CHAPTER 4

RESULTS

This chapter describes the results of the study. The results are divided into the following subsections: participants' demography, cognitive function assessments, differentially expressed genes (DEGs), pathway analysis and immunoassay analysis.

4.1 Participants' Demographic

Forty-six patients that have been recruited and completed the study. We recorded their basic demographic information such as age, gender, race, education level, income, body mass index, etc. The mean age of our patients is 57.9 ± 12.76 years old. For gender, 63% of our patients are male, while Malay races have the highest percentage, with 80.4%. The mean education level of our patients in years is 11.15 ± 3.27. They were also classified into no formal education, primary, secondary and tertiary. The mean body mass index (BMI) of our patients is 26.8 ± 4.16 kg/m^2, which is in the overweight group. For household income, the majority of patients come from low-income groups (71.7%). As for working status, 43.5% of patients were pensioners, 30.4% were currently working, and the rest were unemployed. The results are tabulated in Table 4.1 below.

Table 4.1: Patient’s demography (n=46).

Variables	Frequency, n / Mean	Percentage, % / SD
Age*[1]	57.9	12.76
<40 years old	5	10.9
40-65 years old	29	63.0
>65 years old	12	26.1
Gender		
Male	29	63
Female	17	37
Race		
Malay	37	80.4
Chinese	4	8.7
Indian	5	10.9
Education (years)	11.15	3.27
No formal education	1	2.2
Primary	4	8.7
Secondary	26	56.5
Tertiary	15	32.6
Body Mass Index (kg/m^2)*[1]	26.8	4.16
Underweight (<18.5)	1	2.2
Normal (18.5-24.9)	14	30.4
Overweight (25.0-29.9)	19	41.3
Obese (≥30.0)	12	26.1
Household Income (MYR)*[2]	4731.82	5471.69
Low (<4850)	33	71.7
Middle (4850-10,959)	10	21.7
High (≥10,960)	3	6.5
Marital Status		
Single	2	4.3
Married	42	91.3
Divorced/widowed	2	4.3
Working Status		
Work with employer	8	17.4
Self-employed	6	13.0
Pensioner	20	43.5
Unemployed	12	26.1

*Note: [1]Based on WHO classification; [2]Based on the Department of Statistic Malaysia official portal classification.

4.2 Cognitive Function Assessment

Five instruments were used for cognitive function assessment. The instruments were Mini-Mental State Examination (MMSE), Trail Making Test (TMT) part A and part B, Digit Span, Digit Symbol Substitution Test (DSST), and Clock Drawing Test (CDT). Overall, mean postoperative scores were significantly lower than preoperative ($p<0.05$), except for TMT. TMT has a higher mean in postoperative due to the time taken needed to complete the test. Higher time taken means slower execution in completing the task. The mean scores for each test at preoperative and postoperative are listed in Table 4.2.

Table 4.2: Preoperative and postoperative scores of cognitive tests.

Test	Preoperative scores (mean ± SD)	Postoperative scores (mean ± SD)	*p*-value
MMSE	27.85 ± 2.41	25.96 ± 5.22	0.001*
TMT part A (min)	50.77 ± 20.84	52.98 ± 27.06	0.377
TMT part B (min)	111.07 ± 49.38	128.09 ± 56.89	0.007*
Digit Span	13.14 ± 3.43	11.91 ± 3.13	0.005*
DSST	49.39 ± 18.02	43.89 ± 19.45	<0.001*
Clock Drawing	1.89 ± 0.379	1.72 ± 0.66	0.058

**p*-value significant at <0.05 using paired sample t-test

4.2.1 POCD Prevalence

POCD was calculated using the 1 SD method. We calculated our sample mean and standard deviation. Our sample 1 SD is equal to 2.5 scores. Hence, to classify patients with POCD, patients who have a reduction of scores of more than 2.5 in postoperative tests are considered as POCD. The results show that 11 out of 46 patients have a more than 1 SD reduction. This means 23.9% of our patients develop early POCD. We classify POCD and non-POCD patients from the results to compare their postoperative scores in other instruments. Based on the comparison, POCD patients' scores were significantly lower than non-POCD patients (*p*<0.05). Negative percentage differences denote that reduction of scores in all tests postoperatively, except TMT. The method of calculating mean differences is mentioned in Chapter 3. For Trail Making Test (TMT) scoring, positive percentage differences mean patients took more time to complete the test during the postoperative assessment. This indicates a reduction in each domain of the cognitive function being tested. The results showed that POCD groups have higher percentage differences across all tests (Table 4.3).

Table 4.3: Comparison between postoperative scores of POCD and non-POCD patients.

Cognitive Test	**Non-POCD, n=35 (mean ± SD)**	**Mean % differences[1]**	**POCD, n=11 (mean ± SD)**	**Mean % differences**	***p*-value**
MMSE	27.91 ± 2.28	-1.80	19.73 ± 7.00	-25.96	0.003*
TMT part A (min)	46.38 ± 21.65	-8.64	75.40 ± 32.53	48.51	0.002*
TMT part B (min)	110.68 ± 47.72	-0.35	187.30 ± 45.55	68.63	<0.001**
Digit Span Test	12.50 ± 3.12	-4.87	9.9 ± 2.33	-24.65	0.019*
DSST	48.97 ± 18.62	-0.85	26.60 ± 10.31	-46.14	<0.001**
Clock Drawing	1.86 ± 0.43	-1.58	1.27 ± 1.00	-32.80	0.08

Note: [1]Percentage differences were calculated by subtracting postoperative to preoperative scores.
**p*-value significant at *p*<0.05 using independent t-test
***p*-value significant at *p*<0.001 using independent t-test

4.2.2 Preoperative and Intraoperative Details

We differentiate the preoperative and intraoperative details between POCD and non-POCD patients. The findings show a significant difference (p<0.05) in age and education level between POCD and non-POCD groups. POCD patients (65.00 ± 6.78) were found to be in the older age group compared to non-POCD patients (55.7 ± 13.43). For education level, POCD patients have significantly lower years of education compared to the non-POCD group. Other risk factors include comorbidities such as hypertension, diabetes, hypercholesterolemia, chronic kidney disease (CKD) and smoking. Having hypertension, diabetes and CKD were found to be significantly associated with POCD (p<0.05).

For intraoperative details, we recorded the bypass and cross-clamp time. Both results show a shorter time taken in the POCD group, but insignificant. A significant difference (p<0.05) was found for the amount of blood loss where POCD (300 ml ± 350 ml) lost more blood than non-POCD patients (200 ml ± 103 ml). For ICU stays, the POCD group had a longer ICU stay (5 ± 11) than the non-POCD group (2 ± 3), although it was not significant (p=0.060). On the other hand, the postoperative stay in POCD patients (16 ± 14) is significantly longer duration of stay (p<0.05) compared to non-POCD patients (8 ± 7). All results were tabulated in Table 4.4.

Table 4.4: Preoperative and intraoperative details between POCD and non-POCD groups.

Variables	Group (mean ± SD)/n(%)		*p*-value
	Non-POCD (n=35)	**POCD (n=11)**	
Preoperative variables			
Age (years)	55.7 ± 13.43	65.0 ± 6.78	0.004*^{a}
Education level (years)	11.91 ± 2.58	8.73 ± 4.13	0.004*^{a}
No formal education	0 (0)	1 (100)	0.010*^{b}
Primary	1 (25)	3 (75)	
Secondary	20 (76.9)	6 (23.1)	
Tertiary	14 (93.3)	1 (6.7)	
Body Mass Index (kg/m^{2})	26.4 ± 4.14	27.9 ± 4.25	0.326
Underweight (<18.5)	1 (100)	0 (0)	0.934
Normal (18.5-24.9)	11 (78.6)	3 (21.4)	
Overweight (25.0-29.9)	14 (73.7)	5 (26.3)	
Obese (≥30.0)	9 (75.0)	3 (25)	
Preoperative Risk Factors			
Hypertension			
No	15 (100)	0 (0)	0.006*^{c}
Yes	20 (64.5)	11 (35.5)	
Diabetes			
No	24 (88.9)	3 (11.1)	0.019*c
Yes	11 (57.9)	8 (42.1)	
Hypercholesterolemia			
No	13 (92.9)	1 (7.1)	0.077
Yes	22 (68.8)	10 (31.3)	
Smoking			
No	25 (86.2)	4 (13.8)	0.084
Yes	8 (61.5)	5 (38.5)	
Chronic kidney disease			
No	19 (90.5)	1 (9.5)	0.038*^{c}
Yes	16 (64.0)	9 (36.0)	
EuroSCORE II			
Low Risk	7 (100)	0 (0)	0.079
Medium Risk	5 (50.0)	5 (50.0)	
High Risk	3 (75.0)	1 (25.0)	

table 4.4 continued

table 4.4 continued

Variables	Group (mean ± SD)/n(%)		*p*-value
	Non-POCD (n=35)	**POCD (n=11)**	
Dyspnea status (NYHA)			
Class 1	9 (81.8)	2 (18.2)	0.590
Class II	14 (70.0)	6 (30.0)	
Class III	1 (50.0)	1 (50.0)	
Class IV	0 (0)	0 (0)	
Angina status (CSS)			
Class 0	16 (76.2)	5 (23.8)	0.895
Class 1	4 (66.7)	2 (33.3)	
Class 2	3 (75.0)	1 (25.0)	
Class 3	0 (0)	0 (0)	
Intraoperative Variables			
Procedure (%)			
CABG only	6 (60.0)	4 (40.0)	0.022*[c]
CABG + valve	15 (68.2)	7 (31.8)	
Valves only	14 (100.0)	0 (0)	
Cerebral Oximetry Usage			
Not use	27 (79.4)	7 (20.6)	0.250
Use	7 (63.6)	4 (36.4)	
Bypass time (minutes)	127.42 ± 75.30	124.30 ± 28.30	0.899
Cross-clamp time (minutes)	99.42 ± 65.75	89.00 ± 28.47	0.632
Blood loss (ml) (median ± IQR)	200 ± 103	300 ± 350	0.038*[d]
Duration of ICU stay (days) (median ± IQR)	2 ± 3	5 ± 11	0.060
Duration of post-op stay (days) (median ± IQR)	8 ± 7	16 ± 14	0.049*[d]

*[a] *p*-value significant at $p<0.05$ using independent t-test
*[b] *p*-value significant at $p<0.05$ using chi-square test
*[c] *p*-value significant at $p<0.05$ using fisher exact test
*[d] *p*-value significant at $p<0.05$ using Mann-Whitney U test
**some values may differ due to missing data

4.3 Differentially Expressed Genes

For DEGs analysis, 16 samples were randomly selected, and grouped accordingly to our preferred comparisons (refer Chapter 3, subheading 3.5.4). Each sample is classified into two grouping conditions as which are POCD and CeOx group. To summarize, there are 6 POCD sample (3 pre and 3 post), 10 non-POCD samples (5 pre and 5 post), 6 CeOx samples (3 pre and 3 post), and 10 non-CeOx samples (5 pre and 5 post). Sample label, purity, concentration and RNA integrity number (RIN) were tabulated in the Table 4.5.

Table 4.5: Purity and concentration of RNA samples.

Sample no/ID	Sample Label	Purity	Concentration (ng)	RIN number
1/D053	pre POCD, non-CeOx	1.832	43.2	8.80
2/D057	pre POCD, non-CeOx	1.855	61.6	6.80
3/D073	pre POCD, non-CeOx	1.857	46.8	8.00
4/D053	post POCD, non-CeOx	1.892	25.2	8.80
5/D057	post POCD, non-CeOx	1.842	135.6	8.00
6/D073	post POCD, non-CeOx	1.816	63.2	8.40
7/D051	pre non-POCD, non-CeOx	1.844	56.8	9.10
8/D074	post non-POCD, non-CeOx	1.880	37.6	8.90
9/D076	pre non-POCD, CeOx	1.898	77	7.00
10/D076	post non-POCD, CeOx	1.888	148	8.30
11/D099	pre non-POCD, CeOx	2.056	29	8.30
12/D099	post non-POCD, CeOx	1.925	82	8.20
13/D104	pre non-POCD, CeOx	1.963	63	9.50
14/D104	post non-POCD, CeOx	1.880	75	7.70
15/D066	pre non-POCD, non-CeOx	1.979	38	7.40
16/D066	post non-POCD, non-CeOx	1.869	125	8.70

*POCD, postoperative cognitive dysfunction; CeOx, cerebral oximetry; RIN, RNA integrity number.

