CHAPTER 3

MATERIALS

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METHODS

3.0 Materials and Methods

3.1 Recruitment of subjects

A total of 672 participants were enrolled in the present study, 464 participants were from the Wellness Programme of the University of Malaya (UM) which is an annual health screening program for all the staff of UM, and 208 participants were from the villagers of the Bera district of Pahang. The Wellness Program was an annual voluntary health screening program for the staff of the university (Moy & Bulgiba, 2010). All the staff older than 35 years were invited to participate in this health screening. Those who attended the Wellness Programme were approached for their willingness to participate in this research. Ethnicity of each participant was identified by his /her name in the identification card (IC) and self-reports. All the participants were Malays for at least 3 generations as claimed by self-report.

The WHO cut-off for obesity was applied. Subjects with BMI of 30 kg/m^2 were categorized as obese and those with BMI below 30 kg/m^2 were categorized as non-obese. A total of 500 non-obese and 172 obese participants participated in this study. Medical Ethics Committee of University Malaya Medical Center approved (MEC reference number: 672.23) the study protocol and written informed consent was obtained from all the participants. MEC ethics approval, informed consent and patient information documents are attached in the Appendix A.

3.2 Sample size estimation

Sample size estimation was performed using the Quanto Version 1.2.4 software. Decision about sample size is an important step in any quantitative and genetic study design. For the sample size estimation, the mean BMI of Malaysian Malays was obtained from the data provided Malaysian Adult Nutrition Survey (MANS). The mean BMI was 24.86 ± 13.84 Kg/m^2 (Azmi MY, 2009). For SNPs that have MAF ranges from 0.02 to 0.50, the sample size calculation includes the following specific parameters:

Outcome :	Continuous
Design :	Independent individuals
Hypothesis :	Gene only
Desired power :	80%
Significance :	0.05
Mode of inheritance :	Additive
Allele frequency :	0.02-0.50
Continuous trait settings (logBMI) (Mean ± SD) :	3.21 ± 2.63
Marginal R^2:	0.05
Sampe size (N) :	153

For the estimated effect size, it was estimated that sample of 153 per group was needed to yield power of 80 %. Therefore 306 samples were estimated for this study. Eventually, sample size was increased in order achieve HWE. HWE indicates the sample size is large enough to represent the population that being studied.

3.3 Blood collection

Blood samples were collected for measurement of blood lipids and biomarkers. All blood samples were drawn in a sitting position. BD vacutainer® tubes were used for blood collection. The EDTA lavender top tubes were used for blood plasma collection. EDTA (Ethylenediaminetetraacetic acid) is important as it binds to calcium in the blood and prevents the blood from clotting. Serum red top tube is a plain vacutainer tube used to collect the serum samples. Blood was taken from overnight fasting participants for routine chemistries. A volume of 10ml to 15ml of blood was collected from the participants. Blood was centrifuged immediately and plasma was divided into aliquots stored in 1.5ml microcentrifuge tubes at -80°C. Plasma was used for leptin, resistin and adiponectin detections. Blood from red top tubes were left to sit at room temperature for 30-60 minutes for clot formation. Blood was centrifuged after clot formation and red cells were discarded. Supernatant was transferred and labeled as serum. Total serum cholesterol, total triglyceride, serum High-Density Lipoprotein cholesterol (HDL), serum Low Density Lipoprotein cholesterol (LDL) and triglyceride levels were measured using standard clinical laboratory techniques by clinical diagnostic laboratory of the University of Malaya Medical Center (UMMC).

3.4 Clinical measurements

Anthropometric measurements such as height, body weight, body mass index (BMI), waist circumference (WC), hip circumference (HC), waist–hip ratio (WHR), systolic blood pressure (SBP), and diastolic blood pressure (DBP) were measured. Height and weight were measured using calibrated stadiometers and weighing scales. Waist and hip measurements were made by a circumference measurement tape. WC was measured at the midpoint between the lower border of the rib cage (costal margin) and the iliac crest. HC was measured at the widest circumference over the buttocks and below the iliac crest. Recommended cutoff for Asians (>90 cm for men and >80 cm for

women) was used to define central obesity (Moy & Bulgiba, 2010). Using a digital automatic blood pressure monitor (Omron HEM-907, Omron Healthcare, Kyoto, Japan), blood pressure was measured.

