CHAPTER 3

METHODOLOGY

3.1 Research Design

This was a prospective cohort study to explore the gene profiling and regulatory pathway of POCD occurrences among high-risk CABG patients. Subjects for this study were randomly selected from patients that were scheduled for coronary artery bypass graft procedures. The study was conducted at a single center specifically the National Heart Institute (Institut Jantung Negara, IJN), Malaysia. IJN is the largest referral center for cardiac cases in Malaysia. It is estimated around 4000 coronary artery bypass graft procedures are being conducted per year.

The study has been approved by Institut Jantung Negara Research Ethics Committee (IJNREC), IJNREC NO: IJNREC/441/2019. All ethical consent from the patients was obtained prior to data collection. During the recruitment process, patients were fully informed by the doctor in charge of the study purposes, risks, and benefits. On top of that, they were also being explained their responsibility, confidentiality rights and the right to withdraw at any time point. They were allowed to discuss with their family members before participation. There was no discrimination in patient management regardless of their participation in the study. All subjects were requested to sign the consent form upon voluntarily agreeing to participate in the study.

3.2 Sampling and Sample Size Calculation

The subjects were randomized based on their name in the operation theatre booking list. The booking list contains information about the patients' upcoming procedure for the next day of operation that was booked by the surgeon in charge. Inclusion and exclusion criteria were adopted in the study. The criteria were as listed below:

3.2.1 Inclusion Criteria

1. Patients aged 40 and above.
2. Undergoing high-risk CABG procedure, with any of the following criteria (as evaluated by the surgeon and anaesthesiologist in charge);
   i. Multiple valve surgery (≥ 2 valves ± CABG).
   ii. Single valve ± CABG with known moderate to severe pulmonary hypertension.
   iii. Preoperative mechanical support including balloon pump or ventricular assist device.
   iv. A procedure involving the thoracic aorta with planned hypothermic circulatory arrest.
   v. Any cardiac surgery patient having a combined operation with an Estimated GFR of 16-40 ml/min/1.72m^2.
   vi. Or any post-cardiac surgery patient who on admission to ICU is expected to need ventilator support >48 hours, receiving very high dose vasopressor (as judged by the intensivist in charge), on mechanical support other than elective intra-aortic balloon pump or expected to require new dialysis in the first 24 hours after surgery.
3. Subjects are able to speak, read and write in either Malay or English language.

UNIVERSITI SAINS ISLAM MALAYSIA ISLAMIC SCIENCE UNIVERSITY OF MALAYSIA

3.2.2 Exclusion Criteria

1. Patients with planned off-pump cardiac surgery.
2. Salvage surgery
3. Robotic surgery
4. eGFR <15 or >40 ml/min/1.72m^2
5. Dialysis dependent patients
6. Severe hepatic impairment (Child C liver disease)
7. Pregnant women

3.2.3 Sample Size Calculation

Phase 1: Cognitive Assessment and Gene Profiling

Calculation of sample size for phase 1 matched our specific objective number 1 until 3. The sample size was calculated using the mean difference between two dependent means in G Power 3.1.9.2. The value of power, effect size, confidence interval and alpha value was determined in previous project. By using a confidence interval of 95%, an alpha value of 0.05, effect size 0.5, and a power of 0.85, the calculated sample size was 38. Considering the 20% dropout in our sample collection due to high-risk patients, the total sample size for phase 1 was 46 subjects.

We performed RNA concentration and purity check for all samples. Samples with sufficient RNA quality (absorbance ratio A260/280 between 1.9 and 2.1), concentration (>25 ng) and RNA integrity number of ≥8 were further used for microarray profiling. For DEG analysis, out of 46 patients, 10 out of 35 non-POCD and 6 out of 11 POCD samples met the requirement of RNA quality check and homogeneity of patients.

Patient selection

We performed a homogenous patient selection to reduced biased when comparing between POCD and non-POCD groups. The factors that we check for homogeneity in both groups includes age group, gender, education level, type of surgery, and comorbidities. After matching the factors and performed RNA quality check, 16 samples were finalized for microarray profiling.

Phase 2: Protein Identification from Plasma

Phase 2 sample size was for objective 4. For this, we recalculated the sample needed to performed ELISA. The samples were from the same pool patients with the same inclusion and exclusion criteria as mentioned before. Sample size was calculated using methods from Krejcies (Krejcie & Morgan, 1970). The formula is as follows;

$$s = \frac{\chi^2 NP(1-P)}{d^2(N-1) + \chi^2 P(1-P)}$$

Where;
s = required sample size
$\chi^2 = 3.841$ for 0.95 confidence level
N = given population size
P = the population proportion (prevalence)
d = the degree of accuracy

(3.1)

The confidence level was set at 95%, therefore χ value was 1.96. Prevalence was determined in our phase 1 analysis, resulting in prevalence of 23.9% (0.23) of POCD in high-risk patient population. For ELISA analysis, only 96 samples can be analyses in one plate. Hence, the given population size is 96. The degree of accuracy was expressed as a proportion of 0.05. Hence, the number of samples required was 72 patients. Considering 20% dropout in our sample, the total sample size for phase 2 is 88 patients.

UNIVERSITI ISLAM ANTARABANGSA MALAYSIA / INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

3.3 Recruitment Process and Analyses

The process of patient recruitment and in-hospital data collection was described in Figures 3.1 and 3.2. There were two distinct processes involved in this study specifically; subjects' cognitive evaluation and gene expression analyses. Based on the cognitive evaluation, subjects were assigned to the POCD and non-POCD groups. Subsequently, 10 ml of blood were withdrawn for RNA extraction purposes. The samples were randomly selected for microarray analysis according to two main classifications – 1) POCD and non-POCD groups, 2) Usage and non-usage of cerebral oximetry. For cerebral oximetry, the usage on patients depends on clinician's judgment based on patient conditions in the operating theatre. Hence, patients were divided into two groups, either use or do not use cerebral oximetry.

Microarray analysis was performed to identify the differentially expressed genes (DEGs) and pathway analysis. Based on the DEGs and pathways analysis, the protein that was identified to be involved in the pathway was selected. Further, enzyme-linked immunosorbent assay (ELISA) analysis was done to identify the difference in the protein levels between the POCD and non-POCD subjects.

