CHAPTER 2

Materials and Methods

2.1 Subjects

The population of this current study consisted of 78 healthy young adults that fit the inclusion criteria for recruitment. Written informed consent was obtained from every subject. Subjects were divided into a control group and an overweight and obese (OW/OB) group, with 39 subjects placed in each group based on sex and BMI.

2.1.1 Control group

healthy males and 27 healthy females, having no history or existing of chronic diseases or liver injury. BMIs in the normal range as defined by WHO (15.8-24.9 kg/m^2). Ages are between 19-35 years.

2.1.1 Overweight and obese (OW/OB) group

healthy males and 15 healthy females, having no history or occurrence of chronic disease or liver injury. With BMIs in the overweight and obese ranges as defined by WHO (>25 kg/m^2). Ages between 19-35 years.

The inclusion and exclusion criteria were as follows

Inclusion criteria

Control group

1) Age 19-35 years old
2) Thai nationality

3) Subjects are literate, and are capable of communicating with no problem with hearing or understanding. Consent to participate the research and ready to cooperate was obtained.
4) BMI between 18.5-24.9 kg/m^2
5) Subjects are healthy with no history of chronic diseases or liver injury.
6) Alcohol consumption < 5 times/week (<30 g/day in man and < 20 g/day in women) [14].
7) Not a vegetarian or have dietary practices that involve abstaining from certain types of food.
8) Not on any drugs that alter lipid, glucose or metabolic levels.
9) Subjects have agreed to participate in the study with written informed consent.

OW/OB group

1) Age 19 – 35 years old
2) Thai nationality
3) Subjects are literate, capable of communicating with no problem with hearing or understanding. Have consented to participate in research and are ready to cooperate.
4) BMI > 25 kg/m^2
5) Weight does not exceed table weight limit of 250 kg
6) Subjects are healthy with no history of chronic disease or liver injury.
7) Alcohol consumption < 5 times/week (<30 g/day in man and < 20 g/day in women) [14].
8) Not a vegetarian or have dietary practices that involve abstaining from certain types of food.
9) Not on drugs that alter lipid, glucose or metabolic levels.
10) Subjects have agreed to participate in the study with written informed consent.

Exclusion criteria for both group

1) Have a chronic disease, such as type 2 diabetes, high blood pressure or cancer.
2) Have any liver injury, was treated or hospitalized from any liver disease. Have history of Hepatitis B and C virus infection.
3) Pregnant females
4) Claustrophobia
5) Subjects with an implanted surgical device such as pacemaker, aneurysm clip, prosthetic heat valve, metal foreign bodies, or any contraindications for MRI scan.
6) Any contraindications or unable to lay still on the table in MRI machine for more than 1 hour.

All Subjects were given a questionnaire about health and lifestyle to determine if there was a need to include or exclude subjects for the study. Eating and exercise habits, occupation, personal history, and family medical history was provided. All subjects agreed to participate in the study with written inform consent. All procedures were approved by Ethics Committee of the Faculty of Associated Medical Science, Chiang Mai University, Chiang Mai, Thailand (AMSEC-61EX-016).

2.2 Anthropometry

Every subject was measured by the same examiner. Subjects wore only an examination cloth. Height and bodyweight were measured to the nearest 0.5 cm and 0.1 kg respectively. Hip circumference (HC) and waist circumference (WC) was acquired while subjects were instructed to breathe out mildly. Both measurements were done using non-elastic tape. WC was measured at midpoint of lower margin of rib and top of iliac crest. HC was measured at widest section of buttocks. Waist to hip ratio (W/H ratio) was calculated from WC divided by HC.

2.3 MRI images acquisition

MRI images of liver were obtained for ^{1}H MRS voxel localization using the following procedures:

2.3.1 Subjects lying on the MRI table in the head first supine position. The respiration gate was used to prevent respiratory artefacts.

2.3.2 A SENSE Cardiac coil was placed over upper abdomen with localizer laser over xiphoid process.

2.3.3 Transverse and coronal MRI images of liver were obtained by MRI 1.5 T Achieva, Philips Medical Systems, Best, The Netherlands as using protocols described in Table 1.

Table 1 Protocols for transverse and coronal MRI images of liver

Image plane	**Pule sequence**	**Repetition time (TR)**	**Echo time (TE)**	**Slice thickness**	**Flip Angle**
Transverse	T2-weighted TSE	871 ms	80 ms	6 mm	90◦
Coronal	T2-weighted TSE	829 ms	80 ms	6 mm	90◦

2.4 LFC assessment by ^{1}H MRS

Liver metabolite spectra were obtained by Proton magnetic resonance spectroscopy technique (^{1}H MRS). A voxel was placed in the right lobe of the liver (Couinaud lobe segment V-VIII), carefully avoiding any large vessels and bile ducts. Next, liver metabolite signals without water suppression were obtained and were analyzed for metabolized quantification. ^{1}H MRS protocol is shown in Table 2.