3.5 Buccal swab collection

It was ensured that the person providing the sample did not consumed any food or drink in the 30 minutes prior to sample collection. For collection of buccal swab samples, a cotton swab was firmly scraped against the inside of each cheek 6 times. The swab was then air-dried for at least 2 hours after collection. Cotton swabs were cut from the stick by scissors.

3.6 DNA isolation from human buccal swabs

Buccal swabs were collected and genomic DNA extraction was performed using i-genomic CTB DNA extraction kit (iNtRON Biotechnology, Inc). The buccal swabs were placed into a 1.5ml microcentrifuge tube. The cotton swab was then pre-treated by adding 200 µl of PBS solution and vortexed vigorously. The microcentrifuge tube was centrifuged briefly to remove drops from inside the lid.

This DNA extraction consists of 6 main steps: Pre-lysis, lysis, precipitation, DNA binding, washing and elution steps by using buffers, Proteinase K and RNase A. In the lysis step, 200 µl Buffer CG, 10 µL proteinase K and 3 µl RNase A Solution was transferred into sample tubes and mixed by vortexing vigorously. The lysate was incubated at 65°C in a preheated heat block for 60 minutes. After the lysis was complete, the sample tube was centrifuged to remove un-lysed tissue particles. Following that, 180~200 µl of the supernatant was carefully transferred into a new 1.5 ml tube.

For the DNA binding step, 250 µl Buffer CB was added to the lysate after the lysis was complete. The lysate was mixed by pipetting 5~6 times. After mixing, the tube was spun down to remove drops from the inside of the lid. A total of 250 µl 80% of

ethanol was added to the lysate and mixed by pipetting 5~6 times. After mixing, the tubes were spun down to remove drops from inside of the lid. The whole mixture from the previous step was carefully pipetted to the spin column (inserted in a 2 ml collection tube) without wetting the rim. The tube was centrifuged at 13,000 rpm for 1 minute. The flow-through and collection tube was discarded. In the washing step, the spin column was placed into a new 2.0 ml collection tube. A total of 700 µl Buffer CW (washing buffer) was added to the spin column and the tube was centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded and again centrifuged for an addition of 1 minute to dry the membrane. The flow-through and collection tube were then discarded. The spin column was placed into a new 1.5 ml collection tube. A total of 50 µl Buffer CE (elution buffer) was added directly onto the membrane. The spin column was incubated at room temperature for 1 minute and then centrifuged at 13,000 rpm for 1 minute to elute the DNA.

3.7 DNA measurement

The concentration and purity of DNA were measured using the nanodrop reader at 260nm and 280 nm wavelength.

3.8 SNPs selection

SNPs selection was performed based on results derived from GWAS, meta-analysis, gene-linkage studies and knowledge of tagging SNPs using HapMap database.

3.9 Genotyping of SNPs

3.9.1 Real-Time PCR for genotyping

The presence of MC4R rs2229616 SNP, ADRB2 rs1042713 SNP, ADRB2 rs1042714 SNP, ADRB2 rs1042717 SNP, ADRB2 rs1042718 SNP, ADRB2 rs1042719 SNP, INSIG2 rs7566605 SNP, ADIPOQ rs17366568 SNP and SDC3 rs2491132 SNP were genotyped using pre-designed Taqman® single nucleotide polymorphism genotyping assays (Applied Biosystems, Foster City, CA, USA) on Applied Biosystem StepOne™ Real-Time Polymerase Chain Reaction System.