Lastly, statistical analysis was done to analyze our results statistically. The flow of the methodology mentioned above was illustrated in Figure 3.1.

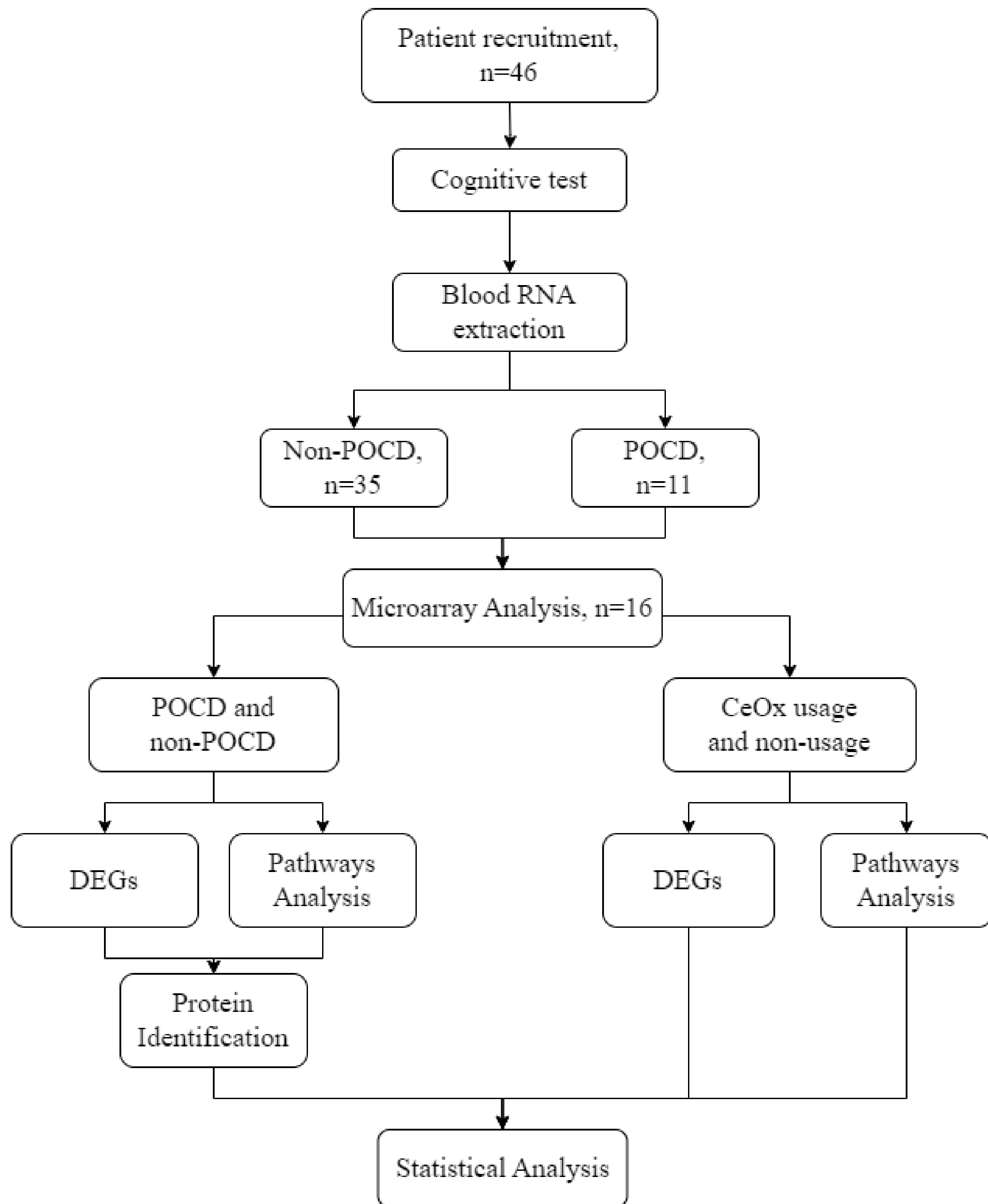

Figure 3.1: Workflow of the study. Starting with patient recruitment, cognitive test and RNA extraction were performed on all patients. The outcome classified into POCD and non-POCD groups. Only 16 samples proceed to microarray analysis, where profiling were made based on comparisons between POCD and non-POCD groups, as well as cerebral oximetry usage and non-usage. Protein identification was done in POCD group, before the last statistical analysis.

