	112	CC
23	CC	68	AA	113	CC
24	CC	69	CC	114	CC
25	CC	70	CC	115	CA
26	CA	71	CA	116	CC
27	CC	72	CA	117	CC
28	CC	73	CC	118	CC
29	CC	74	CC	119	CC
30	CC	75	CC	120	CC
31	CC	76	CA	121	CC
32	CC	77	CC	122	CC
33	CA	78	CC	123	CA
34	CC	79	CC	124	CC
35	CC	80	CC	125	CC
36	CC	81	CC	126	CC
37	CC	82	CC	127	CC
38	CA	83	CC	128	CC
39	CC	84	CC	129	CC
40	CC	85	CC	130	CC
41	CA	86	CC	131	CC
42	CC	87	CC	132	CC
43	CC	88	CC	133	CA
44	CC	89	CC	134	CC
45	CC	90	CC	135	CC

*CC: homozygous wildtype; CA: heterozygous; AA: homozygous mutant.

Subject	Genotype	Subject	Genotype	Subject	Genotype
136	CA	184	CA	232	CC
137	CA	185	CC	233	CA
138	CC	186	CC	234	CC
139	CC	187	CC	235	CC
140	CA	188	CC	236	CA
141	CA	189	CC	237	CC
142	CC	190	CC	238	CC
143	CC	191	CC	239	CC
144	CC	192	CC	240	CC
145	CC	193	CC	241	CC
146	CC	194	CC	242	CC
147	CC	195	CC	243	CC
148	CC	196	CC	244	CC
149	CA	197	CC	245	CC
150	CC	198	CC	246	CA
151	CC	199	CC	247	CC
152	CA	200	CC	248	CC
153	CA	201	CA	249	CC
154	CC	202	CC	250	CC
155	CC	203	CC	251	CC
156	CC	204	CC	252	CC
157	CC	205	CC	253	CC
158	CC	206	CC	254	CC
159	CC	207	CC	255	CC
160	CC	208	CC	256	CC
161	CC	209	CC	257	CC
162	CC	210	CC	258	CC
163	CC	211	CA	259	CC
164	AA	212	CC	260	CC
165	CC	213	CC	261	CC
166	CA	214	CC	262	CC
167	CA	215	CC	263	CC
168	CC	216	CC	264	CC
169	CA	217	CC	265	CA
170	CC	218	CC	266	CC
171	CC	219	CC	267	CC
172	CC	220	CC	268	CA
173	CA	221	CC	269	CC
174	CC	222	CA	270	CC
175	CA	223	CC	271	CC
176	CC	224	CC	272	CC
177	CA	225	CC	273	CC
178	CC	226	CC	274	CC
179	CC	227	CC	275	CC
180	CC	228	CC	276	CA
181	CA	229	CC	277	CA
182	CA	230	CC	278	CC
183	CC	231	CC	279	CC

*CC: homozygous wildtype; CA: heterozygous; AA: homozygous mutant.

Subject	Genotype	Subject	Genotype	Subject	Genotype
280	CC	328	CC	376	CC
281	CA	329	CC	377	CC
282	CA	330	CC	378	CC
283	CC	331	CC	379	CC
284	CA	332	CC	380	CC
285	CC	333	CC	381	CC
286	CC	334	CC	382	CC
287	CC	335	CA	383	CC
288	CC	336	CC	384	CC
389	CC	337	CA	385	CC
290	CC	338	CC	386	CA
291	CC	339	CC	387	CC
292	CC	340	CC	388	CC
293	CC	341	CC	389	CA
294	CC	342	CC	390	CC
295	CC	343	CC	391	CC
296	CC	344	CC	392	CA
297	CC	345	CC	393	CC
298	CA	346	CC	394	CA
299	CA	347	CC	395	CC
300	CC	348	CC	496	CC
301	CC	349	CC	397	CC
302	CC	350	CC	398	CA
303	CC	351	CC	399	CC
304	CA	352	CC	400	CC
305	CC	353	CC	401	CC
306	CC	354	CC	402	CC
307	CA	355	CC	403	CC
308	CC	356	CC	404	CC
309	CC	357	CC	405	CC
310	CC	358	CC	406	CC
311	CC	359	CC	407	CC
312	CC	360	CC	408	CA
313	CC	361	CA	409	CC
314	CC	362	CC	410	CC
315	CC	363	CC	411	CC
316	CC	364	CA	412	CA
317	CC	365	CC	413	CC
318	CC	366	CC	414	CC
319	CC	367	CA	415	CA
320	CC	368	CC	416	CC
321	CC	369	CC	417	CC
322	CC	370	CC	418	CC
323	CC	371	CA	419	CC
324	CC	372	CC	420	CC
325	CC	373	CC	421	CC
326	CC	374	CC	422	CA
327	CA	375	CA	423	CC

*CC: homozygous wildtype; CA: heterozygous; AA: homozygous mutant.

Subject	Genotype	Subject	Genotype	Subject	Genotype
424	CC	434	CC	444	CC
425	CC	435	CC	445	CC
426	CA	436	CC	446	CC
427	CA	437	CC	447	CC
428	CC	438	CC	448	CC
429	CC	439	CC	449	CC
430	CC	440	CC	450	CC
431	CC	441	CC	451	CC
432	CC	442	CC	452	CC
433	CC	443	CA	453	CC

*CC: homozygous wildtype; CA: heterozygous; AA: homozygous mutant.