There are four comparisons were analysed to see the DEGs in different conditions. The comparison was simplified in Figure 4.1. The subsections that follow will show the results of sample quality check, DEGs, and qPCR validation according to their grouping comparison.

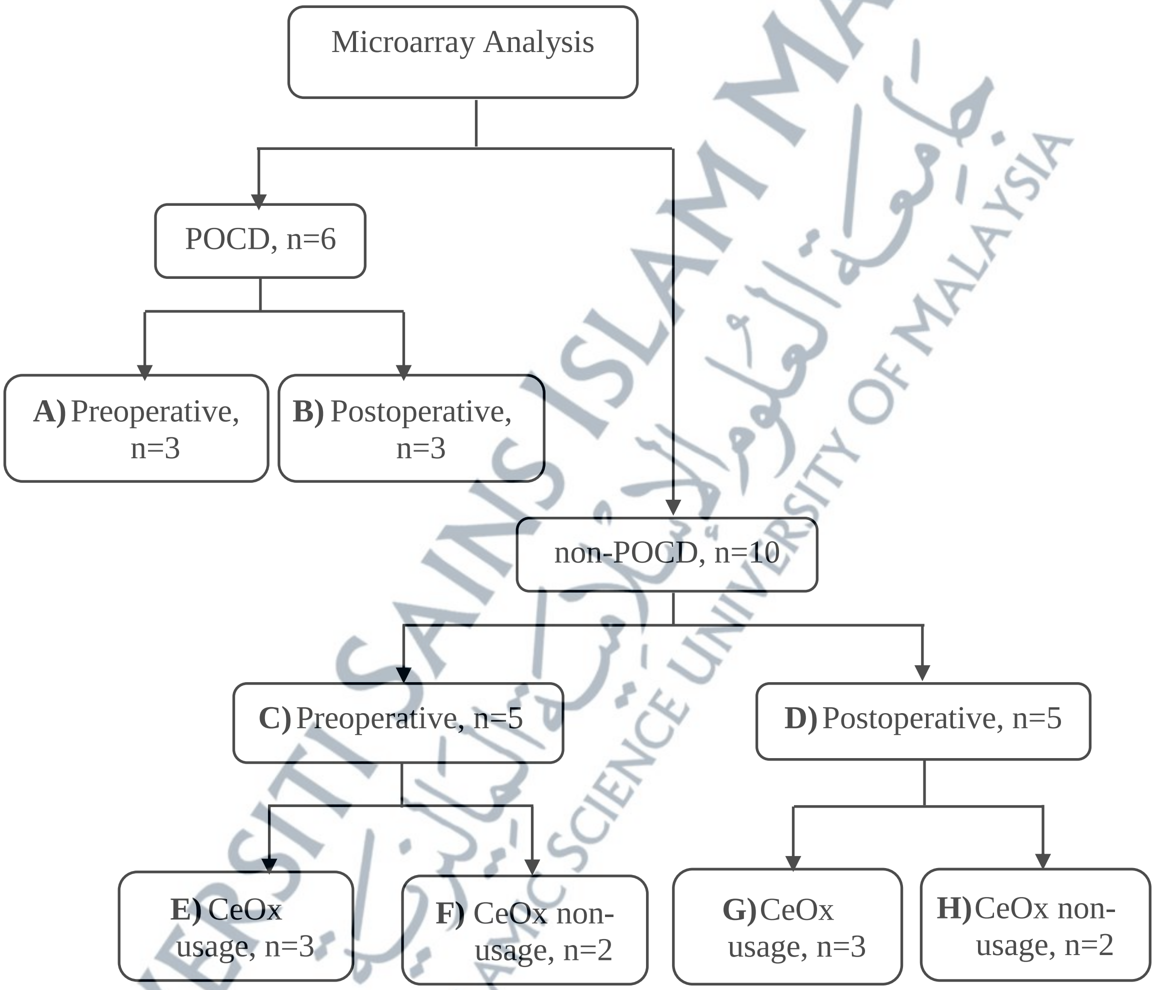


Figure 4.1: The comparisons for DEGs analysis. 1- preoperative versus postoperative samples in POCD patients (**A vs B**), 2- POCD versus non-POCD patients (**B vs D**), 3- CeOx usage versus CeOx non-usage (**G vs H**), 4- preoperative versus postoperative samples in non-POCD patients (**C vs D**). POCD, postoperative cognitive dysfunction; CeOx, cerebral oximetry.

4.3.1 Comparison 1: POCD Patients, Preoperative vs Postoperative Samples

In this comparison, 6 samples were used (refer Figure 4.1). They were sample number 1, 2 and 3 for preoperative samples, while number 4, 5 and 6 for postoperative samples (refer table 4.5). Outliers and correlations were detected in the following principal component analysis (PCA) plot and correlation plot respectively (Figure 4.2). The PCA plot shows the distribution of the samples, where samples were clustered according to groups, without prominent outliers. For correlation plot, darker red shows a higher correlation between samples.

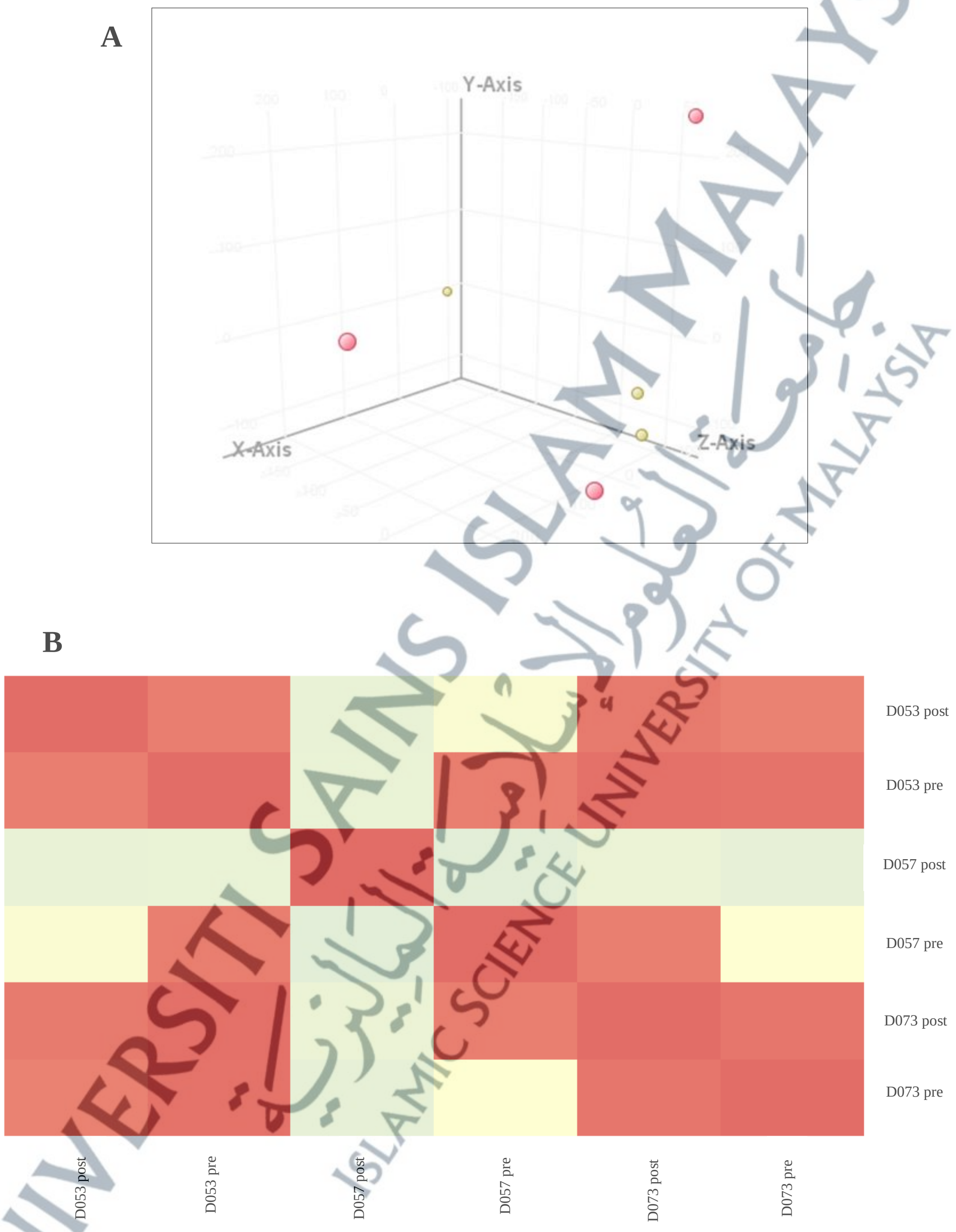


Figure 4.2: PCA plot (**A**) and correlation plot (**B**) for Comparison 1.

i) Differentially expressed genes

The results show that there are 5 differentially expressed genes. There are 4 downregulated and 1 upregulated gene detected in our samples. The details of the differently expressed genes are listed in Table 4.6.

Table 4.6: Differentially expressed genes in POCD groups.

No	Fold Change	*p*-value	Expression (up/down regulated)	Gene Symbol	Gene description	Gene Function
1	-15.38	0.016	Down	KIR3DL2	killer cell immunoglobulin like receptor, three Ig domains and long cytoplasmic tail 2, transcript variant 2	Inhibit interferon-gamma production and cytotoxic function
2	-12.64	0.033	Down	LIM2	lens intrinsic membrane protein 2, transcript variant 1	Lens development and cataractogenesis
3	11.60	0.011	Up	ERFE	erythroferrone	Controls plasma iron level and total body iron
4	-21.73	0.010	Down	KIR2DS3	killer cell immunoglobulin like receptor, two Ig domains and short cytoplasmic tail 3	Protection against acute myeloid leukemia
5	-11.38	0.033	Down	KIR2DS2	killer cell immunoglobulin like receptor, two Ig domains and short cytoplasmic tail 2, transcript variant 1	Identifies NK cells with enhanced anticancer activity

A volcano plot is produced for graphical representation of the DEGs, as it plots the significance and fold changes. The volcano plot shows the upregulated genes in red on top right and downregulated genes in blue on the top left of the plot. The heatmap shows the upregulated genes in red and downregulated genes in blue for postoperative and preoperative conditions (Figure 4.3).