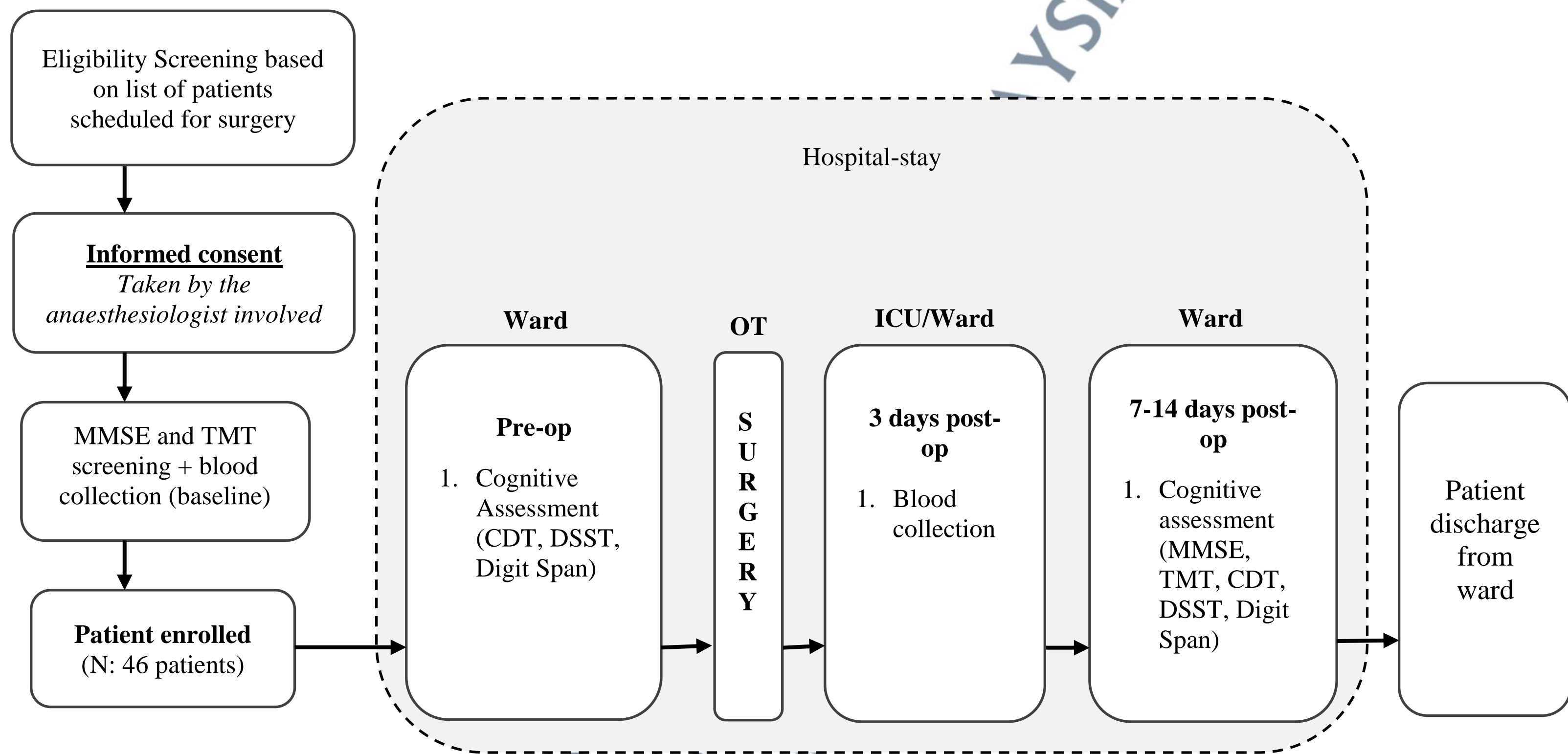

Figure 3.2: The flow of patient recruitment. Eligibility screening was done using the operation theatre booking list one day before surgery. If the patient is eligible, informed consent was taken by the anaesthesiologist in charge. Mini-Mental State Examination (MMSE), Trail Making Test (TMT) test, and blood collection were done if the patients agreed to participate in the study. Additional preoperative cognitive assessment was done which includes Clock Drawing Test (CDT), Digit Symbol Substitution Test (DSST), and Digit Span Test. Surgery was done on the next day, and blood collection for RNA extraction was collected 3 days postoperatively. Postoperative cognitive assessment was done within 7-14 days after surgery or upon discharge, whichever comes first.

3.4 Research Instruments

Postoperative cognitive dysfunction is assessed via a series of cognitive function assessments. The assessment was conducted at two time points (preoperative = 1 day before CABG procedure; postoperative = around 7-14 days after surgery in ward). The assessment includes five tests that cover different domains of cognitive functions. The average time taken to complete the cognitive assessment was approximately 10-15 minutes. The instruments used in each component were explained in detail as follows.

3.4.1 Mini-Mental State Examination (MMSE)

MMSE is a common tool used to assess cognitive impairment. The test covers a range of cognitive domains including orientation, language, concentration, working memory, memory recall, and visuospatial skills. The Malay version of MMSE has been validated previously with Cronbach alpha 0.76 (Zarina et al., 2007). There are 11 task items with a total score of 30. For this study, MMSE was used as the screening tool to detect cognitive impairment. According to Gluhm et al. (2013), a total score of <24 is considered as having mild cognitive impairment. Hence, the cut-off point for the eligibility screening in the study was a score of more than 24.

Classification of POCD is made based on the 1 SD method, taking into account the MMSE preoperative and postoperative scores. Based on this method, the patient who has a decline of 1 standard deviation of MMSE scores postoperatively when compared to his/her preoperative score is defined to have POCD. The calculation of 1 SD was based on published or sample norms (Greaves et al., 2019). In this study, the calculated SD is based on our population sample norms.

To see the difference between preoperative and postoperative scores clearly, percentage differences were calculated. The formula for the calculation of percentage differences is:

$$\text{Percentage differences} = \frac{(A-B)}{B} X\ 100$$

Where;
A = postoperative scores
B = preoperative scores

(3.2)

3.4.2 Trail-Making Test Part A and Part B

Another test being used for eligibility screening was Trail-Making Test (TMT) Part A and Trail-Making Test Part B. TMT has been validated as a test that can measure visuoperceptual abilities in Part A. Part B represents secondary task switching ability and working memory. Both tests become the indicator of executive control abilities (Sánchez-Cubillo et al., 2009). Patients were required to make a trail to connect the numbers (part A) and combination numbers and alphabets (part B). The time taken for the patient to complete each part was recorded. The rule of thumb for the time taken in part A was 90 seconds, while in part B was 3 minutes. If the patients fail, they cannot proceed with other cognitive tests.

3.4.3 Digit Span

The Digit Span test is a test to assess attention and working memory (de Paula et al., 2016). Digit span includes two parts, Digit Span Forward and Digit Span Backward. In the digit span forward, the patient listened to a series of numbers before repeating them in the correct

sequences. For example, if the numbers were 1-2-3, the patient must repeat 1-2-3. On the other hand, digit span backward requires the patient to repeat the numbers in a backward manner. For instance, the researcher said the numbers 3-4-5, and the patient must repeat it as 5-4-3. Each correct trial will give one score, and the test ends when the patients fail to give the correct answer in two consecutive trials.

3.4.4 Digit Symbol Substitution Test

Digit Symbol Substitution Test (DSST) is a tool in clinical neuropsychology that is valid and sensitive to assess motor speed, attention, and manual dexterity (Jaeger, 2018). It is a pen and paper test where there are nine digits, each with respective symbols. Patients were required to draw the symbols according to the digits given as much as they can. The time given for patients to draw the symbols was 120 seconds. The number of correct matching symbol to the digits were scored.

3.4.5 Clock Drawing Test

Clock Drawing Test is commonly used in detecting dementia where it assesses visuo-constructive abilities. It has adequate sensitivity and specificity to identify mild cognitive impairment (Duro et al., 2018). Patients were given a piece of blank paper with a pen. They were required to draw a clock, set with numbers, and the hands at 10 after 11. Interpretation of the clock drawing test was based on the correct order of numbers in the clock, the spacing between the numbers was correct and the hands shows the correct time.

