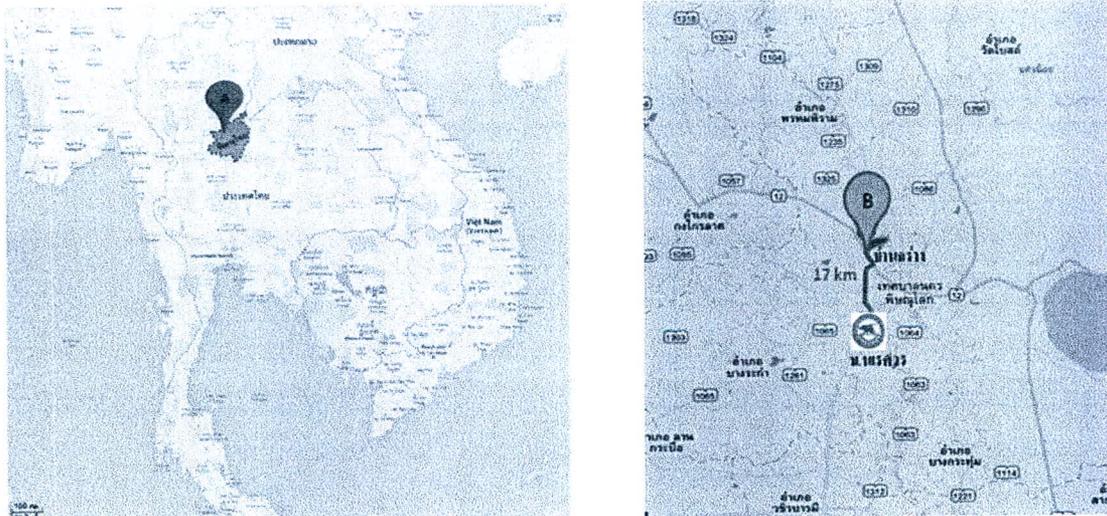


## CHAPTER III

### RESEARCH METHODOLOGY

This study involves the measurements of glucose, total cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), high sensitivity C-reactive protein (hs-CRP), blood pressure, waist circumference, and vascular indexes e.g. ankle-brachial index (ABI), and pulse wave velocity (PWV) in healthy adults at the Ban Krang primary care unit, Muang district, Phitsanulok province, Thailand.

The study was take place in this area because the officers of primary care unit had high competency and skill; there were enough medical data records and amounts of healthy adults that had the similarity of occupation, and lifestyle, furthermore, easy to communication and transportation. The distant from Ban Krang primary care unit to Narasuan University is 17 kilometers as show in figure 7



**Figure 7 A, Phitsanulok province, Thailand; B, The distant from Ban Krang primary care unit to Naresuan University**

Source: <https://maps.google.co.th/maps?q=thailand>

## Population and Subject

### Sample size

The minimum amounts of healthy adults are calculated by the formula below, and using 32 % of the prevalence of high TG level (TG > 150 mg/dL) in lower northern Thailand, that report by Wanvisa Boonlert et.al. (Wanvisa Boonlert, 2006).

$$n = \frac{Z^2 pq}{\sigma^2}$$

$$Z = 1.96, p = 0.32, q = 0.68, \sigma = 0.05$$

$$= \frac{(1.96)^2 (0.32) (0.68)}{(0.05)^2}$$

$$= 334.375$$

Therefore the minimum amounts of healthy adults used in this study are at least 335.

Five hundred and forty-five healthy adults were randomly selected from the health survey data in the year 2006 – 2007 of the Ban Krang Primary Care Unit, Muang district, Phitsanulok province, Thailand and invited to participate in this study. Medical histories of each subject were obtained by a questionnaire at the first time of health examination at Bang Krang Primary Care Unit, Budhachinaraj Hospital. There were 398 participants passed the inclusion and exclusion criteria. Sixteen participants, who had hs-CRP concentration over 10 mg/L were rejected from study. Thirty-five subjects, again, were kept out of this study, because their brachial were bigger than standard cuff of VP-1000 analyzer so cannot be measured vascular indexes. The final number of participants was 347; that obtain completely data of medical histories, anthropometric data, biochemistry analytes, and vascular indexes.