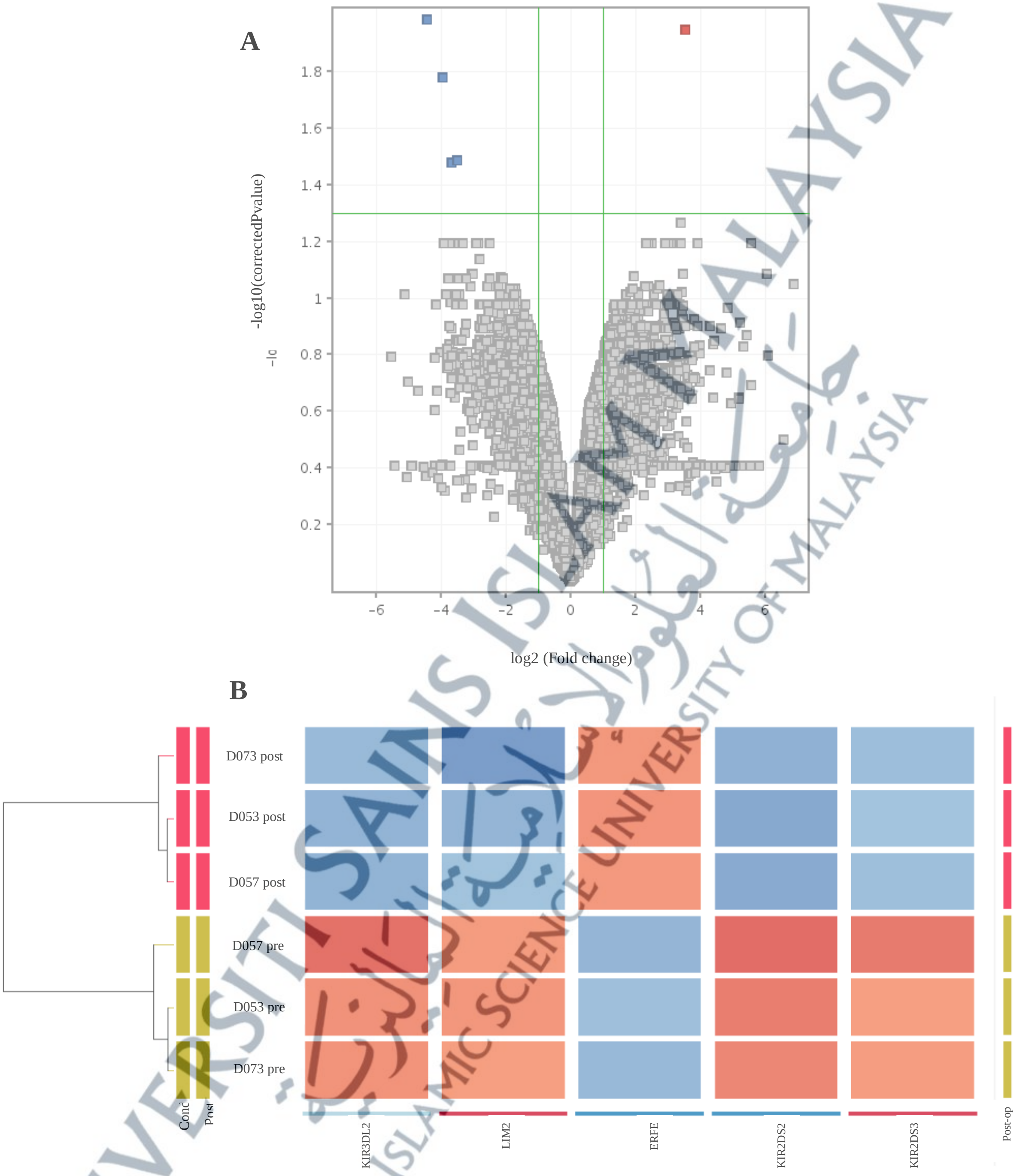


Figure 4.3: Volcano plot (**A**) and heatmap (**B**) for Comparison 1.

ii) qPCR validation

From 5 dysregulated genes for this comparison, we chose 3 genes of interest for qPCR validation. LIM2 gene was related to cataractogenesis, which is not of our interest. Another gene, KIR2DS2, has a limited sample available for qPCR. Hence, we proceed with other 3 genes which are ERFE, KIR2DS3, and KIR3DL2. The figures below show the amplification curve for each gene and their housekeeping gene, beta-actin (Figure 4.4 – Figure 4.6).

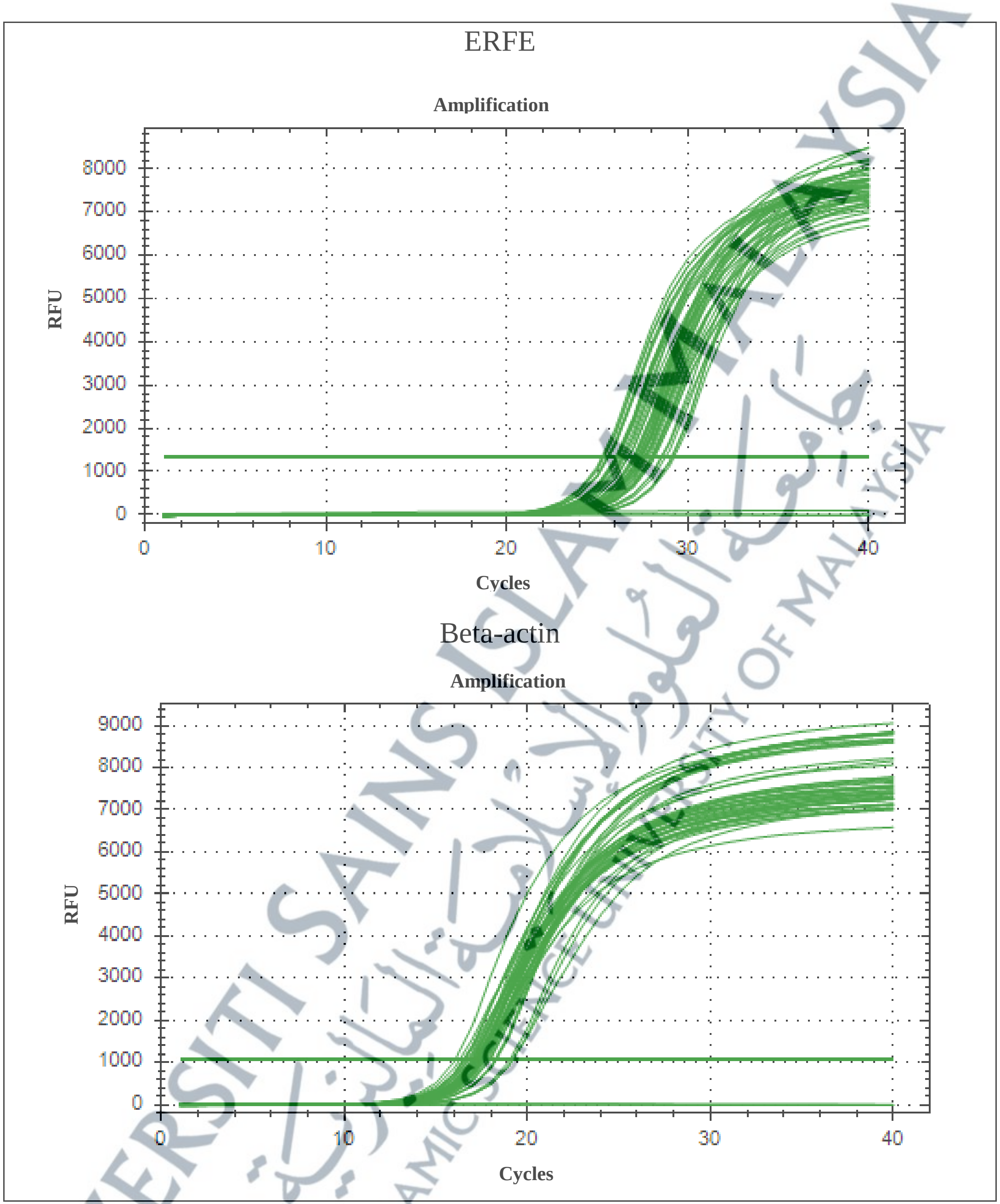


Figure 4.4: Amplification curve for ERFE gene and beta-actin.

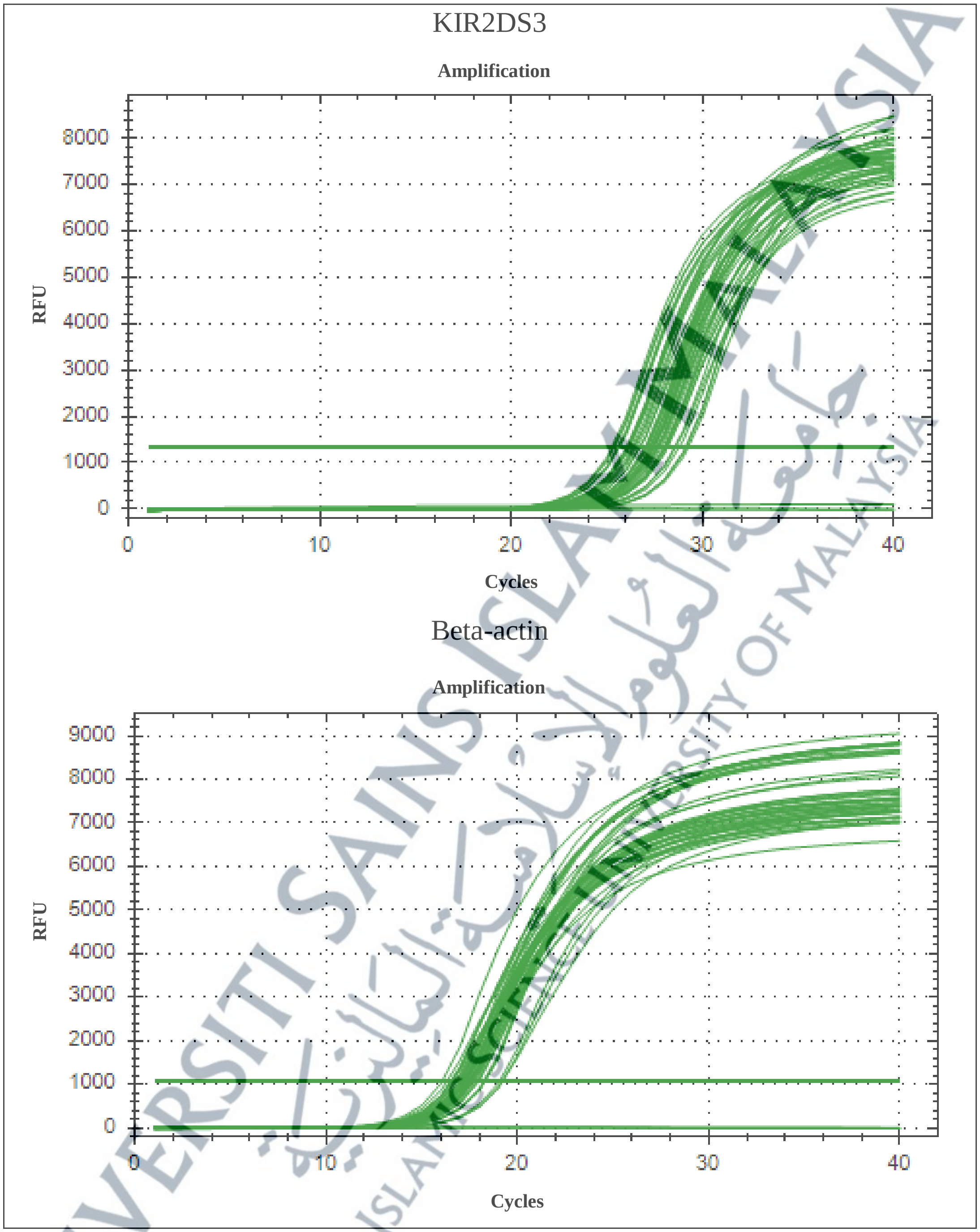
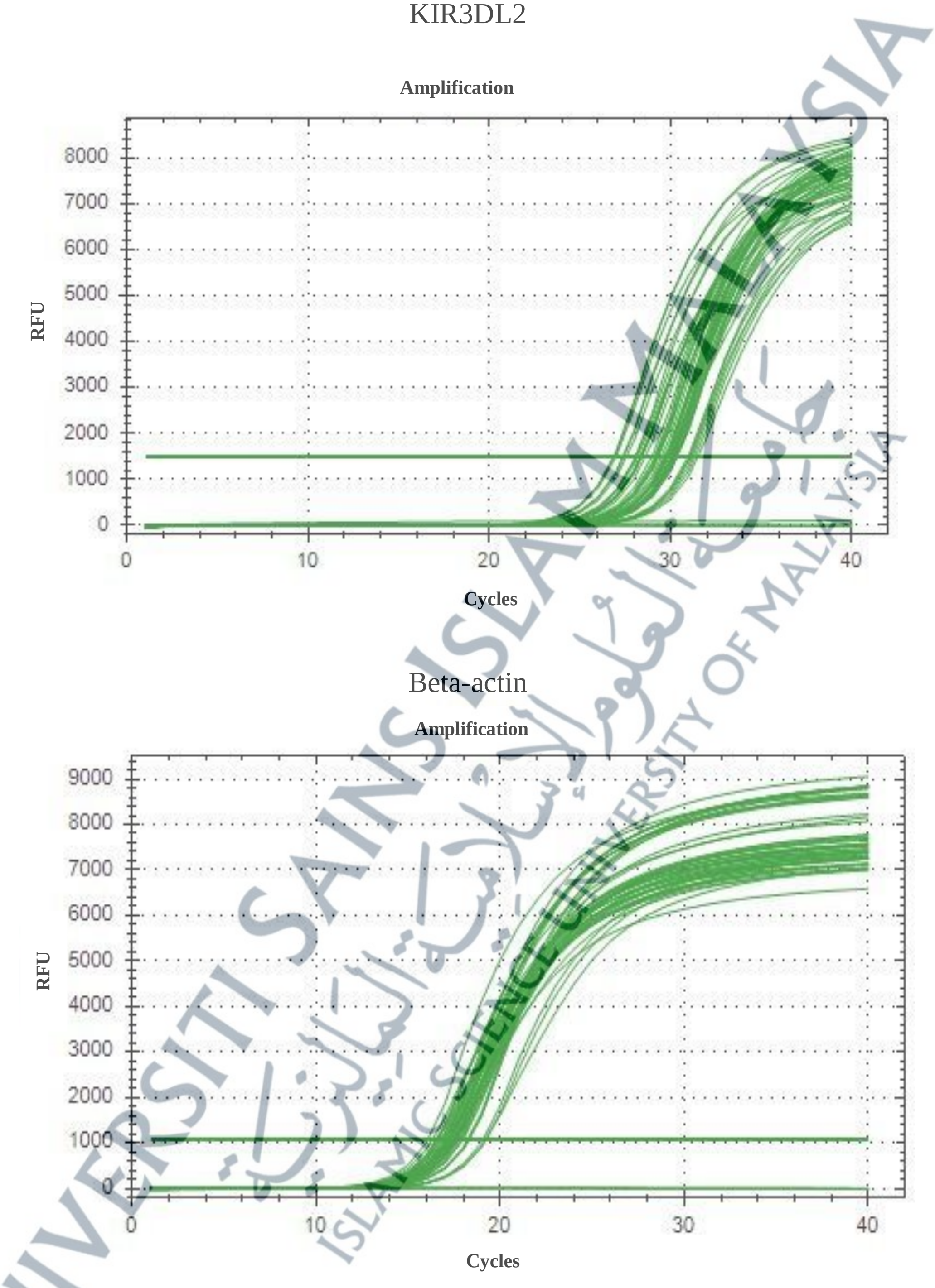