3.5 Gene Expression Analysis

3.5.1 Blood Collection

Blood samples were collected from all subjects at two-time points, preoperatively and on day-3 postoperatively. The blood sample was collected by a nurse or trained phlebotomist. A total of 10 ml of blood were collected in anticoagulant tubes. The blood is then processed to extract the RNA on the same day.

3.5.2 RNA Extraction

The RNA extraction protocol was done according to the manufacturer's kit [GeneJET™ Whole Blood RNA Purification Mini Kit] (Thermo Scientific, USA). The protocol for extraction started with centrifuging 50-500 µL of blood for 5 minutes, 400 x g at 4°C, then the supernatant was discarded. The pellet was resuspended in 600 µL of Lysis Buffer, using vortex to mix well. Then, 450 µL of ethanol (96-100%) was added and mixed by pipetting or vortexing.

Half of the prepared lysate was transferred to a column inserted in a collection tube. The column was centrifuged for 1 min at 12000 x g. The flow-through solution was discarded, then the column and collection tube were reassembled. The remaining lysate was transferred into the column and centrifuged as before. The collection tube containing the flow-through solution was discarded. The column was placed into a new 2 mL collection tube. Next, 700 µL of Wash Buffer 1 was added, then centrifuged for 1 min at 12000 x g. The flow-through was discarded and the purification column was placed back into the collection tube. Then, 500 µL of Wash Buffer 2 was added to the purification column, then centrifuged for 1 min at 12000 x g.

After that, 500 μL of Wash Buffer 2 was added to the purification column, then centrifuged for 2 min at 12000 x g. The collection tube was emptied, then placed the purification column back into the tube and the column was re-spin for 1 min. at maximum speed (>20000 x g). The collection tube containing the flow-through solution was discarded and the purification column was transferred to an RNase-free 1.5 mL microcentrifuge tube. Finally, 50uL of nuclease-free water was added to the center of the purification column membrane and centrifuged for 1 min at 12000 x g (Figure 3.3a). The purification column was discarded. The purified RNA was immediately used for an RNA integrity check (Figure 3.3b). The RNA was kept on ice after extraction and while working on it.

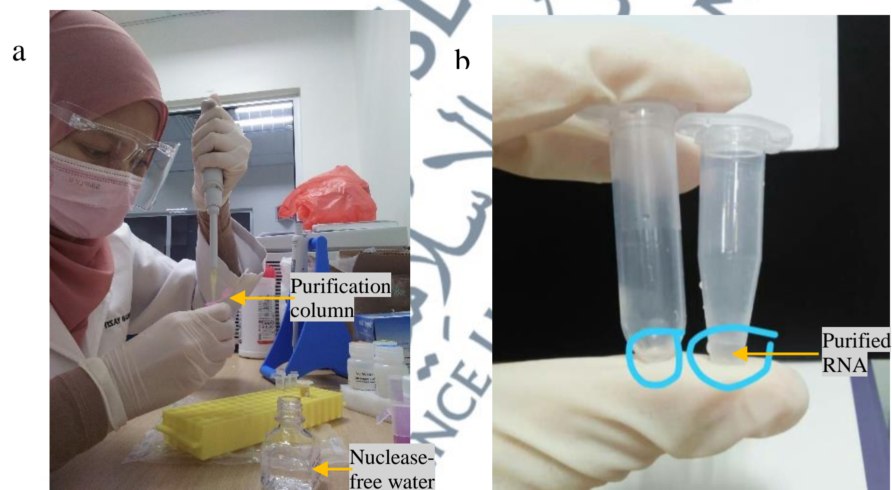

Figure 3.3: a) Adding nuclease-free water into the purification column membrane.
b) The purified RNA in the Eppendorf tube is ready for RNA integrity check.

3.5.3 RNA Integrity Check

The assessment of RNA quality control was done using RNA Integrity Number (RIN). It is a standardized quality control check for RNA integrity by Agilent 2100 Bioanalyzer System. The RIN software algorithm used a numbering system from 1 to 10, where 1 is the most degraded and 10 is the most intact RNA (Mueller et al., 2016). The extracted RNA was stored at -70°C until further analysis. Apart from the RIN number, sample concentration, and purity were also checked. Only samples with a concentration >25ng and purity ~1.8 were accepted and further analyzed.

3.5.4 Microarray Analysis

3.5.4.1 Sample Selection and Group Comparison

To perform microarray analysis, we selected the samples based on group comparison of interest. There are 16 samples that were finalized for the analysis, and each sample belongs to groups which are, - 1) POCD, 2) Non-POCD, 3) CeOx Usage, and 4) Non-CeOx Usage. Out of these four groups, each sample will have a pre- and postoperative sample. Hence, these are the four comparison groups we analyzed;

1) POCD patients (Postoperative vs Preoperative samples)

2) Postoperative samples (POCD vs Non-POCD patients' samples).

3) Postoperative samples (Patients use CeOx during surgery vs Non-usage of CeOx).

4) Non-POCD patients (Postoperative vs Preoperative samples).

3.5.4.2 Microarray Workflow

Sample preparation and microarray hybridization were performed based on the protocol using G3 Human Gene Expression 8x60k slide v3 microarray (Agilent, USA). The whole procedure takes around 3 days. The workflow was as follows:

1) Sample Preparation

   Spike-In solution was done before other procedures using RNA Spike-In Kit, One-Color. Spike-In was used to bind with an RNA molecule with a matching sequence, also known as a control probe. Prior to the preparation, all components in the kits were thawed and briefly spun. The spike mix stock was diluted according to the starting amount of RNA (25ng). Firstly, four (4) 1.5mL microcentrifuge tubes were labelled as 'Spike Mix First Dilution', 'Spike Mix Second Dilution', 'Spike Mix Third Dilution', and 'Spike Mix Fourth Dilution' respectively.