3.9.1.1 PCR amplification

A reaction mix with total reaction volume of 9 µl containing 5 µl Taqman® GTXpress™ Master Mix (Applied Biosystem), 0.5 µl assay mix and 3.5 µl ultrapure water was prepared. The SNP reaction mix was aliquoted into MicroAmp™ fast reaction optical tubes. A total of 9 µl PCR reaction mix was added into each well of the DNA reaction plate. All the wells were inspected for uniformity of volume. A total of 1 µl of genomic DNA was delivered to the final reaction mix. Negative controls and duplicates were added to each batch of samples to ensure genotypic quality. The plate was sealed with the appropriate MicroAmp™ strip optical cover. The plate was vortexed to mix contents in the wells. The plate was centrifuged briefly to spin down the contents and eliminate any air bubbles. The PCR reaction was performed with the following thermal cycling condition (Table 3.1).

Table 3.1 : Thermal cycling conditions for real-PCR

Number of Cycle	Duration	Temperature (°C)	Step
	30 sec	25	Holding
	20 sec	95	Holding
45	3 sec	94	Denaturation
	20sec	60	Annealing/Extension
	30 sec	25	Holding

3.9.1.2 Allelic discrimination plate read and analysis

After PCR amplification, an endpoint plate reading was performed using an Applied Biosystems Real-Time PCR System. The Sequence Detection System (SDS) Software uses the fluorescence measurements made during the plate reading to plot fluorescence (Rn) values based on the signals from each well. The plotted fluorescence signals showed which alleles were in each sample.

3.9.2 Sequenom MassARRAY® iPLEX platform (MALDI-TOF)

The genotyping of FTO, ADIPOQ, RETN, MC4R and LEP SNPs were performed using Sequenom MassARRAY® iPLEX platform, and this service was outsourced due to the unavailability of the equipment. The MassARRAY® Designer software designs both PCR and iPLEX single base extension primers for multiplexed assays. iPLEX assay is a primer extension process designed to detect sequence differences at the single nucleotide level. A single termination mix and universal reaction conditions was used for genotyping of all SNPs. The SpectroCHIP® arrays are placed into the MALDI-TOF mass spectrometer and the mass correlating genotype is determined in real time. A SpectroCHIP® typically carried out the process in 45-60 minutes. Through the use of MALDI-TOF mass spectrometry, the mass of the extended primer is determined (Gabriel, Ziaugra, & Tabbaa, 2009).

3.9.2.1 Preparation of the DNA samples

Genomic DNA samples were diluted to 10 ng/µL concentration. The A260/A280 ratios of the samples were between 1.7-2.0. For quality check, 4 duplicate check controls and 6 randomly selected samples for each DNA sample was run on 1% agarose gel along with 100bp size marker. DNA samples of 5 µl per assay group (double volume for duplicate samples) were transferred in the 96-well skirted plate. A total of 7 wells were used for 5 duplicates, 1 control well and 1 blank well.

3.9.2.2 Amplification of target loci by PCR

An optimal multiplex PCR reaction was performed to uniformly amplify many individual loci of DNA with minimal nonspecific byproducts. The purified amplicons are then used as templates for the primer extension reaction. Robotic workstation was used to dispense 2 µl of DNA sample per well of 384-well PCR plate. A total of 4 µl of PCR master mix was added into each well of the 384-well reaction plate. PCR master mix consists of 2.22 µl of H_2O, 0.83 µl of PCR buffer (10x), 0.43 µl of $MgCl_2$, 0.13 µl dNTP mix, 0.66 of primer mix (forward and reverse primer, 1 µM) and 0.22 µl of HotStarTaq Plus was added to each well. The plates were vortexed on a vortexer. The plate was centrifuged for 1 minute at 425 x g, room temperature, in a pre-PCR tabletop centrifuge with microtiter plate carriers, to bring the solutions to the bottom of the wells. The plate was placed in the ABI (Applied Biosystems) 384-well block. Lid mode was set as constant at 100°C. PCR was performed with the cycling programmes as shown in Table 3.2. The plate was centrifuged for 1 minute at 425 x g, room temperature to bring the solutions to the bottom of the wells, and then stored at 4°C until ready for SAP clean procedure.

