CHAPTER II

MATERIALS AND METHODS

Study Population

Sixty-six patients who were admitted at Maharaj Nakorn Chiang Mai hospital with acute myocardial infarction were included in this study. Normal controls were twenty-one age and gender-matched healthy. All participants were obtained with ethical approval. Written informed consent was obtained from each subject prior to study entry. All patients received standard treatments during the study. Participants in control group were collected for blood samples and received echocardiography examination only one time in the participated day.

Criteria for acute myocardial infarction patients

Patients with acute myocardial infarction were defined as the presence of typical prolonged chest pain (>30 min) accompanied by 1) serial changes on the standard 12-lead electrocardiogram (ECG) indicative of ischemia (i.e. ST segment changes) or 2) detection of significant (two-fold more than the upper normal range) increase in traditional myocardial damage markers (creatine kinase or cardiac troponin T).

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Criteria for participants

1. All of the participants must be older than 21 years and have a normal sinus rhythm.

2. The participants in control group must not have any recent history of acute ischemic stroke, aortic aneurysm, severe uncontrolled hypertension and also other neoplastic, hepatic, autoimmune or inflammatory diseases
3. The participants must not have any surgical procedure in the preceding 6 months.
4. The participants must not have diabetic mellitus which may affect the clinical outcome in the prognostic probability study.

Endpoint

In order to assess the predictive probability for adverse outcome and mortality of urocortins levels, the endpoint was set at 1 year after discharge, consisting of mortality, reinfarction, need for urgent revascularization or readmission with heart failure. The time duration from discharge day to the endpoint of patient was recorded (days) and then used for predictive probability examination.

Plasma sample collection

Participant's blood was drawn by a professional nurse for 3 ml in each day, at the same time of day in each experimental day. The blood sample collection started on admission day, which must be within 24 hours after the onset of acute myocardial infarction (i. e. day 0). After that, the blood samples were collected continuously on day 1, 3 and 5 after onset. Patients were called for a check up and blood samples collection at 1, 3 and 6 months after discharge. Patients were followed up for 6 months after complete blood collection in order to record the death, readmission to the hospital and any adverse events. Lost follow-up patients during this period were

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excluded from the correlation study for adverse events but their plasma urocortins level data were included into the plasma profile study. The participants in control group were not followed for the follow-up data. The diagrams of blood sample collection and follow up protocol were shown in figure 2.

The blood samples were collected and kept in the blood collection tubes (3 ml) which contained ethylenediamine tetraacetic acid (EDTA) as anticoagulant. Within thirty minutes after blood was drawn, the tubes were centrifuged at 5,000 rpm for 10 minutes at 4ºC (Sigma 2-16K, Sartorius AG, Germany) and then the plasma was separated from blood and stored at -70ºC until assay of urocortins. The blood samples were also tested for plasma BNP level on the discharge day by using the routine procedure of the Maharaj Nakorn Chiang Mai hospital's central laboratory and plasma BNP level was used as a reference marker compared with urocortins.

Echocardiography

Echocardiography was performed by cardiologists at Maharaj Nakorn Chiang Mai hospital. Both patient and control groups received echocardiography in order to record the parameters of cardiac function. Left ventricular wall motion index (LVWMI) and area of infarction were derived. Left ventricular ejection fraction (LVEF), left ventricular end diastolic volume (LVEDV) and left ventricular end systolic volume (LVESV) were calculated and then tested for the relationship between left ventricular function and urocortin level.

LVWMI is used for defining left ventricular systolic function. The left ventricle was divided into 16 segments, each of which was given a score for its motion (-1 for dyskinesia, 0 for akinesia, 1 for hypokinesia, 2 for normokinesia, and 3 for

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hyperkinesia). LVWMI is the mean score of all segments.[94] LVEF is the fraction of blood ejected by the ventricle relative to its end-diastolic volume.[95] It is used as a measure of the function of the left ventricle and as the index of the extent of left ventricular fiber shortening.[95;96] The ejection fraction is the percentage of blood ejected from the left ventricle with each heart beat. LVEDV reflects the lusitropic properties of the relaxed heart. It is the volume of amount of blood at the start of systole. LVESV, the volume of blood left behind from the previous systole, was defined as the inotropic properties of the contracting ventricle.[97]

Killip classes

Killip classes of patients were determined by cardiologists at Maharaj Nakorn Chiang Mai hospital, in order to risk stratify the patients with acute myocardial infarction. Patients were ranked by Killip class in the following criteria:[98]

1. Killip class I includes individuals with no clinical signs of heart failure.
2. Killip class II includes individuals with rales or crackles in the lungs, an S3 gallop, and elevated jugular venous pressure.
3. Killip class III describes individuals with frank acute pulmonary edema.
4. Killip class IV describes individuals in cardiogenic shock or hypotension (measured as systolic blood pressure lower than 90 mmHg), and evidence of peripheral vasoconstriction (oliguria, cyanosis or sweating).