   Into the 1st Dilution tube, 2 µL of Spike Mix stock was added to 38 µL of Dilution Buffer (1:20 dilution factor). The tube was then mixed well and briefly spun. For the 2nd Dilution tube, 2 µL of 1st Dilution was added together with 48 µL of Dilution Buffer (1:25 dilution factor). The tube was then mixed well and briefly spun. For the 3rd Dilution tube, 2 µL of 2nd Dilution was added together with 38 µL Dilution Buffer (1:20 dilution factor). The tube was then mixed well and briefly spun. For the 4th Dilution tube, 10 µL of the 3rd Dilution was added together with 30 µL of Dilution Buffer (1:4 dilution factor). The tube was then mixed well and briefly spun. Finally, 2 µL of the 4th Dilution was added to 25 ng of RNA sample and proceeded with cyanine 3 labeling (Figure 3.4).

Figure 3.4: Preparation for labeling reaction. All Spike Mix Dilution has been prepared and is ready to be put into samples.

2) Labelling

The labeling protocol started with the addition of 10 to 200 ng total RNA samples to a microcentrifuge tube (final volume: 1.5 µL), followed by adding 2 µL of diluted Spike Mix (total volume: 3.5 µL). T7 Primer Mix (0.8 µL of T7 Primer mixed with 1 µL of nuclease-free water) was added to the microcentrifuge tube for incubation in 65ºC water bath for 10 minutes, followed by incubation on ice for 5 minutes (total volume of 5.3 µL). Next, cDNA Master Mix was prepared according to the guideline and added to a 1.5 mL microcentrifuge tube then briefly spun. To each sample tube, 4.7 µL of cDNA Master Mix was added and mixed by pipetting. The samples were incubated in a 40ºC circulating water bath for 2 hours, then in 70ºC circulating water bath for 15 minutes (Figure 3.5).

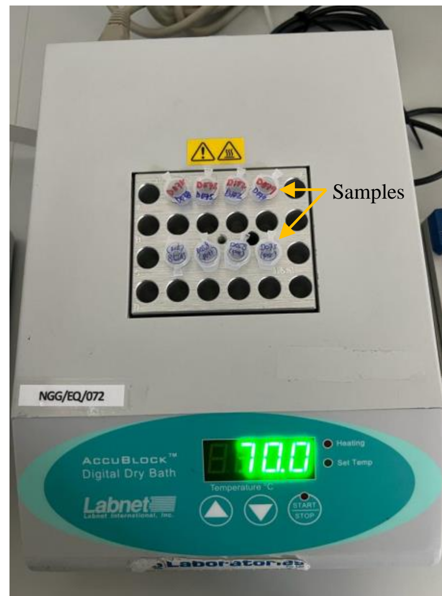

Figure 3.5: Incubation during labelling reaction in 70°C water bath.

The samples were moved on ice to be incubated for 5 minutes, then briefly spun. Finally, Transcription Master Mix was prepared and 6 µL was added to each sample tube. The tubes were mixed by pipetting and incubated in a circulating water bath at 40°C for 2 hours (Figure 3.6).

Figure 3.6: Incubation during labelling reaction in 40°C water bath.

The labeled RNA was then purified by adding 85 µL of nuclease-free water to the samples. 350 µL of Buffer RLT was added and mixed by pipetting, followed by 250 µL ethanol and mixed by pipetting. The sample was transferred to RNeasy Mini Spin Column in a 2 mL Collection Tube. The tube was spun at 4ºC for 30 seconds at 13000 rpm then discard the flow-through solution and collection tube. The RNeasy column was transferred to a new collection tube and 500 µL of Buffer RPE was added. The tube was spun at 4ºC for 30 seconds at 13000 rpm then discard the flow-through solution and reuse the collection tube. Another 500 µL of Buffer RPE was added, spun at 4ºC for 30 seconds at 13000 rpm then discard the flow-through solution and collection tube. The RNeasy column was transferred to a new collection tube and 30 µL of RNase-free water was added directly onto the filter membrane. After 60 seconds, the tube was spun at 4ºC for 30 seconds at 13000 rpm. The sample was put on ice but discard the RNeasy column. The cRNA was quantify using NanoDrop ND-1000 UV-VIS Spectrophotometer (Figure 3.7).

Figure 3.7: cRNA quality check using NanoDrop UV-VIS Spectrophotometer.

3) Hybridization

Firstly, a 10x blocking agent was prepared by adding 1250 µL of nuclease-free water to the vial containing a lyophilized large volume of 10x Gene Expression Blocking Agent. The vial was then gently mixed on a vortex, then spun in a centrifuge for 5 to 10 seconds. On the other hand, the Fragmentation mix was prepared according to 8-pack microarray formats, then incubate at 60°C for exactly 30 minutes. It was cool on ice for one minute immediately, and 2x Hi-RPM Hybridization Buffer was added. Mix well by pipetting then spun for 1 minute at room temperature at 13000 rpm. The sample was then put on ice and loaded onto the array immediately (Figure 3.8).

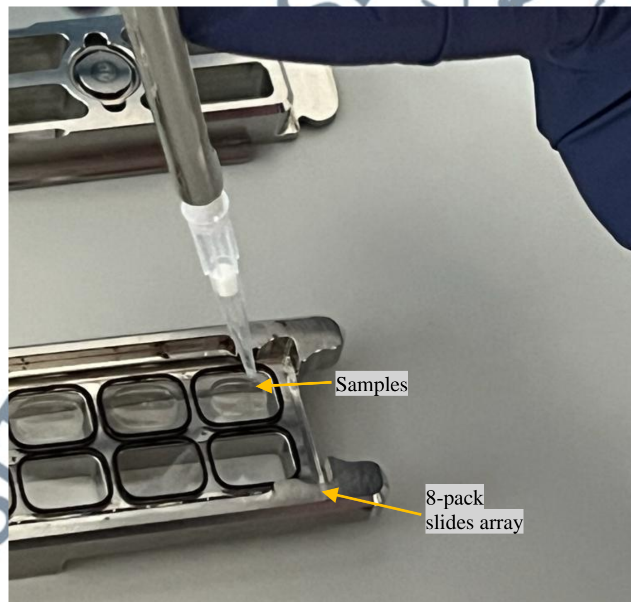

Figure 3.8: Sample loading into slides array.