Figure 4.5: Amplification curve for KIR2DS3 gene and beta-actin.


KIR3DL2

Beta-actin

Figure 4.6: Amplification curve for KIR3DL2 gene and beta-actin.

The table below shows the selected genes’ cycle threshold (CT) value (Table 4.7). The fold changes of the expression (2^(-ΔΔCt)) was then compared between the preoperative and postoperative group. The result shows that gene ERFE has a higher fold change in the postoperative group, reflecting its upregulation. The wide standard error mean value in the preoperative group for ERFE was due to sample number 3 that has higher 2^(-ΔΔCt) compared to other samples in preoperative group. For genes KIR2DS3 and KIR3DL2, the downregulation of genes can be seen as postoperative groups has lower fold changes, and it was significant at $p<0.05$. The bar charts are illustrated in Figure 4.7.

Table 4.7: CT value and fold change for ERFE, KIR2DS3 and KIR3DL2 genes

Group	Sample no	Avg GOI CT	Avg HG CT	ΔCT	ΔΔCT	2^(-ΔΔCt)	Log 2^(-ΔΔCt)	SEM	*p*-value
ERFE									
Pre	1	30.94	17.18	13.76	2.82	0.14	-0.85	0.493	0.67
	2	28.48	17.50	10.98	0.03	0.98	-0.01		
	3	27.27	19.18	8.09	-2.85	7.23	0.86		
Post	4	27.36	16.80	10.55	-0.35	1.28	0.11	0.059	
	5	27.86	17.92	9.94	-1.00	2.00	0.30		
	6	27.33	17.25	10.08	-0.87	1.82	0.26		
Average pre				10.94					
KIR2DS3									
Pre	1	26.39	17.18	9.21	0.68	0.63	-0.20	0.105	0.02*
	2	25.56	17.50	8.07	-0.48	1.39	0.14		
	3	27.52	19.18	8.34	-0.20	1.15	0.06		
Post	4	28.10	16.80	11.30	2.76	0.15	-0.83	0.154	
	5	27.71	17.92	9.79	1.25	0.42	-0.38		
	6	28.60	17.25	11.35	2.81	0.14	-0.85		
Average pre				8.54					
KIR3DL2									
Pre	1	27.43	17.18	10.25	0.02	0.98	-0.01	0.146	0.04*
	2	26.88	17.50	9.39	-0.85	1.78	0.26		
	3	30.24	19.18	11.06	0.83	0.56	-0.25		
Post	4	31.34	16.80	14.54	4.31	0.05	-1.29	0.146	
	5	31.03	17.92	13.11	2.88	0.14	-0.87		
	6	30.31	17.25	13.06	2.82	0.14	-0.85		
Average pre				10.23					

**p*-value significant at $p<0.05$ using paired sample t-test. CT, cycle threshold; GOI, gene of interest; HG, housekeeping gene; SEM, standard error mean.

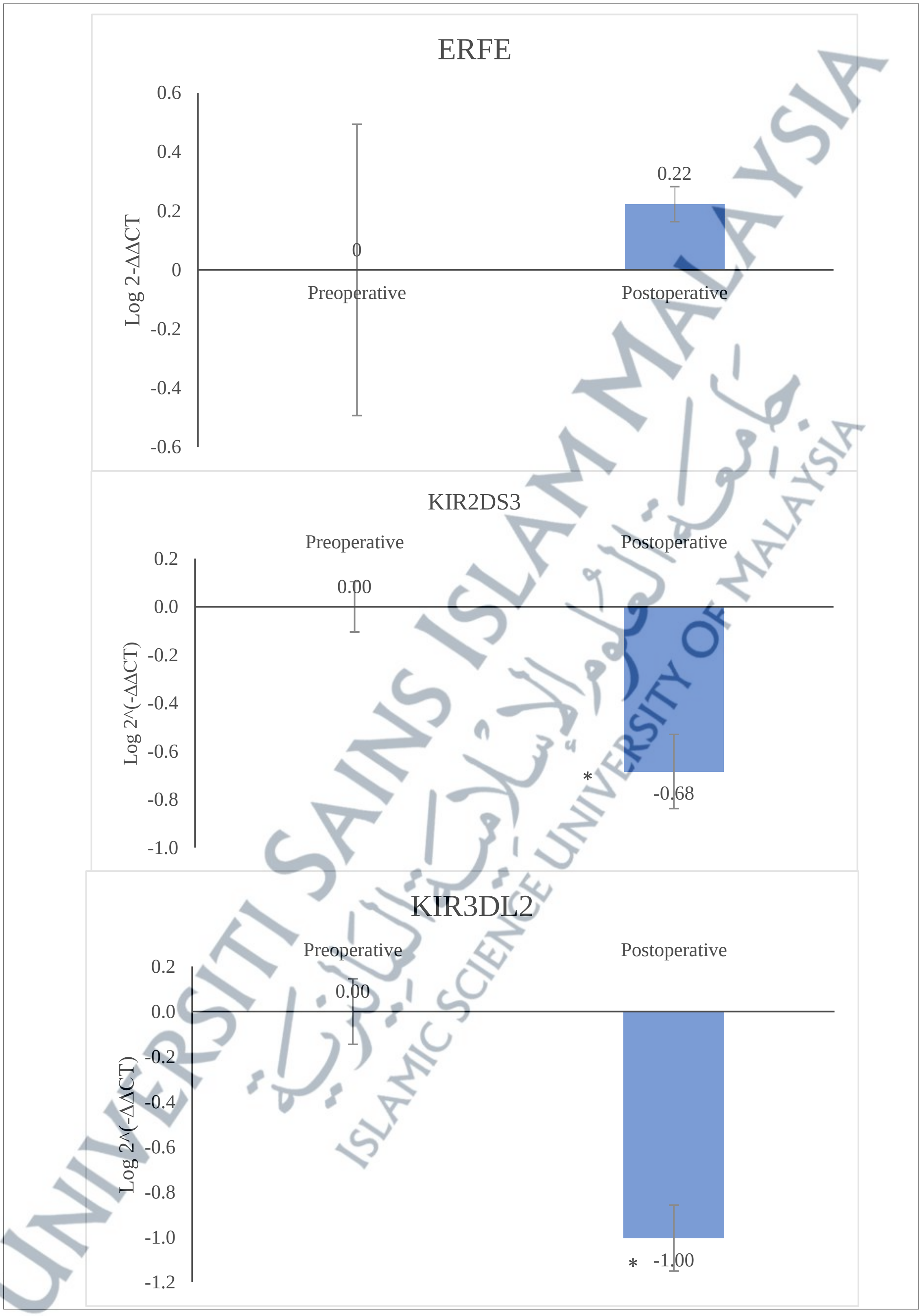


Figure 4.7: Bar chart showing fold change of each genes. *p-value significant at $p<0.05$ using paired sample t-test.

4.3.2 Comparison 2: POCD vs Non-POCD Patients

For this comparison, we use 5 samples (refer Figure 4.1). They were sample number 4, 5, and 6 for the POCD group, while samples 8 and 16 were for the non-POCD group (refer Table 4.5). Outliers and correlations were detected in the following PCA plot and correlation plot respectively (Figure 4.8). The PCA plot shows the distribution of the samples, where samples from POCD group in yellow were dispersed from each other. For correlation plot, darker red shows a higher correlation between samples.

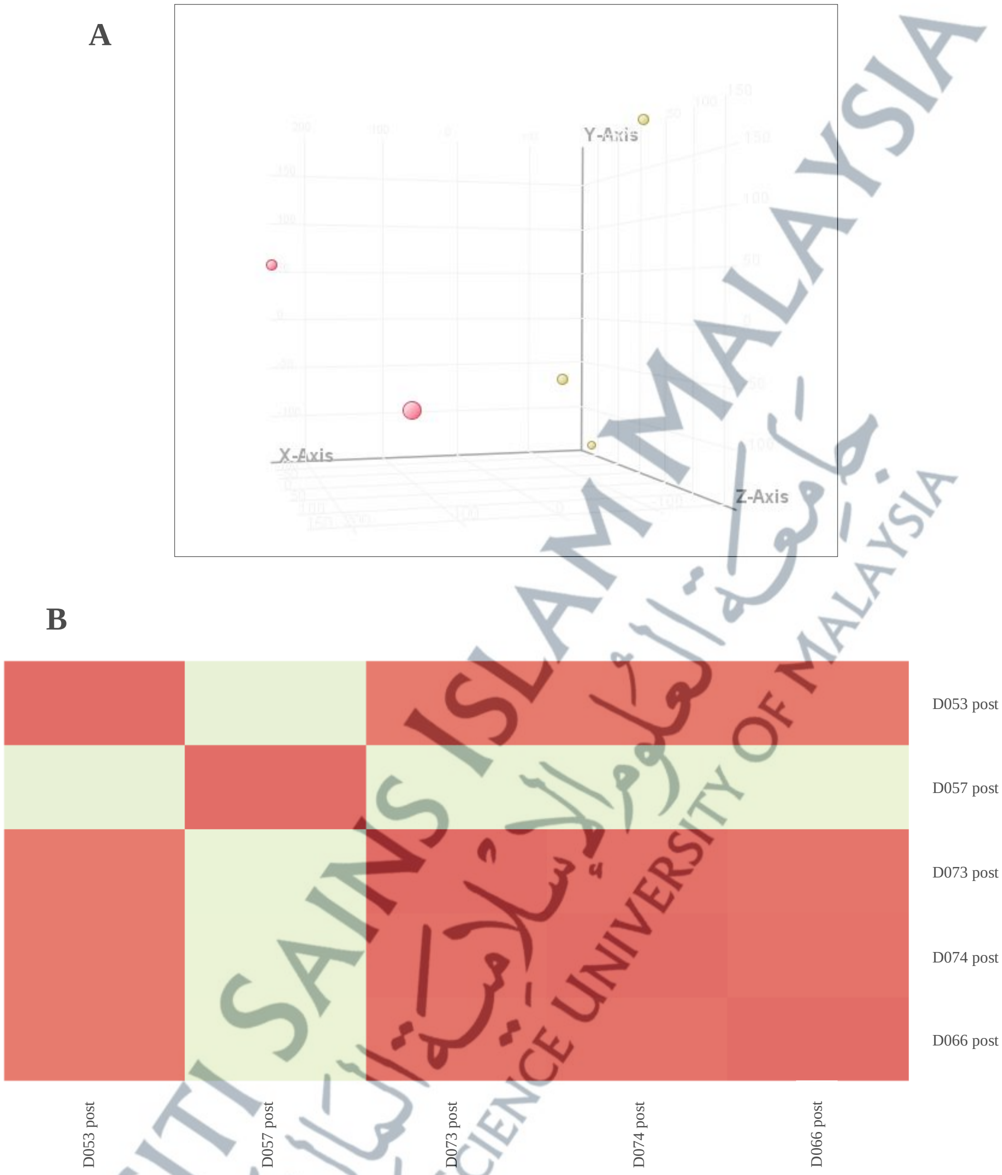


Figure 4.8: PCA plot **(A)** and correlation plot **(B)** for Comparison 2.

i) Differentially expressed genes

The results for this comparison showed one significant DEG, as shown in Table 4.8. The volcano plot shows the downregulated genes in blue on the top left of the plot. The heatmap shows the downregulated genes in blue for POCD groups (Figure 4.9).