Table 3.2 : PCR cycling conditions for amplification of target loci

Number of Cycle	Duration	Temperature (°C)	Step
1	5 min	94	Initial denaturation
45	20 sec	94	Denaturation
	30 sec	56	Annealing
	1 min	72	Extension
1	3 min	72	Final extension
Final step	indefinite	4	Hold

3.9.2.3 Post-PCR: SAP reaction cleanup

Post-PCR step performed with Shrimp alkaline phosphate (SAP) cleanup reaction. This procedure is crucial to remove the remaining unincorporated dNTPs from amplification products. The SAP dephosphorylates unincorporated dNTPs by cleaving the phosphate groups from the 5' termini. This procedure was performed on a post-PCR 96-tip robot. A total of 2 µl of the SAP cocktail was dispensed into each individual well of the 384-well post-PCR reaction plate. The SAP reaction cocktail consists of 1.53 µl H_2O, 0.17 µl of SAP buffer (10x) and 0.3 of SAP (1.7 U/µl) was added to each individual well. After the SAP cocktail was added, the plates were removed from the robot and centrifuged. Treated plates were placed in a 37°C incubator for 50 min and right after this, the plates were placed in an 85°C incubator for an additional 20 minutes to inactivate SAP enzyme.

3.9.2.4 Primer extension

The primer extension or iPLEX is a method used for detecting SNP. The primer was extended by one mass-modified nucleotide during the iPLEX reaction. A total of 2 µl of primer Extend Master Mix with Sequenom Termination Mixes was added to each well of the reaction plate. The primer Extend Master Mix with Sequenom Termination Mixes consist of 0.49 µl of iPLEX buffer (10x), 0.2 µl of iPLEX extension mix, 1.04 µl of Probe mix (5 µM to 15 µM) and 0.04 µl of iPLEX enzyme was added in each individual well. The plates were vortexed on vortexer. The plates were centrifuged for 1 minute at 425 x g, room temperature, in a post-PCR tabletop centrifuge with microtiter plate carriers, to bring the solutions to the bottom of the wells. The plates were placed in the ABI (Applied Biosystems) 384-well block. Primer extension was performed with the following cycling programs (Table 3.3).

Table 3.3 : PCR cycling conditions for primer extension reaction

Number of Cycle	Duration	Temperature (°C)	Step
1	30 sec	94	Initial denaturation
45	5 sec	94	Denaturation
5	5 sec	52	Annealing
	5 sec	80	Extension
1	3 min	72	Final extension
Final step	indefinite	4	Hold

3.9.2.5 Primer extension reaction resin cleanup

Resin slurry was made by adding 80 ml of ultrapure water to spectroCLEAN resin from two containers (28 g each). The plate was centrifuged for 1 minute at 425 x g, at room temperature, in a post-PCR tabletop centrifuge with microtiter plate carriers, to bring the solutions to the bottom of the wells. A total of 16 µl of resin suspension (containing 7mg resin) was added to each well of the 384-well reaction plates. Plates were sealed and rotated on plate rotator for 30 minutes. The plates were centrifuged for 3 minute at 425 x g, at room temperature, in a post-PCR tabletop.

3.9.2.6 Spotting primer extension products on spectroCHIPs

The extended analyte products from 384-well microtiter plates were arrayed on 384-sample spectroCHIPs. The oligonucleotides were consecutively incorporated with the correct matrix for MALDI-TOF, to a volume of 25 nl then was arrayed onto existing matrix spots on the silica chips. Once the chip is spotted, the plate was covered with adhesive sealer.

3.9.2.7 Detection of primer extension products by mass spectrometry

The extended products were detected using MassARRAY Compact mass spectrometer and Sequenom real-time detection software. The spotted SpectroCHIP was placed into the mass spectrophotometer. Each spot was subsequently shot with a laser under vacuum by matrix-assisted laser desorption ionization–time-of-flight (MALDI-

TOF) method. Once the sample molecules were vaporized and ionized, they were transferred electrostatically into a time-of-flight mass spectrophotometer (TOF-MS). Here, the molecules were separated from the matrix ions, individually detected based on their mass-to-charge (m/z) ratios and examined. The resulting spectra were analyzed by SpectroTyper software which combines the base caller with a clustering algorithm.