A clean gasket slide was loaded into the Agilent SureHyb chamber base, with the label facing up. The volume of hybridization sample was slowly dispensed (to avoid air bubbles) onto the gasket well according to the volume for the 8-pack format. The 'active side' of the slide was put down, parallel to SureHyb gasket slide by gripping the end of the slide, making sure the sandwich-pair is properly aligned (Figure 3.9). The cover was put on the chamber and clamped the slide assembly firmly. The assembled chamber was rotated vertically to wet the gasket and assess the mobility of bubbles. Then, the assembled chamber was loaded into the oven rotator rack to hybridize at 65°C for 17 hours.

Figure 3.9: Assembling the chamber to the slide containing the sample.

UNIVERSITI ISLAM MALAYSIA

4) Washing

This step began with prewarm the Gene Expression Wash Buffer 2 by dispensing 1000 mL directly into a sterile storage bottle, tightly capped, and put in a 37°C water bath a night before washing arrays. Next, solvent wash and Milli-Q water wash were prepared according to the guidelines. Then, a slide-staining dish was filled with Wash Buffer 1. One hybridization chamber was removed from the incubator, disassembled, and submerged the array-gasket sandwich was into the slide-staining dish prepared previously (Figure 3.10).

Figure 3.10: Disassembling the slide from the chamber for washing.

UNIVERSITI ISLAM MALAYSIA

The sandwich was pried open from the barcode end when it is completely submerged in the buffer. After opening, all slides were placed into a slide rack in another slide-staining dish and stirred using a magnetic stir plate with setting number 4 for 1 minute. Meanwhile, Gene Expression Wash Buffer 2 was taken out from the water bath and poured into another slide-staining dish. The slide rack was transferred to a slide-staining dish with Wash Buffer 2 and stirred at moderate speed for 1 minute (Figure 3.11). Once done, the slide rack was removed slowly, and discard the used wash buffer. The slides were then placed carefully into a slide holder for scanning immediately (Figure 3.12).

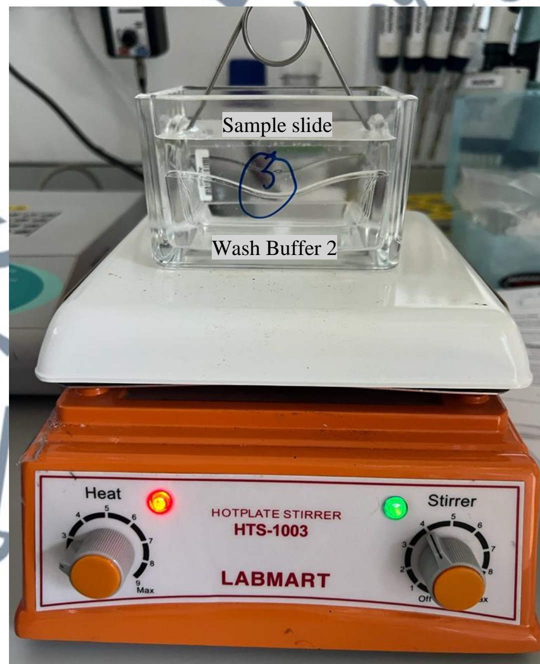

Figure 3.11: Washing step using Wash Buffer 2.

Figure 3.12: Placing the slide into the slide holder for scanning.

5) Scanning and Feature Extraction

Agilent SureScan Microarray Scanner was used for this study. The process began by putting the assembled slide holders into the scanner cassette, then the appropriate scanner protocol was selected (AgilentG3_GX_1color). Click Start Scan when the scanner status showed Scanner Ready. After the scanner generate the microarray scan images, the images were extracted using Feature Extraction Software. The software generated the quality control report. From the report, further analysis of the differentially expressed genes was done using Agilent Genespring GX Bioinformatics Software.

3.5.5 Validation of Targeted Genes

3.5.5.1 cDNA Synthesis for mRNA

cDNA synthesis was conducted using reagents from Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Scientific, USA). The protocol began with thawing, mixing, and briefly centrifuging all components in the kit, followed by adding those reagents in order into an RNase-free tube. The reagents include 1 µL of 10X dsDNase Buffer, 1 µL of dsDNase, 0.1 pg – 500 ng poly(A) mRNA, and lastly put nuclease-free water up to 10 µL. After gently mixing and centrifuging, the mixture was incubated for 2 min at 37°C in a water bath, then chilled on ice. Next, other components were added to the tube which were 4 µL of 5X Reaction Mix, 2 µL of Maxima Enzyme Mix, and 4 µL of nuclease-free water. After mixing gently and centrifuging, the tube was incubated for 10 min at 25°C, then 15 min at 50°C. The reaction was terminated by heating at 85°C for 5 minutes. The cDNA synthesis was now ready to use in qPCR.

3.5.5.2 Primer Synthesis

Primer design is an important step in PCR. The primer sequences used in this study were designed using Primer 3, in which the sequences were based on coding region referred from GenBank® data. Primer design was determined to have annealing temperature around 50-60°C and 100 to 200 base pair in length. The housekeeping gene used in this study is beta-actin.

Prior to qPCR, optimization tests were conducted for all genes. This includes primer performance, primer efficiency test and primer specificity test. For primer efficiency test, the slope of the standard curve indicates the PCR efficiency. The PCR efficiency should be

between 80-110%, or slope value between -3.6 ≥ slope ≥ -3.3. The efficiency can be calculated using a formula after the standard curve has been generated from the PCR software program (Thermo Scientific, USA). The standard curve for efficiency test of each genes were shown in Appendix. The calculation of efficiency is as follows;

$$\text{Efficiency} = 10^{(-1/\text{slope})} - 1 \quad (3.3)$$

The details of the primer sequences and efficiency test of housekeeping and target genes were tabulated in Table 3.1.

UNIVERSITI SAINS ISLAM MALAYSIA
ISLAMIC SCIENCE UNIVERSITY OF MALAYSIA

Table 3.1: Primer sequences for target genes.