Table 4.8: Differentially expressed genes in POCD groups compared to non-POCD groups.

No	**Fold Change**	***p*-value**	**Expression (up/down regulated)**	**Gene Symbol**	**Gene description**	**Gene Function**
1	-475.16	<0.001	Down	BTNL3	butyrophilin like 3	Enable signaling receptor binding activity

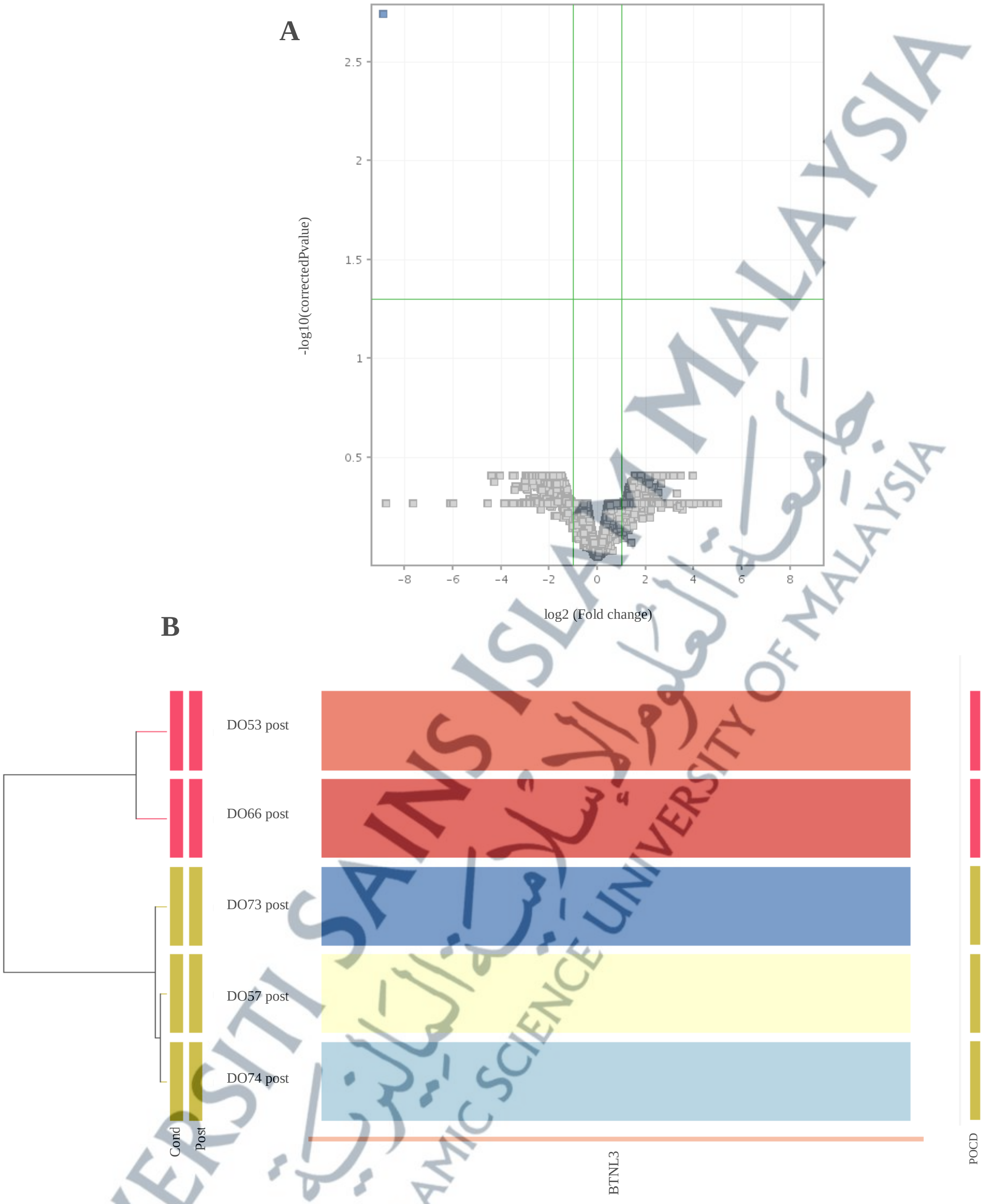


Figure 4.9: Volcano plot (A) and heatmap (B) for Comparison 2.

ii) qPCR validation

Validation was done for BTNL3 gene. The figure below shows the amplification curve of BTNL3 with the housekeeping genes, beta-actin (Figure 4.10) and CT value (Table 4.9).

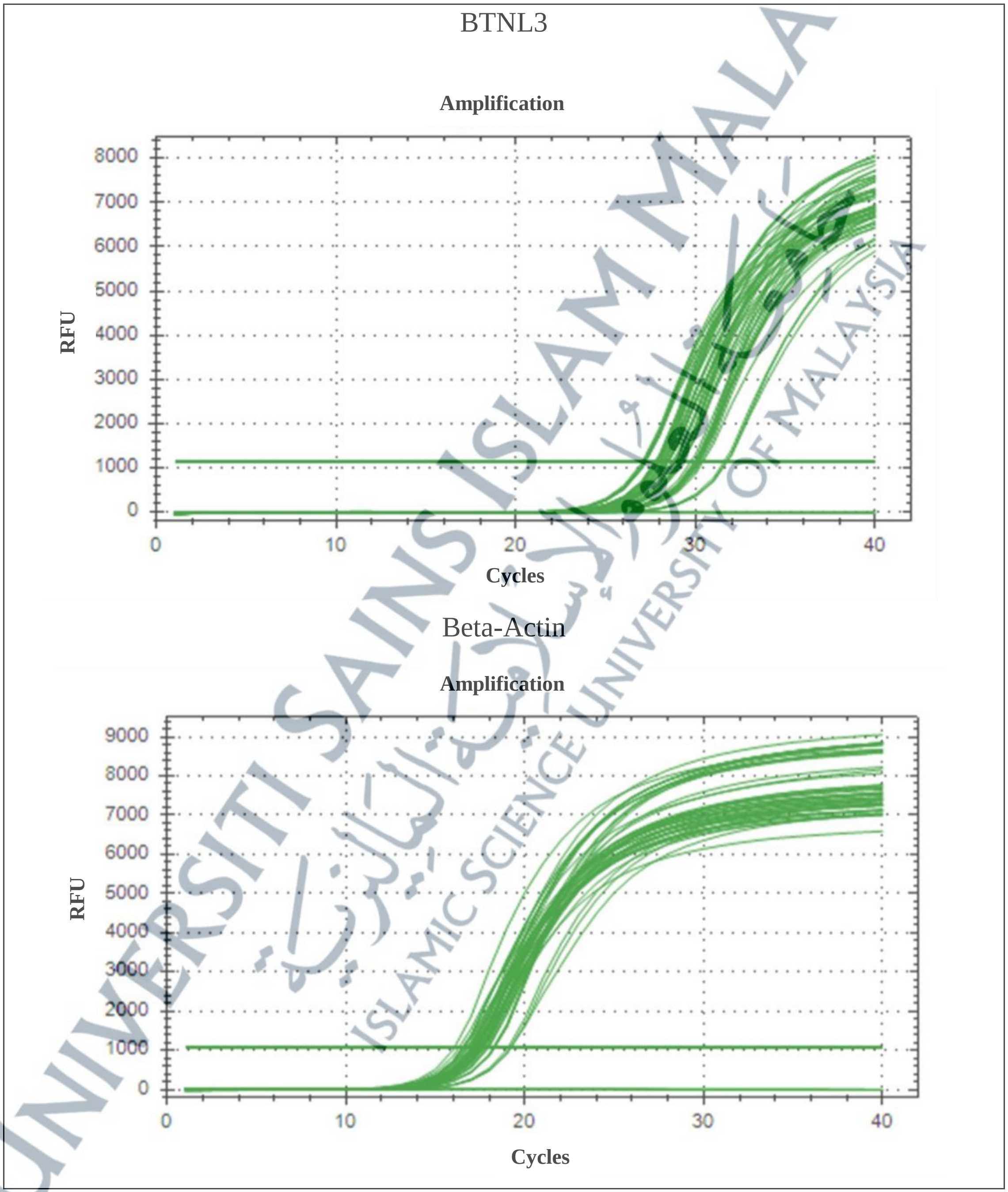


Figure 4.10: Amplification curve for BTNL3 gene and beta-actin.

For both groups, the mean fold changes were almost equal, implying the downregulation of BTNL3 in both groups. This contradicting result from microarray may cause by unequal number of samples in both groups, causing a discrepancy in statistical analysis. Besides, the frequent freeze thaw cycle of the samples may reduce the sample quality throughout the process. The fold change graph was illustrated in Figure 4.11.

Table 4.9: CT value and fold change for BTNL3 gene.

Group	**Sample no**	**Avg GOI CT**	**Avg HG CT**	**ΔCT**	**ΔΔCT**	**2^(-ΔΔCt)**	**log 2^(-ΔΔCt)**	**SEM**	***p*-value**
POCD	4	30.19	16.82	13.36	1.17	0.44	-0.77	0.276	0.90
	5	29.26	17.92	11.34	-0.85	1.80	-1.61		
	6	28.50	17.26	11.24	-0.95	1.93	-0.79		
Non-POCD	8	28.91	17.22	11.68	-0.51	1.42	-0.98	0.118	
	16	30.12	17.43	12.70	0.51	0.70	-1.21		
Average non-POCD				12.19					

CT, cycle threshold; GOI, gene of interest; HG, housekeeping gene; SEM, standard error mean.

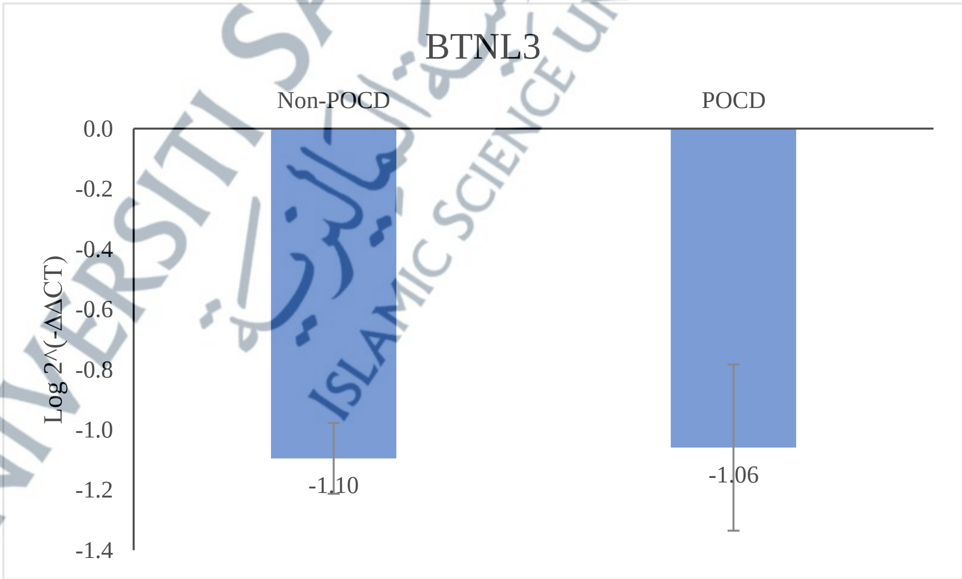


Figure 4.11: Bar chart showing fold change of BTNL3 genes.