### Inclusion criteria

The subjects in this study consist of men and women that were non-smoking, age  $\geq 40$  years, BMI  $\leq 30$ , no recent illness and conditions such as tissue injury, infection, autoimmune disease, cancer, general inflammation or chronic inflammation, and taken any medication.

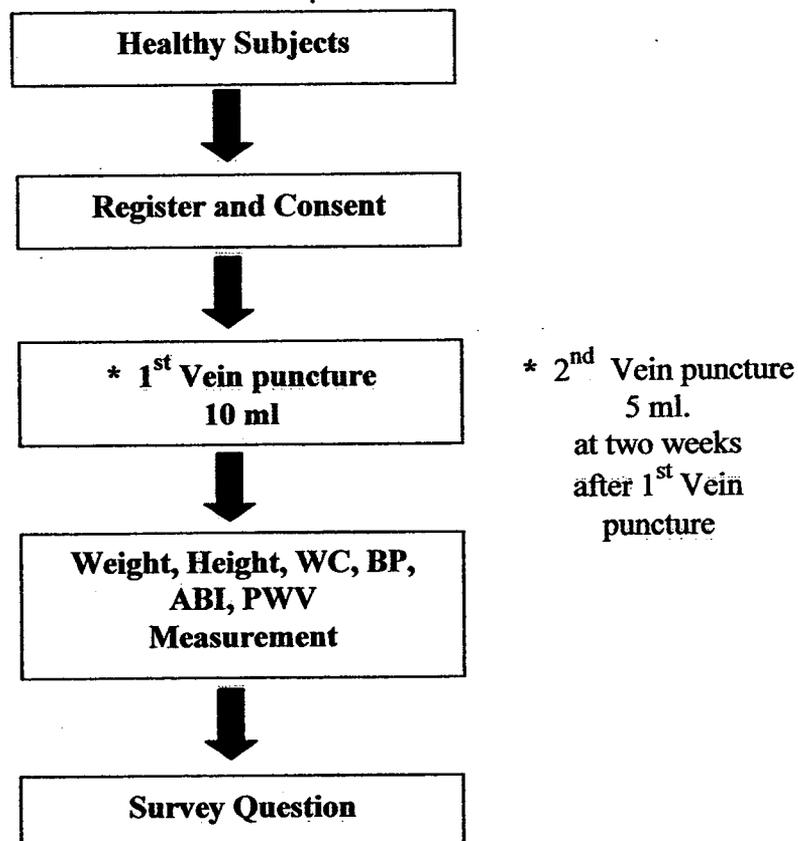
### **Exclusion criteria**

This study exclude the participants who is diabetes mellitus, hypertension (Blood pressure > 140/90 mmHg), cardiovascular disease, history of alcohol abuse (intake  $\geq$  500 g/week in the last year (PubMed Health. A.D.A.M. Retrieved 3 December 2012.)), current smoking, and any medication uses related to current diseases, and subjects who could not take blood specimens or didn't provide the consent form were excluded from the study.

### **Classifications of obesity, non-central obesity, and central obesity:**

Normal weigh, over weigh, and obesity were defined when BMI < 25.00, BMI=25.00-29.99, and BMI  $\geq$  30, respectively. Non-central obesity was defined when WC is < 90 cm and < 80 cm for men and women, respectively, while central obesity was defined when WC is over above (Florez H., 2006; Ethiraj Dhanaraj May, 2009).

### **Methods**



**Figure 8 Summary of the methods**

### **Summary of the methods**

This study was approved by the Institute Review Board of the Naresuan University for Human Research Study. Project schematic (Figure 8) represented summary of the methods of this study. All invited subjects were registered at the first time by interview, data information sheet and sign the consent form. The first vein puncture was done (second vein puncture at 2 weeks after), after that measured weight, height, WC, BP, ABI, PWV, finally asked the survey question.