No	Gene	Primer		Efficiency test	
		Forward	Reverse	Slope value	Efficiency (%)
1	Beta-actin (housekeeping gene)	TCCACCGCAAA TGCTTCT	AGCCATGCCAA TCTCATCTT	-3.3901	97.2
2	BTNL3	GCGTGTCTCTCT AAGGCTAAA	GTGGCCTCCTC ATCGTAAAT	-3.4079	94.9
3	KIR2DS3	CAGGAGGTGTC ATACGCATAAT	TACATGCTGCT ATCTGTTGGG	-3.5295	90.5
4	KIR3DL2	GTGAATAGGCA GGACTCTGATG	CTGAGAAGGGC GACTGATTT	-2.3537	169
5	ERFE	GGCCCAGCTTC TAGTTCTAATG	GCCTTGGCTGT GAAGATTTG	-3.8038	81.9
6	MORN4	CCAGAACTGGA CCACAGAAA	GCACATAAGGG TTAGGGAAAGA	-3.8974	81.9
7	CCL23	AGAATGCTGAA GCTGGACAC	GGAAGGTAGTT GAGGCAAGAAA	-3.4419	94.9
8	GPR37L1	AGTCAGACCCC TCAACATCC	ACCATCTCTCCC CCTTCATCTC	n/a	n/a
9	ADAMTS2	CACGATGAATA CCACGATCAC	CACAAACGCTG AGGAGAAG	-4.7903	58.4
10	DCANP1	GCATAGCAGCA GAAGGAAGA	GTGAACTGTGA ACGGGTAGAG	-3.7748	81.9

n/a, not available.

UNIVERSITI SAINS ISLAM MALAYSIA
ISLAMIC SCIENCE UNIVERSITY OF MALAYSIA

3.5.5.3 Master Mix Preparation and PCR Protocol for Target-Gene Quantification

Mastermix preparation was done by using Brilliant 3 SYBR Green (Agilent, USA). The reference dye used was SYBR Green 1 dye which is light sensitive, hence the preparation of the master mix was done away from light. Prior to mixing the reagent, the reference dye was diluted with nuclease-free PCR-grade H_2O with a ratio of 1:500. All other reagents were mixed in order into a single reagent mixture, until a final volume of 20 µL (including our sample cDNA). The reagent mixture was listed in Table 3.2. The mixture was then gently mixed without creating bubbles and distributed into individual PCR reaction tubes. The reactions were briefly centrifuged and placed in the instrument. The Agilent AriaMx platform was used, hence, the program was set according to the guidelines in Table 3.3. The report generated was then analyzed to validate our targeted genes.

Table 3.2: Reagent mixture for target-gene PCR.

Component	Volume for one tube
Nuclease-free PCR-grade H_2O	6.7 µL
2X SYBR Green QPCR master mix	10 µL
cDNA	1 µL
Forward primer	1 µL
Reverse primer	1 µL
Diluted reference dye (optional)	0.3 µL

Table 3.3: qPCR cycling program for target-gene quantification.

Cycles	Duration of cycles	Temperature
1	3 minutes	95°C
40	5 second	95°C
	5-10 seconds	60°C

3.5.5.4 Calculation of Relative Fold Gene Expression

Report generated by qPCR shows the cycle threshold (CT) value of each sample. The CT is the cycle number of fluorescence that is generated from the PCR, that is discrete from the background noise. From the CT value, we calculated the relative fold gene expression of the samples using delta-delta CT (ΔΔCt) method. The formula for this method is 2^-(ΔΔCt). The steps for calculating the expressions are as follows.

Firstly, the CT value of each samples were averaged from their technical triplicate. This yield in one CT value for each sample only. Then, we calculated the delta CT for each sample. The ΔCt is calculated by subtracting the CT value of gene of interest (GOI) to CT value of housekeeping gene (HG);

$$\Delta Ct = Ct\ (GOI) - CT\ (HK) \quad (3.4)$$

After obtaining ΔCt value for each sample, we averaged the CT value according to the sample grouping. For example, there are 3 samples in POCD and non-POCD group, hence there will be one CT value for each group. In each comparison, one group will be the reference or the control group. We then calculated the delta-delta Ct value (ΔΔCt) by subtracting the ΔCt value of each sample to the average ΔCt value of the control group.

$$\Delta\Delta Ct = \Delta Ct\ (sample) - \Delta Ct\ (control\ average) \quad (3.5)$$

Finally, we calculated the relative fold gene expression using the formula $2^{-(\Delta\Delta Ct)}$. The final value will be log transform prior to statistical analysis.

3.6 Identification of Protein in POCD Patients

From the gene expression analysis, 6 genes were differentially expressed in POCD groups (refer chapter 4, comparison 1 and 2). From the 6 genes, we analyze their function and association with POCD condition. One gene showed promising function and possible association with POCD, which is erythroferrone (ERFE). Hence, ERFE (also known as FAM132B) protein was selected for protein level assessment using enzyme-linked immunosorbent assay (ELISA). We used FineTest Human FAM132B (Protein FAM132B) ELISA Kit (FineTest, China). The protocol of the assay was described below.

3.6.1 Reagent Preparation

All reagents were brought to room temperature 20 minutes prior of usage. For wash buffer, 30 ml of Concentrated Wash Buffer was added to 750 ml of Wash Buffer with deionized or distilled water to dilute it. Unused solution was stored back to 2-8°C. For preparation of standards solution, 1 ml of Sample Dilution Buffer was added into one standard tube (labelled as zero tube), leave for 10 minutes at room temperature and mixed thoroughly. Next, 7 Eppendorf tube (labelled 1st tube to 6th tube, and 1 blank) were prepared by adding 0.3ml of Sample dilution buffer to each tube. From the zero tube, 0.3 ml were taken out and put into 1st tube and mix thoroughly. Then, 0.3 ml of 1st tube was transferred to 2nd tube and mix thoroughly, and so on until 6th tube. The 7th tube were left as blank.

Next, Biotin-labeled antibody working solution was prepared within 1 hour before conducting assay. 110 ml of solution were required for 198 wells, and diluted with 990 ml Antibody Dilution Buffer at ratio of 1:100. The solution was mixed thoroughly. HRP-Streptavidin Conjugate (SABC) Working Solution was prepared 30 minutes before

conducting assay. 110 ml of SABC solution were required for 198 wells, and diluted with 990 ml SABC Dilution Buffer at ratio of 1:100. The solution was mixed thoroughly.