4.3.3 Comparison 3: Cerebral Oximetry vs Non-Cerebral Oximetry

For this comparison, 5 samples were used (refer Figure 4.1). They were sample number 10, 12 and 14 for the cerebral oximetry group, while sample number 8 and 16 for the non-cerebral oximetry group (refer table 4.5). Outliers and correlations were detected in the following PCA and correlation plot (Figure 4.12). The PCA plot shows the distribution of the samples, where samples were clustered according to groups, without prominent outliers. For correlation plot, darker red shows a higher correlation between samples.

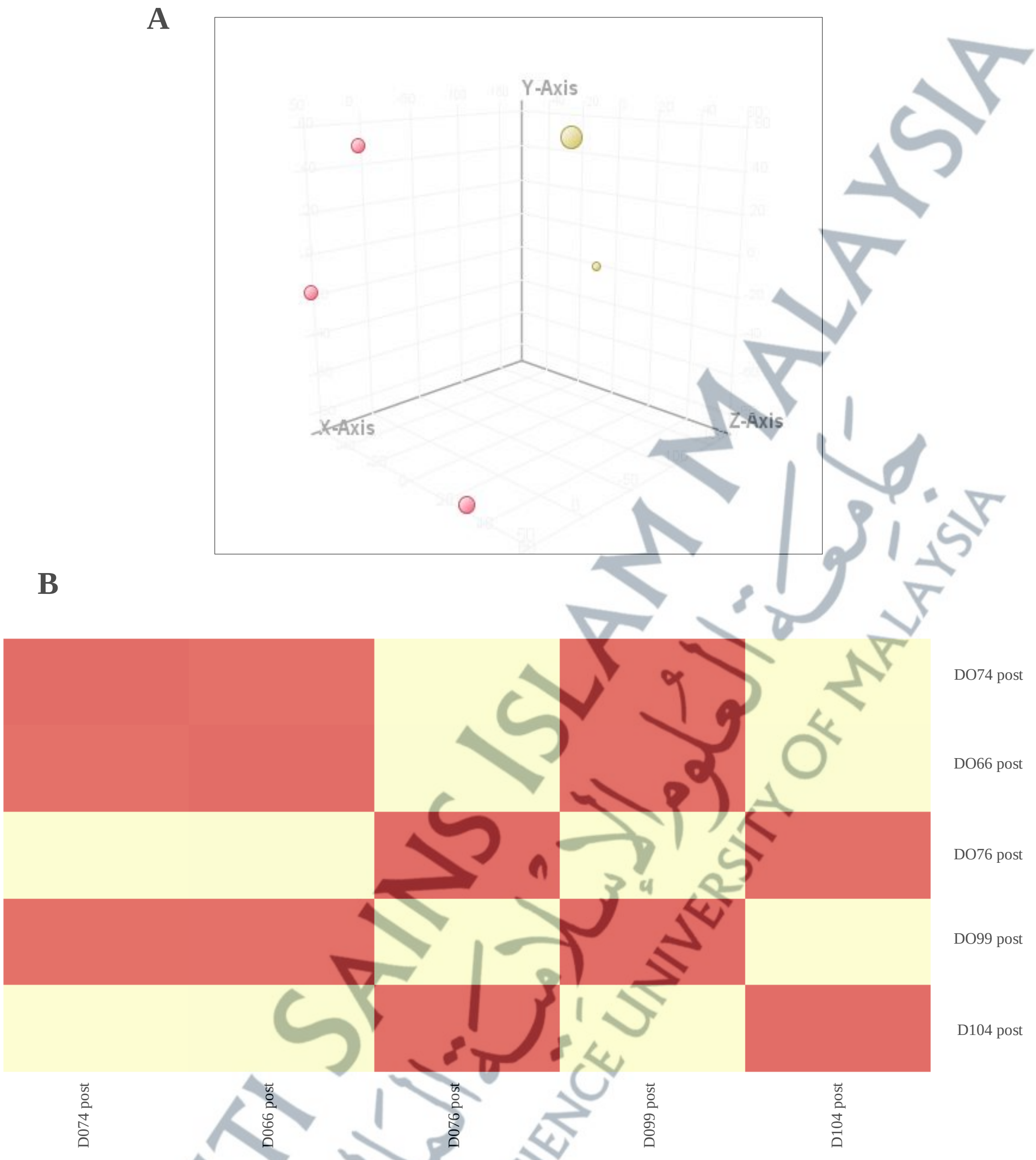


Figure 4.12: PCA plot **(A)** and correlation plot **(B)** for Comparison 3.

i) Differentially expressed genes

The results for this comparison showed that there are 12 dysregulated genes, where 3 upregulated and 9 downregulated genes. The details for the genes are listed in Table 4.10 below.

Table 4.10: DEGs in cerebral oximetry groups compared to non-cerebral oximetry groups.

No	**Fold Change**	***p*-value**	**Expression (up/down regulated)**	**Gene Symbol /GenBank Accession**	**Gene description**	**Gene Function**
1	-146.63	<0.001	down	EIF1AY	eukaryotic translation initiation factor 1A Y-linked	Stabilizing the binding of initiator Met-tRNA to 40S ribosomal subunits
2	48.90	<0.001	up	LERFS	lncRNA negative regulator of fibroblast-like synoviocyte migration, SYNCRIP interacting	Regulates rheumatoid synovial aggression and proliferation
3	-17.36	<0.001	down	MORN4	MORN repeat containing 4	Promoting axonal degeneration following neuronal injury
4	14.48	0.014	up	lnc-GPR123-1	lnc-GPR123-1:1	Regulate blood pressure, immune response, food intake and development
5	-12.19	<0.001	down	ZFY	zinc finger protein Y-linked	Promotes meiotic sex chromosome inactivation
6	-12.16	0.021	down	SMPD3	sphingomyelin phosphodiesterase 3	Release of microRNAs into extracellular space
7	11.27	0.026	up	TCONS_l2_00013783	Homo sapiens clone TCONS_0024998	Novel transcript

					2 lncRNA, complete sequence. [MG828801]	
8	-8.09	0.021	down	DDX3Y	DEAD-box helicase 3 Y-linked	Key spermatogenic factor in the AZFa region
9	-7.87	0.012	down	CCL23	C-C motif chemokine ligand 23	Promoting leucocyte trafficking, directing the migration of monocytes, macrophages, dendritic cells and T lymphocytes to site of injury
10	-7.54	0.041	down	HACD1	3-hydroxyacyl-CoA dehydratase 1	Regulating cardiac development and differentiation
11	-6.67	0.021	down	GPR37L1	G protein-coupled receptor 37 like 1	Role in blood pressure regulation and cardioprotection
12	-6.31	0.033	down	TCONS_00014808	novel transcript [ENST00000523784]	Novel transcript

The volcano plot shows upregulated genes in red on the top right and downregulated genes in blue on the top left of the plot. The heatmap shows the upregulated genes in red and downregulated genes in blue for cerebral oximetry usage conditions (Figure 4.13).

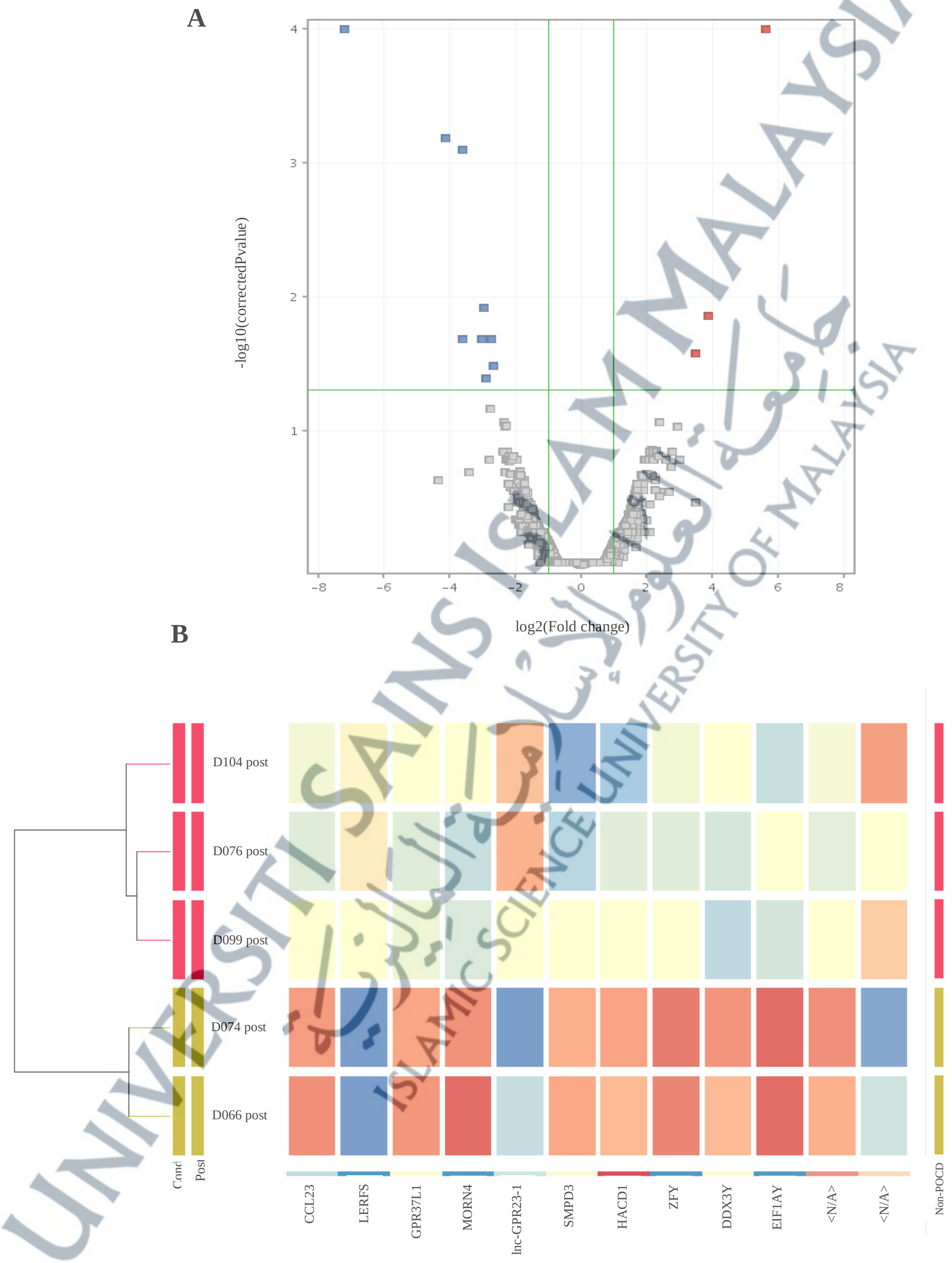


Figure 4.13: Volcano plot **(A)** and heatmap **(B)** for Comparison 3.

ii) qPCR validation

From 12 DEGs, we chose 3 genes of interest for validation, according to their function and possible association with POCD. The genes are MORN4, CCL23, and GPR37L1. For gene GPR37L1, the gene did not pass the primer efficiency test as the expression is too low. Hence, no further validation is made for GPR37L1, leaving with 2 genes, MORN4 and CCL23. The figure below shows the amplification curve of each gene and their housekeeping gene, beta-actin (Figure 4.14 to Figure 4.16).