Table 2 Protocol for liver ^{1}H MRS

Parameter	
Pule sequence	Point resolved spectroscopy (PRESS)
Voxel size	10×10×10 mm^3
Repetition time (TR)	2000 ms
Echo time (TE)	43 ms
Number of signal averages (NSA)	96

Liver metabolite signals without water suppression were obtained and analyzed for metabolized quantification by AMARES algorithms available on jMRUI software [43-45]. Spectrum fitting and quantification was done for water peak (4.7 ppm), and major lipid spectrum peaks (CH_3 = 0.9 ppm, CH_2 = 1.3 ppm, and 2.1 ppm) with prior knowledge and Gaussian line shape was then applied [33]. Signal intensity correction for T2 relaxation using linear least-square equation with was performed to determine T2 of water and fat. Liver fat content (LFC) was calculated by a validated method described elsewhere [42, 46]. NAFLD was determined as LFC >5.56% [42]. The LFC were calculated with the following experimentally determined factors: 1) ratio of lipid proton fitted (0.5 - 3.0ppm) to total number of proton is 0.85; 2) proton density of water and fat are 111 and 111 mol/l, respectively; 3) water per gram of liver is 0.711 g; 4) density of liver and liver fat is 1.051 and 0.90 g/l, respectively.

2.5 Biochemical analysis of blood

Blood collection of subjects was done by The Associated Medical Science Clinical Service Center, Chiang Mai University. 10 ml of Intravenous blood was drawn from antecubital vein for biochemical analysis. The test focused on total cholesterol (Cho), HDL, VLDL, triglyceride (TG), fasting blood glucose (FG) and glycated hemoglobin (HbA1c). Subject were told to fast for 10-12 hours prior to blood examination (after 19.00-20.00). Later, LDL concentration was calculated from novel adjustable LDL estimation equation [47, 48]. Dyslipidemia was described as abnormal levels of Cho level present in plasma. This also included any increasing Tri, LDL and low HDL, as well. The National Cholesterol Education Project (NCEP) Adult Treatment Panel (ATP) III has defined dyslipidemia as Cho $\geq$ 200 mg/dl, Tri $\geq$ 150 mg/dl, LDL $\geq$ 130 mg/dl, HDL $\leq$ 40 mg/dl [49]. Normal FG range should be between 70-100 mg/dl, with FG between 100-125 mg/dl considered to be pre-diabetes. Normal HbA1c levels should be less than 6% [50].

2.6 NMR measurement

Blood samples were centrifuged at 3,500×g for 10 minutes to separate blood serum. The samples were lyophilization prior to NMR study.

2.6.1 Lyophilized serum powder was dissolved with deuterium oxide (Sigma-Aldrich, USA) 500 µl, and then mixed gently. After that, the homogeneous solution was transfered into a 5 mm diameter NMR tube.

2.6.2 NMR signal of serum metabolites was collected at 300 K. All samples were performed on a Bruker AVANCE 400 MHz (Bruker, Germany) with 1D water-suppression pulses using pre-saturation pulse sequence. 90° pulse was applied. Number of signal averages (NSA) = 16 Spectra in 0-8 ppm range were analyzed by TopSpin version 4.0.1 software. The base line and phase were manually corrected. Metabolites were identified by compared to existing literatures [51, 52] and human metabolome database (http://www.hmdb.ca).

2.7 Statistical analysis

Statistical analysis was performed on SPSS using statistical software version 17.0. Particular metabolites of interest were further analyzed by multivarite statitiscal analysis using MetaboAnalyst (http://www.metaboanalyst.ca). Results with P-value < 0.05 were considered statistically significant.

2.7.1 The Kolmogorov-Smirnov test and Shapiro-Wilk test were performed to determine data normality.

2.7.2 Comparison of LFC and blood biochemical examination between groups was performed with unpaired samples t-test.

2.7.3 Relationship between groups was done with Pearson correlation. Multiple stepwise linear regression analysis was used to verify the relationships of between LFC and independence of significant correlate variables.

2.7.4 Mann-Whitney U test was used to compare the difference between groups of NMR serum metabolites.

2.7.5 Partial least squares discriminant analysis (PLS-DA) was performed to identify variable metabolites from control group and OW/OB group.