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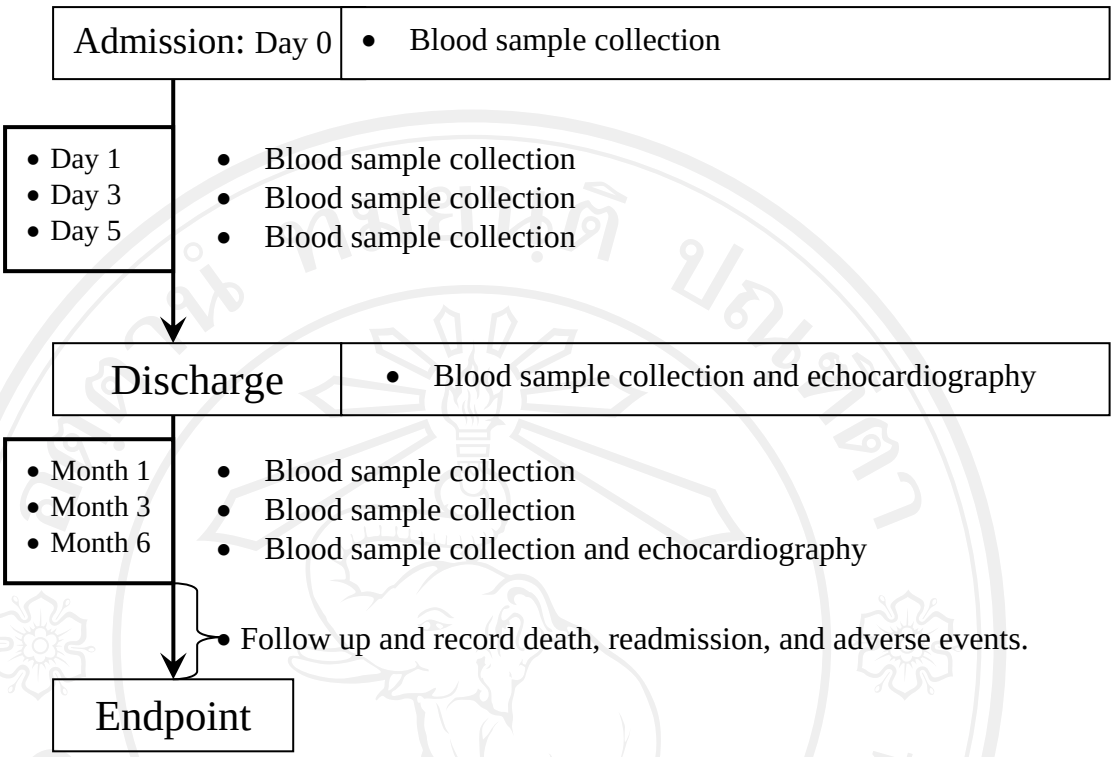

Figure 2 Protocol for the participated acute myocardial infarction patients during the study.

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Urocortins measurement

Plasma peptide was extracted by solid phase extraction (SPE) using buffer A (Code RK-BA-1) and buffer B (Code RK-BB-1) and by passing through C18-SEP column (Code RK-SEPCOL-1, Phoenix Pharmaceuticals, Inc. USA) according to the assay procedure of the commercial urocortin assay kit (Phoenix Pharmaceuticals, Inc. USA). SPE is a chromatographic technique used to extract compounds (called analytes) for subsequent analysis by removing interfering substances that may be present. The sample was dissolved in an appropriate solvent and passed through a small bed of adsorbent of very consistent particle size and shape to maximize separation efficiency. The separation ability of SPE is based on the preferential affinity of desired or undesired solutes in a liquid, mobile phase for a solid, stationary phase through which the sample passed. Impurities in the sample were washed away while the compound of interest was retained on the stationary phase. The compound that was retained on the stationary phase can be eluted from the solid phase extraction cartridge with the appropriate solvent. After the extraction, the eluant was kept in polystyrene tubes then evaporated to dryness by lyophilization (Lyolab, Lyophilization system, Inc. USA). Dried extract was kept at -20ºC until the urocortins assay was performed.

The Enzyme Immunoassay kit (Phoenix Pharmaceuticals, Inc. USA) was used to detect a specific peptide based on the competitive enzyme immunoassay principle. The immunoplate in the EIA kit was pre-coated with secondary antibody which can bind to the Fc fragment of the primary antibody (peptide antibody). Fab fragment of the secondary antibody will be competitively bound by both biotinylated peptide and targeted peptide in samples. The biotinylated peptide was able to interact with

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streptavidin horseradish peroxidase (SA-HRP), which catalyzes the tetramethylbenzidine (TMB) substrate solution composed of 3, 3', 5, 5'-TMB and hydrogen peroxide to produce a blue-colored solution.