3.10 Haplotyping

Linkage disequilibrium block construction and haplotype analysis were performed using Haploview software (version 4.2). Measurement of linkage disequilibrium coefficient (D') was also performed to measure the strength of LD. Permutation test with 5000 replication was used to access the empirical significance. Adjustment for multiple testing was performed by obtaining p-values from permutation test from Haploview software.

3.11 Enzyme-Linked Immunosorbent Assay (ELISA) for quantitative detection of Adipokines

Hormonal concentrations of leptin, resistin and adiponectin were determined using Human Leptin Instant ELISA kit (eBioscience), Human Resistin Platinum ELISA kit (eBioscience) and Human Adiponectin Platinum ELISA kit (eBioscience). Plasma was diluted before the assay. All samples were assayed in duplicates.

3.11.1 Measurement of Human Resistin using ELISA

Buffer concentrates were brought to room temperature and diluted before starting the test procedure. To prepare wash buffer (1x), 50 ml of wash buffer concentrate 20x (PBS with 1% Tween 20) was brought to final volume of 1000 ml with deionized water. The contents were mixed gently to avoid foaming. The wash buffer (1x) was transferred to a clean wash bottle and stored at 2°C to 25°C. To prepare assay buffer (1x), 5 ml of assay buffer concentrate 20x (PBS with 1% Tween 20 and 10% BSA) was brought to final volume of 100 ml with distilled water. The contents were

mixed gently to avoid foaming. The assay buffer (1x) was transferred to a clean wash bottle and stored at 2°C to 8°C.

To prepare human resistin standard, firstly human resistin standard was reconstituted by addition of 600 µl of distilled water. The contents were mixed gently to ensure complete and homogeneous solubilization. The concentration of reconstitute standard was 4000 pg/ml. The reconstituted human resistin standard was allowed to sit for a minimum of 10 minutes. The well containing diluted human resistin standard was mixed well prior to making dilutions. External standard dilution was performed. A total of 7 tubes, one for each standard point were labeled as S1, S2, S3, S4, S5, S6 and S7. Serial dilutions of 1: 2 were prepared for the standard curve. Firstly, 225 µl of Assay buffer (1x) was pipetted into each tube. A total of 225 µl reconstituted standard (4000 pg/ml) was pipetted into the first tube, labeled S1 and mixed. So the concentration of standard 1 is 2000 pg/ml. A total of 225 µl of this dilution was pipetted into the second tube, labeled as S2, and mixed thoroughly before the next transfer. Serial dilutions were repeated 5 more times thus creating the points of the standard curve. Assay buffer was served as blank.

Before starting with the test procedure, the samples were prediluted. Plasma samples were diluted 1:10 with assay buffer (1x). A total of 135 µl of assay buffer (1x) was added to 15 µl of plasma sample. Each sample, standard and blank was assayed in duplicate. Firstly, the microwell strips were washed twice with approximately 400 µl wash buffer per well with thorough aspiration of microwell contents between washes. The wash buffer was allowed to sit in the wells for about 10-15 seconds before aspiration. After the last wash step, wells were emptied and microwell strips tapped on paper towel to remove excess wash buffer. The microwell strips were used immediately after washing. The wells were ensured not to be dry. A total of 100 µl of human resistin standards that were diluted were pipetted into the standard wells accordingly. A total of

100 µl of Assay buffer (1x) was added in duplicate to the blank wells. A total of 50 µl of Assay buffer (1x) was added to the sample wells. A total of 50 µl of each prediluted sample was added in duplicate to the sample wells.

The concentrated Biotin-conjugate anti-human resistin polyclonal antibody was diluted to 1:100 by adding assay buffer (1x) in a clean plastic tube. A total of 50 µl of prediluted Biotin-Conjugate was added to all wells. The microwell plate was covered with an adhesive film and incubated at room temperature (18°C to 25°C) for 2 hours. The concentrated Streptavidin-HRP was diluted to 1:200 by adding assay buffer (1x) in a clean plastic tube**.** Adhesive film was removed and the wells were emptied. Microwell strips were washed for 4 times with approximately 400 µl wash buffer per well with thorough aspiration of microwell contents between washes. The wash buffer was allowed to sit in the wells for about 10-15 seconds before aspiration. After the last wash step, wells emptied and microwell strips tapped on paper towel to remove excess wash buffer. The microwell strips used immediately after washing.