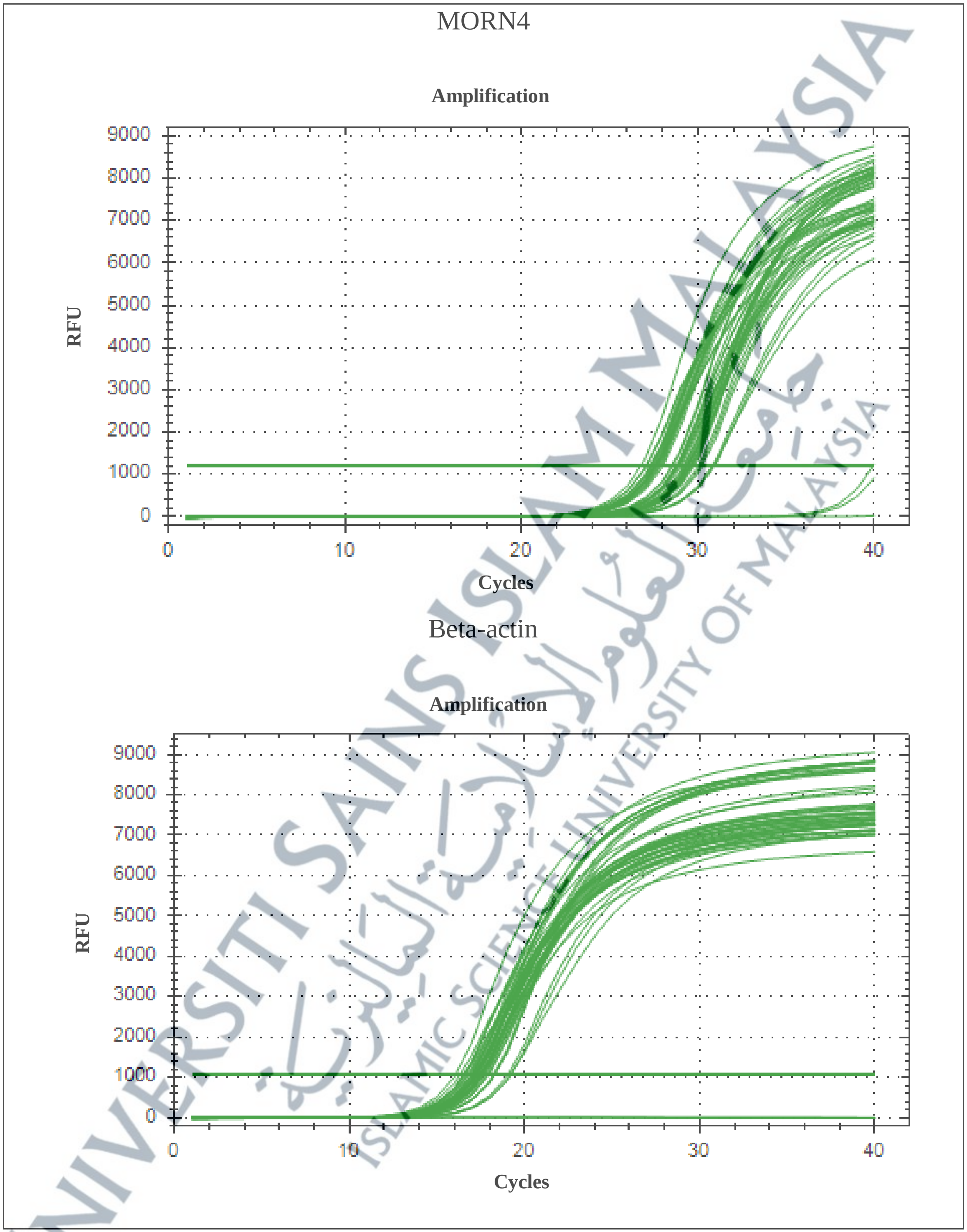
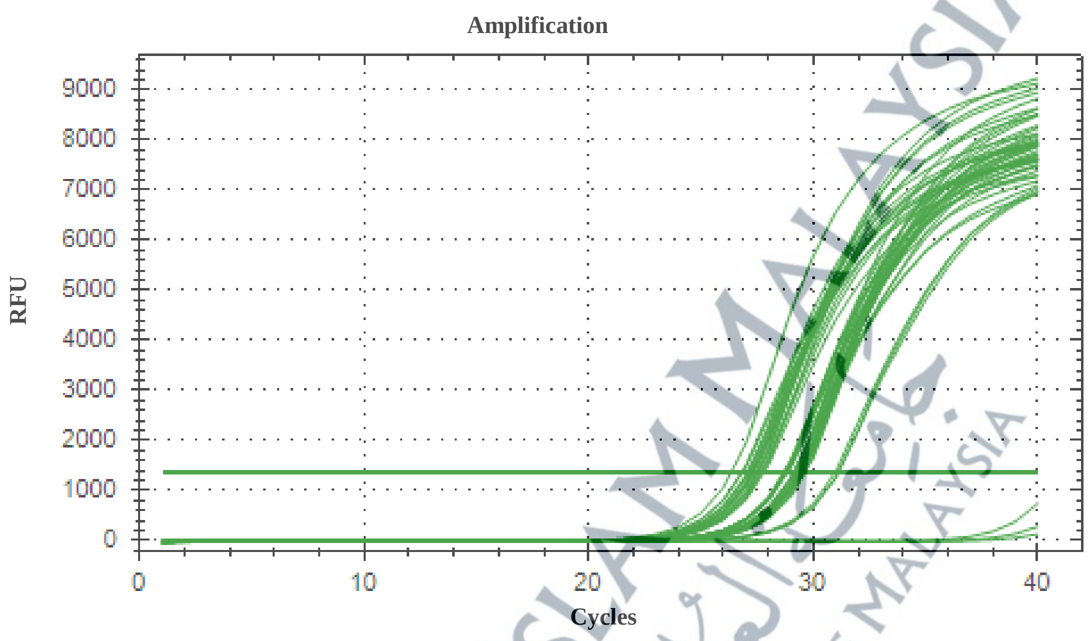


Figure 4.14: Amplification curve for MORN4 gene and beta-actin.

CCL23



Beta-actin

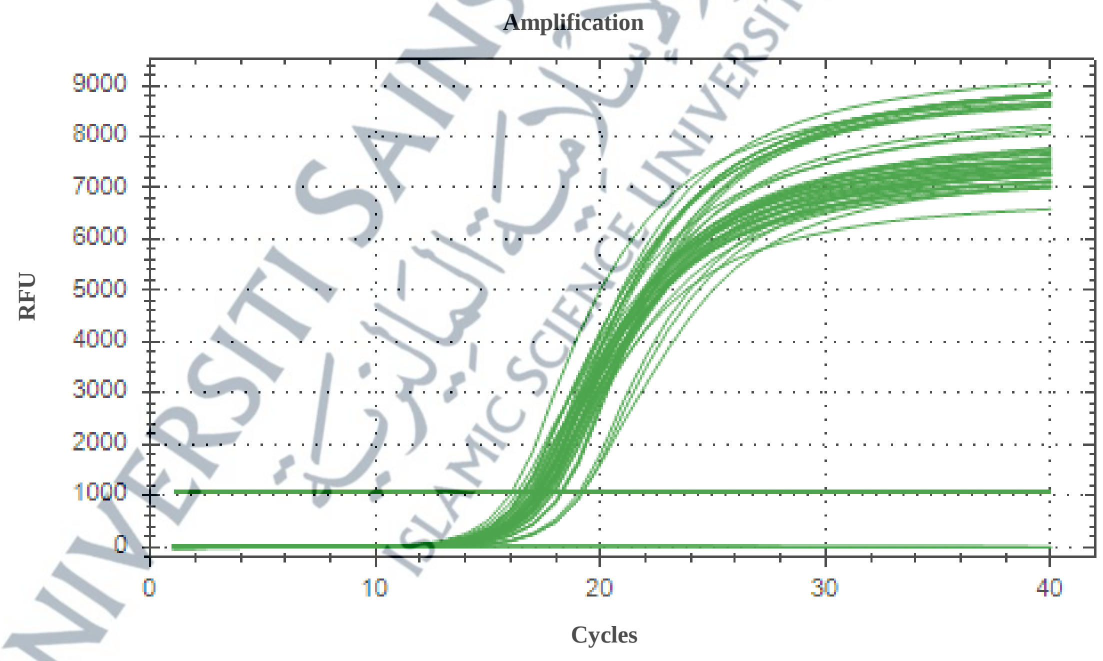
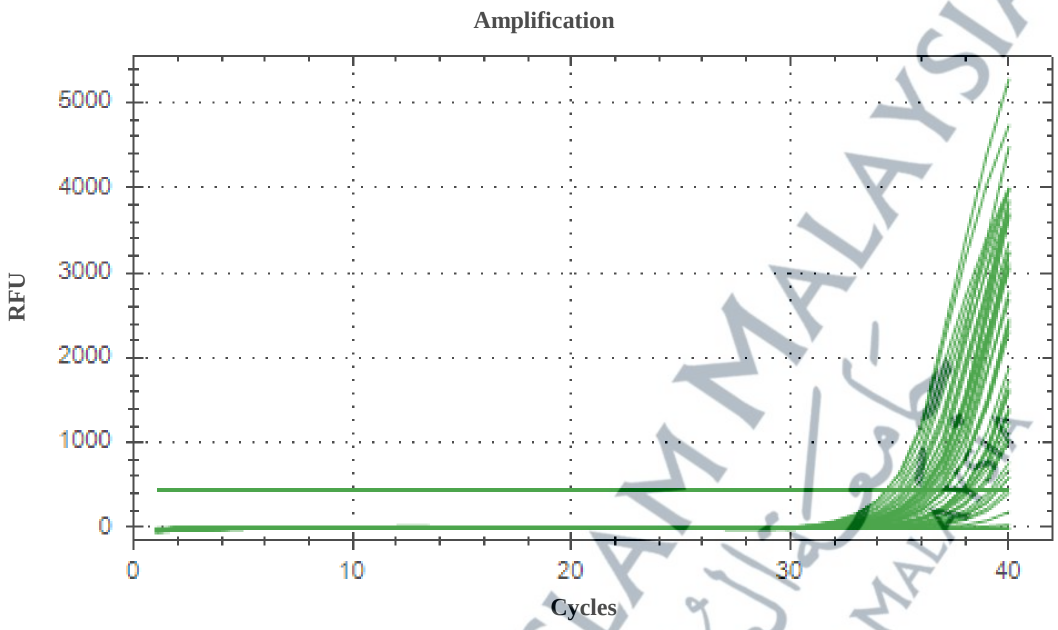


Figure 4.15: Amplification curve for CCL23 gene and beta-actin.

GPR37L1



Beta-actin

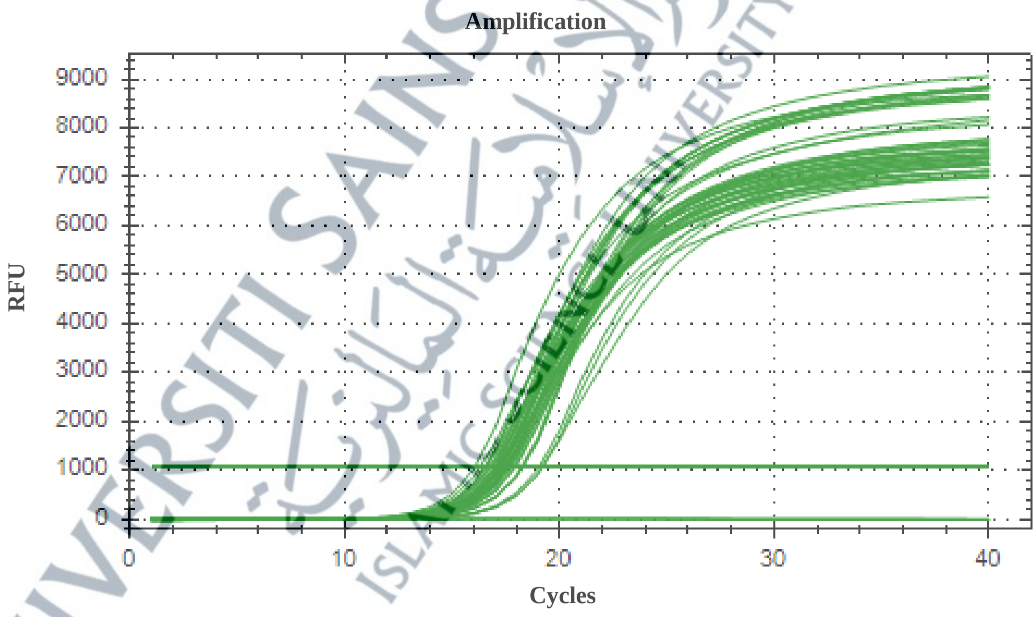


Figure 4.16: Amplification curve for GPR37L1 gene and beta-actin.