The enzyme substrate reaction was stopped by hydrogen chloride (HCl) and the solution turns to yellow. The intensity of the yellow color is directly proportional to the amount of biotinylated peptide-SA-HRP complex, but inversely proportional to the amount of the peptide in standard solutions or samples. Microtiter plate reader (Bio Tek, Science Tech, Inc. USA) was used for reading the O.D. absorbance at a wave length of 450 nm. A standard curve of a peptide with known concentration was established accordingly by using Sigmaplot version 9 (Systat Software Inc.). The peptide with unknown concentration in samples was determined by extrapolation to this standard curve (figure 3). The concentration of peptide was determined by locating the O.D. value of individual sample on the Y-axis and drawing a horizontal line to intersect with the standard curve. A vertical line from that point would intersect to the X-axis at the point which reflected the peptide concentration in the urocortins sample.

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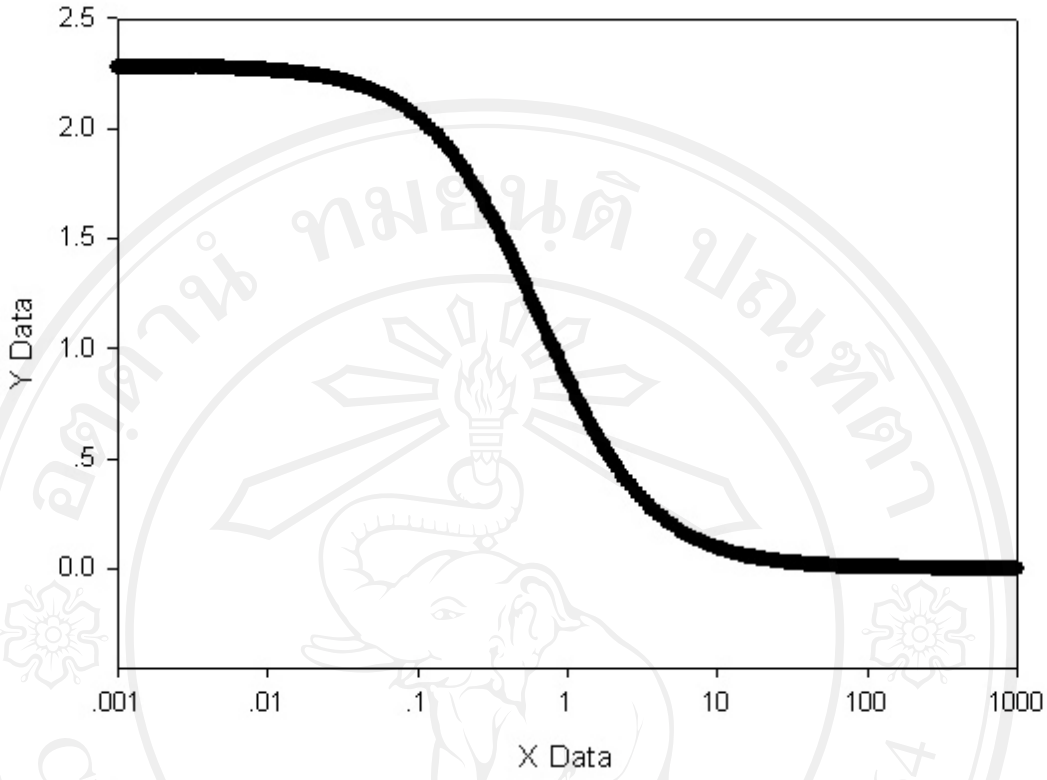

Figure 3 The standard curve for calculation the concentration of urocortins samples. Curve was constructed by plotting the known concentrations of standard peptide on the log scale (X-axis), and its corresponding O.D. reading on the linear scale (Y-axis). The standard curve shows an inverse relationship between peptide concentrations and the corresponding absorbance.

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Statistical Analysis

Statistical analysis was performed on SPSS version 11 (SPSS Inc.) and the entire data were reported in mean ± SEM. The variables in the two independent groups and Killip classes were compared using the Mann Whitney *U*-test. The relationships of factors on urocortin level were examined by bivariate correlation or Spearman's correlations, a non-parametric measure of correlation. Receiver-operating characteristic (ROC) curves were used to investigate the predictive value of urocortins. Kaplan Meier survival curves were used to visualize the relationship between urocortins and the endpoints. P value < 0.05 identified statistical significant difference.

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