A total of 100 µl of diluted Streptavidin-HRP was added to all wells, including the blank wells. The microwell plate was covered with an adhesive film and incubated at room temperature (18°C to 25°C) for 1 hour. Adhesive film was removed and the wells were emptied. The microwell strips were washed for 4 times with approximately 400 µl wash buffer per well with thorough aspiration of microwell contents between washes. The wash buffer was allowed to sit in the wells for about 10-15 seconds before aspiration. After the last wash step, wells were emptied and microwell strips were tapped on paper towel to remove excess wash buffer. The microwell strips were used immediately after washing. A total of 100 µl of TMB substrate solution (tetramethyl-benzidine) was pipetted into all the wells. The microwell strips were incubated at room temperature (18°C to 25°C) for about 10 minutes. The microwells strips were kept away from direct exposure to intense light. The colour development on the plate was

monitored and substrate reaction stopped when the highest standard has developed a dark blue colour. The enzyme reaction was stopped quickly by pipetting 100 µl of the Stop solution (1M phosphoric acid). The results were read immediately after the stop solution was added.

Absorbance of each microwell was read on a spectro-photometer using 450 nm as the primary wave length and 620 nm as the reference wave length. The plate reader was blanked by using the blank wells. The absorbance of both the samples and the standard wells were determined. A standard curve was created by plotting the mean absorbance for each standard concentration. Average absorbance value for each set of duplicate standards and samples was calculated. The concentration of circulating human resistin for each sample was obtained by using the human resistin standard curve. The concentration reading from the standard curve was multiplied by dilution factor (x20) as the samples have been diluted 1:20.

3.11.2 Measurement of Human Adiponectin using ELISA

Buffer concentrates were brought to room temperature and diluted before starting the test procedure. To prepare wash buffer (1x), 50ml of wash buffer concentrate 20x (PBS with 1% Tween 20) was brought to a final volume of 1000 ml with deionized water. The contents were mixed gently to avoid foaming. The wash buffer (1x) was transferred into a clean wash bottle and stored at 2°C to 25°C. To prepare assay buffer (1x), 5 ml of assay buffer concentrate 20x (PBS) with 1% Tween 20 and 10% BSA) was brought to a final volume of 100 ml with distilled water. The contents were mixed gently to avoid foaming. The assay buffer (1x) was transferred into a clean wash bottle and stored at 2°C to 8°C.

To prepare human adiponectin standard, firstly human adiponectin standard was reconstituted by addition of 400 µl of distilled water. The contents were then mixed gently to ensure complete and homogeneous solubilization. The concentration of

reconstitute standard was 100 ng/ml. The reconstituted human adiponectin standard was allowed to reconstitute for a minimum of 10-30 minutes. The well containing diluted human adiponectin standard was mixed well prior to making dilutions. External standard dilution was performed. A total of 7 tubes, one for each standard point were labeled as S1, S2, S3, S4, S5, S6 and S7. Serial dilutions of 1: 2 were prepared for the standard curve. Following that, 225 µl of Assay buffer (1x) was pipetted into each tube. A total of 225 µl reconstituted standard (100 ng/ml) was pipetted into the first tube, labeled S1 and mixed. The concentration of standard 1 is 50 ng/ml. A total of 225 µl of this dilution was pipetted into the second tube, labeled as S2, and mixed thoroughly before the next transfer. Serial dilutions were repeated 5 more times thus creating the points of the standard curve. Assay buffer was served as blank.

Before starting with the test procedure, the samples were pre-diluted. Plasma samples were diluted 1:500 with assay buffer (1x). Firstly, a total of 90 µl of assay buffer (1x) was added to 10 µl of plasma samples. Secondly, total of 10 µl of the prepared diluted samples were diluted further by adding 490 µl of assay buffer (1x). Each sample, standard and blank was assayed in duplicate. Following that, the microwell strips washed twice with approximately 400 µl wash buffer per well with thorough aspiration of microwell contents between washes. The wash buffer was allowed to sit in the wells for about 10-15 seconds before aspiration. After the last wash step, the wells were emptied and microwell strips were tapped on paper towel to remove excess Wash buffer. The microwell strips were used immediately after washing. Wells were ensured not to be dry. A total of 100 µl of human adiponectin standards that were diluted were pipetted in the standard wells accordingly. A total of 100 µl of Assay buffer (1x) was added in duplicate to the blank wells. A total of 50 µl of Assay buffer (1x) was added to the sample wells.