#### **Blood pressure measurement**

All invited subject rested at least 10 minutes before performing of blood pressure measurement. The measurements of systolic and diastolic blood pressure were in term of millimeters of mercury (mmHg) using the digital blood pressure meter. Subjects seated on the chair that has back supported, their leg uncrossed, have their feet on the floor. Removed clothing that cover cuff position, place the cuff on upper arm, used the pillow to support the arm to raise it to the heart level. The AHA recommended doing double determination (rested one minute before the second measurement) and does the third determination if the different between the first to more than 5 mmHg, then recorded the average value (Liz Smith, 2005).

#### **Weight and height measurements**

Weight measurement; used digital standing scale, the scale should be placed on the balanced, smooth, hard-floor, and horizontal surface. The scale must be set at zero before measuring. Participants were asked to remove their excess clothing, shoes, key, mobile phone, and excess luggage. Participant should stands with minimal movement in the center of the platform, record the final digital number.

Height measurement; used standiometer to measure the maximum distance from the floor to the highest point on the head, when the subject was facing directly ahead. Shoes should be off, feet together, and arms by the sides. Heels, calf, buttocks, upper back and occiput should be contact with the wall. Height measurement can change all the day, being highest in the morning, so it should be measured at the same time of day. In this study measure the height and weight in the morning in term of centimeters and kilograms respectively. Height and weight were used for BMI calculation.

### **Waists circumferences measurements**

WC are measured by locate the upper hip bone and the top of the right iliac crest (portion of the pelvic bone at the belt line of the body). Place a measuring tape in a horizontal plane around the abdomen at the level of the iliac crest. Before reading the tape measure, make sure the tape is secure, but not too tight and is parallel to the floor. The reading should be taken at the end of expiration.

Subjects who met the inclusion criteria were selected to enroll in this study and arranged for blood specimen collection and vascular index assessments.

### **Vascular indexes assessment**

Non-invasive vascular parameters, PWV and ABI, were measured using the VP-1000 Analyzer (Colin, Co. Ltd., Komaki, Japan). Details of the methodology were previously described (Yamashina, A., 2002). Each subject was examined while resting in the supine position. Cuffs were wrapped on both brachia and ankles. Pulse volume waveforms at the brachium and ankle were recorded using a semiconductor pressure sensor. PWV and ABI were measured after at least a five minute rest.

Vascular stiffness was assessed by using PWV. The VP-1000 software used age, gender, and disease of individual to calculate PWV and provide peripheral vascular stiffness interpretation. The vascular occlusion was assessed using ABI and occlusion is indicated when ABI lower than 0.9 (Colin Medical Technology Corporation, n.d.; Yamashina A., 2002). Normal vascular was defined when PWV and ABI are normal, while abnormal vascular was defined when ABI or PWV or both are abnormal.

### **Blood specimen collection and preparation**

Blood samples were collected twice. First 10 mL of fasting blood was separated into two tubes, 2 mL was transferred to sodium fluoride tube for plasma glucose determination, and 8 mL was transferred to plain tube (clot blood tube) for determination of serum TC, TG, HDL-C, LDL-C, and hs-CRP. The second 5 mL of non-fasting blood was obtained by two weeks later for the secondary hs-CRP determination. Each blood samples were drawn and immediately centrifuged at 3000 rpm for 10 minutes. Plasma and serum were aliquot 500 micro liters into micro-tubes and stored at -70 °C deep freezer till analyzed. Frozen plasma and serum samples were transported from Naresuan University, Phitsanulok to Police General Hospital,

Bangkok, using dry ice box. The blood samples were immediately stored at -20 °C deep freezer till analyzed.