3.6.2 Assay Procedure

All standards, test samples, and control (blank) were placed into the wells and their position was recorded. For standards, 100 µl of standards from each tube were transferred into standard wells. For test samples, 100 µl of samples that have been diluted at least ½ with Sample Dilution Buffer were added into the test sample wells. The plate was then sealed with a cover and incubated for 90 minutes at 37ºC. Then, the cover was removed and the content of the plate was discarded. The plate was washed 2 times with Wash Buffer without letting the wells completely dry at one time. Next, 100 µl Biotin-labeled antibody working solution was added directly to the bottom of the wells without touching the sidewall. The plate was then covered and incubated for 60 minutes at 37ºC.

After incubation, the plate was washed for 3 times with Wash buffer, while letting the buffer stay in the wells for 1-2 minutes. Next, 100 µl SABC Working solution was added into each well, covered, and incubated at 37ºC for 30 minutes. After incubation, the plate was washed 5 times with Wash buffer, while letting the buffer to stay in the wells for 1-2 minutes. Then, 90 µl TMB Substrate was added to each well, the plate was covered and incubate in dark at 37ºC within 10-20 minutes. The reaction was terminated when apparent gradient appeared in standard wells. To stop the reaction, 50 µl of Stop Solution was added to each well according to the order of adding TMB Substrate. The color turned to yellow immediately (Figure 3.13). The plate was then immediately read the absorbance in Microplate Reader at 450nm.

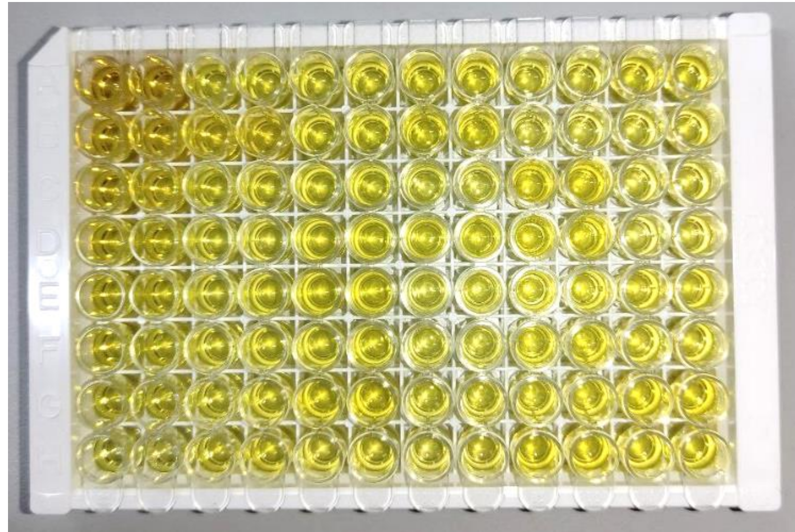

Figure 3.13: ELISA plate turned yellow after adding the stop solution.

3.7 Bioinformatic Analysis

For bioinformatics analysis, GeneSpring GX Bioinformatics Software was used to investigate the differentially expressed RNAs and the target genes. DAVID (Database for Annotation, Visualization and Integrated Discovery) is an online database that allow researchers to study high throughput gene function. It is a centralized location that provides biological information from various data annotations. It consists of DAVID Knowledgebase, DAVID Gene Functional Classification Tool and DAVID Functional Annotation Tool. For this study, we used DAVID Functional Annotation Tool. Gene Ontology was performed to check the genes biological process, molecular functions and cellular components. For pathway analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was used to generate the pathway involved by the target genes, which allow us to predict the role of dysregulated RNAs and their network interaction. The flowchart of bioinformatic analysis was illustrated in Figure 3.14.

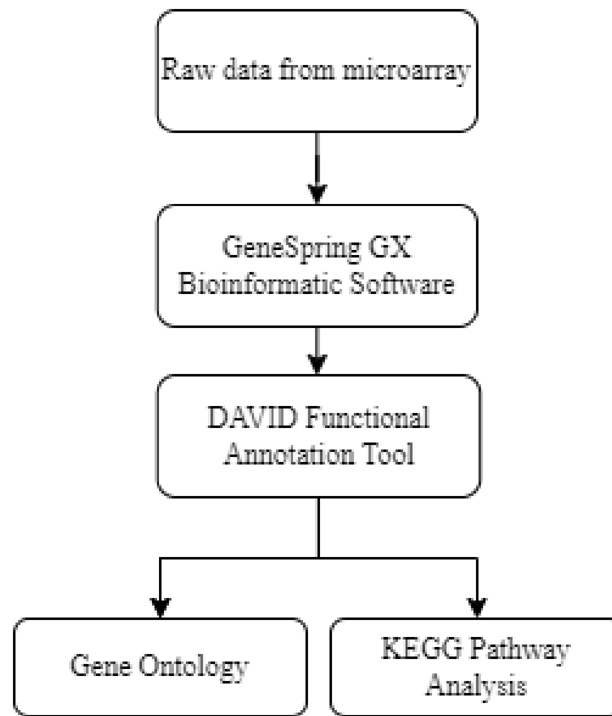

Figure 3.14: Flowchart for bioinformatic analysis.

UNIVERSITI SAINS ISLAM MALAYSIA
ISLAMIC SCIENCE UNIVERSITY OF MALAYSIA

3.8 Statistical Analysis

Statistical analysis for this study was done using SPSS software version 24. Descriptive analysis was done for demographic data, scores of cognitive assessment and gene expression level. Data were presented as mean values ± standard deviation. Test of normality was done using Shapiro-Wilk analysis. Analysis of T-test was used to see the differences between demographic data and cognitive assessments for POCD and non-POCD group. Significant DEGs were analyzed using with p-value set at $p<0.05$. For validation of differentially expressed target genes, qPCR was done and paired t-test was done to analyze its significance at $p<0.05$.

3.9 Data Handling and Storage

3.9.1 Confidentiality

All information and data collected were kept confidential to the researchers. Any information about the participants was noted as numbers instead of names. Participants' study data were analyzed as general, not as individual. Data were shared as reports or publications. Individual data is only to be disclosed upon request from the participants. Relevant permissions were obtained prior to publication.

3.9.2 Storage of Data

The data collected in this research were kept under lock and key, at the study site, which is Institut Jantung Negara. The hardcopy data was kept in steel cabinets with locks. The electronic data was kept in a password-protected computer, in a secured database system. All data were only accessible to the main investigators. The data were stored for 5 years after the end of the research study and then destroyed.