A total of 50 µl of each prediluted sample was added in duplicate to the sample wells. The concentrated Biotin-conjugate anti-human adiponectin polyclonal antibody was diluted to 1:100 by adding assay buffer (1x) in a clean plastic tube. A total of 50 µl of prediluted Biotin-Conjugate was added to all wells. The microwell plate was covered with an adhesive film and incubated at room temperature (18°C to 25°C) for 2 hours. The concentrated Streptavidin-HRP was diluted to 1:400 by adding assay buffer (1x) in a clean plastic tube**.** Adhesive film was removed and the wells were emptied. Microwell strips were washed for 6 times with approximately 400 µl wash buffer per well with thorough aspiration of microwell contents between washes. The wash buffer was allowed to sit in the wells for about 10-15 seconds before aspiration. After the last wash step, wells were emptied and microwell strips were tapped on paper towel to remove excess wash buffer. The microwell strips were used immediately after washing. A total of 100 µl of diluted Streptavidin-HRP was added to all wells, including the blank wells. The microwell plate was covered with an adhesive film and incubated at room temperature (18°C to 25°C) for 1 hour. Adhesive film was removed and the wells were emptied. The microwell strips were washed for 6 times with approximately 400 µl wash buffer per well with thorough aspiration of microwell contents between washes. The wash buffer was allowed to sit in the wells for about 10-15 seconds before aspiration. After the last wash step, wells were emptied and microwell strips were tapped on paper towel to remove excess wash buffer. The microwell strips were used immediately after washing.

A total of 100 µl of TMB substrate solution (tetramethyl-benzidine) was pipetted to all wells. The microwell strips were incubated at room temperature (18°C to 25°C) for about 30 minutes. The microwells strips were kept away from direct exposure to intense light. The colour development on the plate was monitored and substrate reaction was stopped when the highest standard has developed a dark blue colour. The

enzyme reaction was stopped quickly by pipetting 100 µl of the Stop solution (1M phosphoric acid). The results were read immediately after the stop solution was added. Absorbance of each microwell was read on a spectro-photometer using 450 nm as the primary wave length and 620 nm as the reference wave length. The plate reader was blanked by using the blank wells. The absorbance of both the samples and the standard wells were determined.

A standard curve was created by plotting the mean absorbance for each standard concentration. Average absorbance value for each set of duplicate standards and samples was calculated. The concentration of circulating human adiponectin for each sample was obtained by using the human adiponectin standard curve. The concentration read from the standard curve was multiplied by dilution factor (x1000) as the samples have been diluted 1:1000.

3.11.3 Measurement of Human Leptin using ELISA

Buffer concentrates were brought to room temperature and diluted before starting the test procedure. To prepare the wash buffer (1x), 25 ml of wash buffer concentrate (20x) (phosphate-buffered saline with 1% Tween 20) was brought to a final volume of 500 ml with deionized water. The contents were mixed gently to avoid foaming. The wash buffer (1x) was transferred to a clean wash bottle and stored at 2°C to 25°C. To prepare assay buffer (1x), 5 ml of assay buffer concentrate 20x (PBS with 1% Tween 20 and 10% BSA) was brought to a final volume of 100 ml with distilled water. The contents were mixed gently to avoid foaming. The assay buffer (1x) was transferred to a clean wash bottle and stored at 2°C to 8°C.