#### **Blood chemistry biomarker determinations**

The blood samples were thawed at room temperature and mixed by rolling mixer before analysis. Plasma glucose, serum total cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and hs-CRP were determined using an automated clinical chemistry analyzer (OLYMPUS AU 640, Olympus Corporation, Tokyo, Japan). The hs-CRP concentrations of individual subject were averaged and used for data analysis. Subjects that hs-CRP concentrations  $\geq 10$  mg/L were excluded from this study

#### **Equipment, Supplies and Reagents**

##### **Equipment**

-70 °C deep freezer MDF-U32V	Sanyo, Japan
-20 °C deep freezer	Sharp cooperation, Japan
4 – 8 °C refrigerator	Sanden intercool, Thailand
Digital blood pressure meter	Omron, Japan
Sphygmomanometer	ERKA Kallmeyer Medizintechnik GmbH & Co.KG, Germany
Stadiometer	Got creative, Suphanburi, Thailand
Weighting scale	Camry scale, China
Zentrifuge, 2070	Hettich Universal, Germany
OLYMPUS AU640	Olympus Corporation, Tokyo, Japan
VP-1000 Analyzer	Colin, Co. Ltd., Komaki, Japan
Auto-pipette 100-1000 $\mu$ L	ERBA Mannheim GmbH, Germany
Auto- pipette 20-200 $\mu$ L	ERBA Mannheim GmbH, Germany
Rolling mixer	Techno fab, Thailand
Vortex Genie 2, G560E	Scientific Industries, USA

##### **Supplies**

Needle No. 21 and 23	Terumo corporations, Philippines
Syringe 10 ml.	Terumo corporations, Philippines
Tourniquets	Terumo corporations, Thailand

Measuring tape	Hoechstmass, Germany
Sodium fluoride tube 2 ml.	Greiner bio-one, Thailand
Plain tube 10 ml.	Greiner bio-one, Thailand
Microtube 1.5 ml.	Zest-med Co.Ltd, Thailand
Stainless rack for test tube	Unitecscience, Thailand
Pipette tip 100-1000 $\mu$ L	Zest-med Co.Ltd, Thailand
Pipette tip 20-200 $\mu$ L	Zest-med Co.Ltd, Thailand
70 % alcohol	GPO, Bangkok, Thailand
0.85 % NSS	Otsuka.co.ltd., Thailand
Eelectrodes for the VP1000 Analyzer	Colin, Co. Ltd., Komaki, Japan

#### **Chemical reagents**

Glucose reagent	Olympus, AU, Germany
Cholesterol reagent	Olympus, AU, Germany
Triglyceride reagent	Olympus, AU, Germany
HDL-C reagent	Olympus, AU, Germany
LDL-C reagent	Olympus, AU, Germany
hs-CRP reagent	Olympus, AU, Germany
OLYMPUS system calibrator	Olympus, AU, Germany
OLYMPUS HDL-C/LDL-C calibrator level 1-2	Olympus, AU, Germany
OLYMPUS hs-CRP calibrator level 1-5	Olympus, AU, Germany
OLYMPUS control level 1-2	Olympus, AU, Germany
OLYMPUS HDL-C/LDL-C control level 1-2	Olympus, AU, Germany
OLYMPUS hs-CRP control level 1-2	Olympus, AU, Germany
Concentrate detergent cleaning solution	Olympus, AU, Germany

#### **Data analysis**

All data was analyzed using SPSS program. The characteristic variables were presented as mean with standard deviations (SD), but age, TC, TG, HDL-C, LDL-C, and hs-CRP were presented as median with 95% confidence interval (CI).

Obesity, non-central, and central obesity were presented in percentages. The vascular indexes, ABI and baPWV, were used to classify normal and abnormal vascular types following the recommendation criteria of manufacturer.

ANOVA and t-test were used for comparison study of obtainable quantitative data and Chi-square ( $\chi^2$ ) was used for qualitative data analysis. Mann-Whitney test was used to determine the statistic difference between non normal distribution data sets. Stepwise multi regression was performed to investigate the relationship among hs-CRP, PWV, ABI and other independent variables.