Before starting with the test procedure, the samples were prediluted. Plasma samples were diluted 1:25 with assay buffer (1x). A total of 240 µl of assay buffer (1x) was added to 10 µl of plasma samples. A total of 100 µl of distilled was added to the sample wells and 250 µl distilled water added to all standard and blank wells in the

microwell strips. A total of 50 µl of each prediluted sample was added in duplicate, to the designated wells and the contents were mixed. The microwell plate was covered with an adhesive film and incubated at room temperature (18°C to 25°C) for 3 hours. Adhesive film was removed and the wells were emptied. Microwell strips were washed for 6 times with approximately 400 µl wash buffer per well with thorough aspiration of microwell contents between washes. The wash buffer was allowed to sit in the wells for about 10-15 seconds before aspiration. After the last wash step, wells were emptied and microwell strips were tapped on paper towel to remove excess wash buffer. The microwell strips were used immediately after washing.

A total of 100 µl of TMB substrate solution (tetramethyl-benzidine) was pipetted to all the wells. The microwell strips were incubated at room temperature (18°C to 25°C) for about 10 minutes. The microwells strips were kept away from direct exposure to intense light. The colour development on the plate was monitored and substrate reaction stopped when the highest standard has developed a dark blue colour. The enzyme reaction was stopped quickly by pipetting 100 µl of Stop solution (1M phosphoric acid). The results were read immediately after the stop solution was added. Absorbance of each microwell was read on a spectro-photometer using 450 nm as the primary wave length and 620 nm as the reference wavelength. The plate reader was blanked by using the blank wells. The absorbance of both the samples and the standard wells were determined.

A standard curve was created by plotting the mean absorbance for each standard concentration. Average absorbance value for each set of duplicate standards and samples was calculated. The concentration of circulating human leptin for each sample was obtained by using the human leptin standard curve. The concentration reading from the standard curve was multiplied by dilution factor (x50) as the samples have been diluted 1:50.

3.12 Statistical analysis

3.12.1 Hardy-Weinberg equilibrium

Hardy-Weinberg equilibrium in both obese and non-obese were checked (Santiago Rodriguez 2009). Genotype and allelic frequencies among obese and non-obese were calculated.

3.12.2 T-test

T-test is a method used to evaluate the differences in means between two groups. T-test used to test mean difference of obesity parameters between the two BMI groups (obese and non-obese). Prior to the statistical analysis, all continuous data were examined for normality. Data that was non-normally distributed was natural log-transformed for normalization.

3.12.3 Mann-Whitney U test

Mann-Whitney U test was used for non-normally distributed data to test between two groups.

3.12.4 Pearson's correlation

Pearson's correlation was used to examine a correlation between at least two continuous variables. The correlation coefficient, r ranges from +1.00 to -1.00. The value of +1.00 indicates a perfect positive correlation and -1.00 indicates a perfect negative correlation. A value of 0.00 indicates a lack of correlation. Pearson correlation was performed to test correlation between the obesity biomarkers such as leptin, adiponectin and resistin with obesity-related traits.

3.12.5 General Linear Method (GLM)

A general linear method (GLM) is used to test significance for multiple dependent variables. GLM is used for normally distributed data. The GLM method which was adjusted for age and gender was used in assessing effects of SNPs on obesity parameters. The results of association analysis for the SNPs and obesity parameters

indicates that the additive model best-fits the data. All data were given as the mean±standard deviation. Statistical analysis was performed using SPSS 16.0 software.

3.12.6 Bonferroni correction

Bonferroni method used to control overall type 1 error rate in multiple testing so that the error rate does not go beyond α. This correction was achieved simply by setting the cutoff value for stating statistical significance at α/k for each test results, in which k is the number of the tests that were conducted. Since this study involves investigation of many genes, Bonferroni correction was performed for validity of the significance test (Gordi & Khamis, 2004). This procedure was performed to avoid the risk of claiming significant effects of the SNPs with obesity parameters when there is none. Below are the Bonferroni corrections and statistical level used for each gene:

a) FTO

α= 0.05/30 SNPs

= 0.002

b) MC4R

α= 0.05/3 SNPs

=0.017

c) ADRB2

α= 0.05/5 SNPs

= 0.010

d) LEP

α= 0.05/7 SNPs

= 0.007

e) RETN

α= 0.05/3

= 0.017

f) ADIPOQ

$\alpha = 0.05/2$

$= 0.025$