The CT value was calculated in table 4.11. For fold change expression in both genes, the cerebral oximetry group shows a higher relative fold change compared to non-cerebral oximetry group. This result contradicts with microarray that shows downregulation of the genes in CeOx group. However, the number of samples in both groups were unequal that may results in this contradicting outcome. The relative fold changes graph was shown in Figure 4.17.

Table 4.11: CT value and fold change for MORN4 and CCL23 gene.

Group	Sample no	Avg GOI CT	Avg HG CT	ΔCT	ΔΔCT	2^(-ΔΔCt)	Log 2^(-ΔΔCt)	SEM	*p*-value
MORN4									
CeOx	10	28.77	16.55	12.22	-0.27	1.21	0.08	0.161	0.123
	12	27.66	17.29	10.36	-2.13	4.36	0.64		
	14	27.57	16.33	11.24	-1.25	2.38	0.38		
Non-CeOx	8	29.51	17.22	12.29	-0.20	1.15	0.06	0.061	
	16	30.12	17.43	12.69	0.20	0.87	-0.06		
Average non-CeOx				12.49					
CCL23									
CeOx	10	28.72	16.55	12.17	0.11	0.93	-0.03	0.161	0.229
	12	27.66	17.29	10.36	-1.70	3.24	0.51		
	14	27.21	16.33	10.88	-1.18	2.26	0.35		
Non-CeOx	8	29.22	17.22	12.00	-0.06	1.04	0.02	0.018	
	16	29.54	17.43	12.12	0.06	0.96	-0.02		
Average non-CeOx				12.06					

CeOx, cerebral oximetry; CT, cycle threshold; GOI, gene of interest; HG, housekeeping gene; SEM, standard error mean.

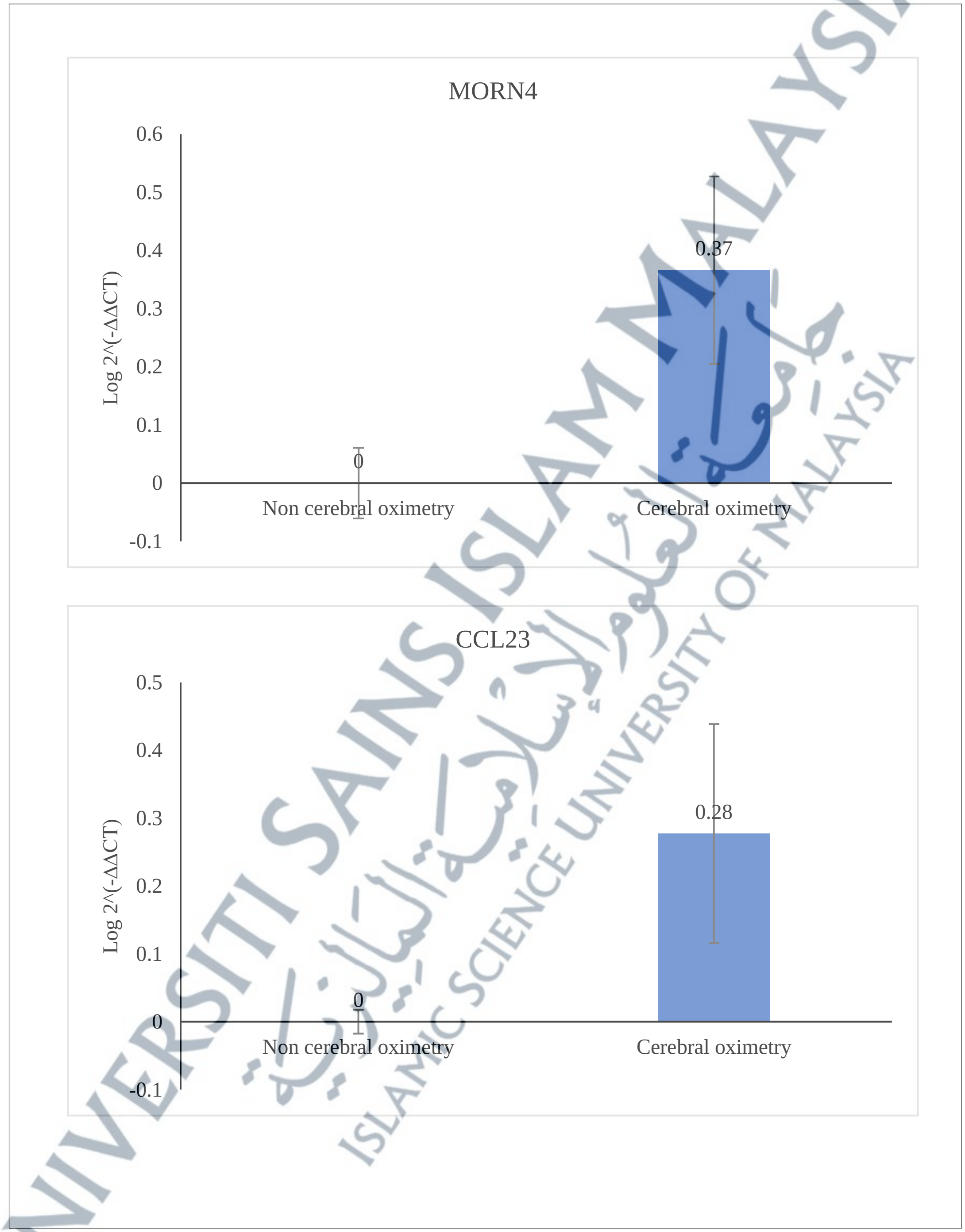


Figure 4.17: Bar chart showing fold change of MORN4 and CCL23 genes.

4.3.4 Comparison 4: Pre vs Postoperative Samples in Non-POCD Patients.

For this comparison, 10 samples were selected (refer Figure 4.1). They were sample number 7, 9, 11, 13 and 15 for the preoperative group, while sample number 8, 10, 12, 14 and 16 for the postoperative group (refer table 4.5). Outliers and correlations were detected in the PCA and correlation plot below (Figure 4.18). The PCA plot shows the distribution of the samples, where samples were clustered according to groups, without prominent outliers. For correlation plot, darker red shows a higher correlation between samples.

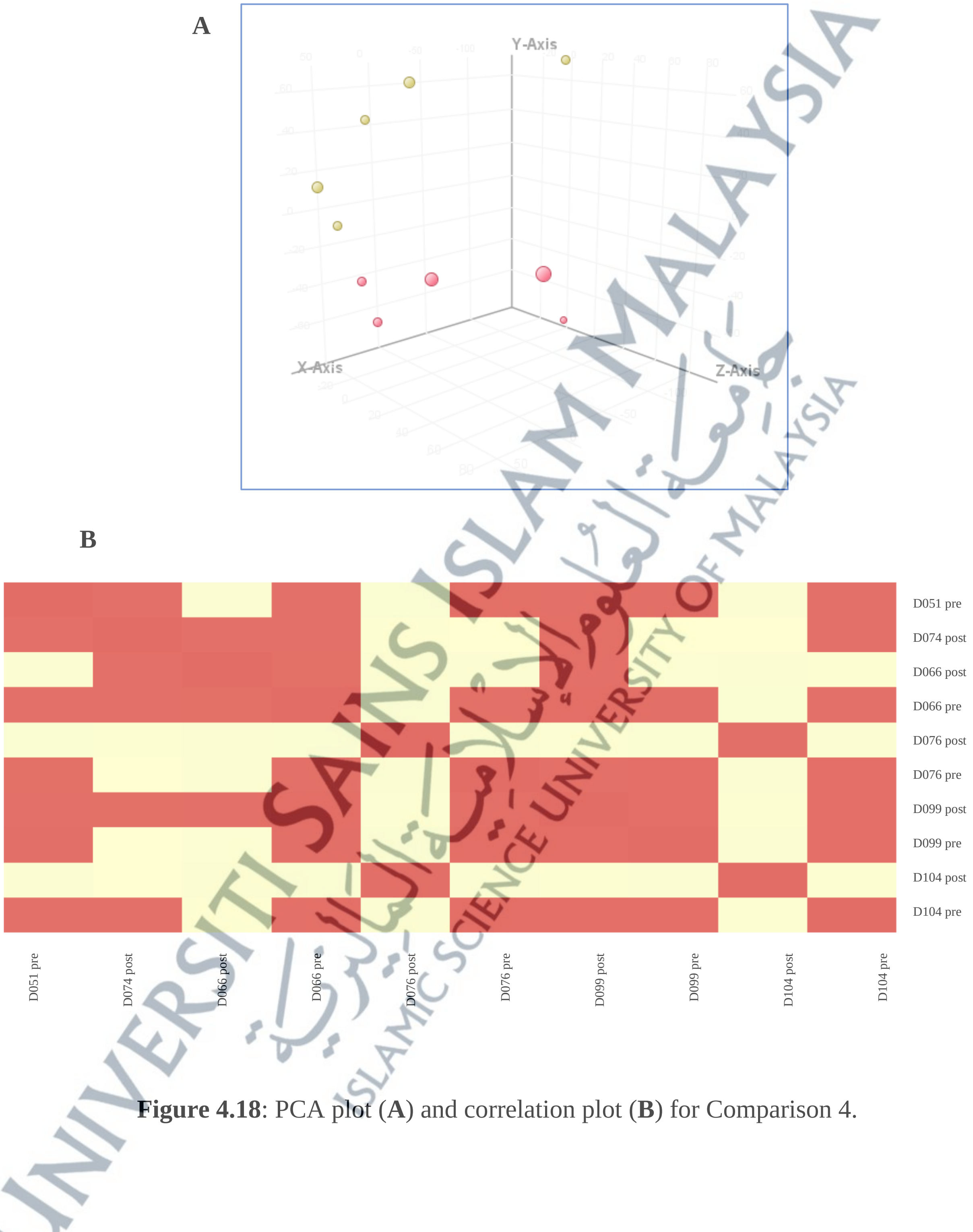


Figure 4.18: PCA plot (**A**) and correlation plot (**B**) for Comparison 4.

i) Differentially Expressed Genes (DEGs)

For this comparison, there are a total of 456 dysregulated genes. There are 225 upregulated genes and 231 downregulated genes. Out of those, we select 10 significant genes of our interest. We selected the genes based on functions and possible association with brain injury or nervous cells. We selected the top 10 genes with highest fold changes value. The genes are listed in Table 4.12. The volcano plot shows upregulated genes in red on the top right and downregulated genes in blue on the top left of the plot. The heatmap shows the upregulated in red and downregulated genes in blue for postoperative and preoperative conditions (Figure 4.19).

Table 4.12: DEGs in postoperative compared to preoperative groups in non-POCD patients.

No	Fold Change	*p*-value	Expression (up/down regulated)	Gene Symbol	Gene description	Gene Function
1	11.05	0.012	up	ADAMTS2	ADAM metallopeptidase with thrombospondin type 1 motif 2	Provides instructions for making an enzyme that processes procollagen molecules
2	-10.84	0.002	down	DCANP1	dendritic cell associated nuclear protein	Activating naïve T cells to initiate antigen-specific immune response
3	9.99	0.002	up	LINC02207	long intergenic non-protein coding RNA 2207	Mediating neutrophil dysfunction in systemic lupus eryhtomatosus
4	8.93	0.018	up	TMCC2	transmembrane and coiled-coil domain family 2	Amyloid precursor protein metabolic process

5	7.69	0.038	up	DSC2	desmocollin 2	Link adjacent cells together in specialized regions known as desmosomes
6	-7.64	0.049	down	SMPD3	sphingomyelin phosphodiesterase 3	Release of microRNAs into extracellular spaces
7	6.95	0.014	up	NSG2	neuronal vesicle trafficking associated 2	AMPAR-binding protein that is required for normal synapse formation and maintenance
8	6.37	0.018	up	ZNF608	zinc finger protein 608	Enable metal ion binding activity
9	-4.03	0.030	down	ADGRG1	adhesion G protein-coupled receptor G1	Central and peripheral nervous systems, reproductive system, muscle hypertrophy, immune regulation, and hematopoietic stem cell generation
10	-3.84	0.019	down	ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif 5	Key protease for versican regulation in murine aortas undergoing dilatation and extracellular matrix remodeling in model of thoracic aortic aneurysms

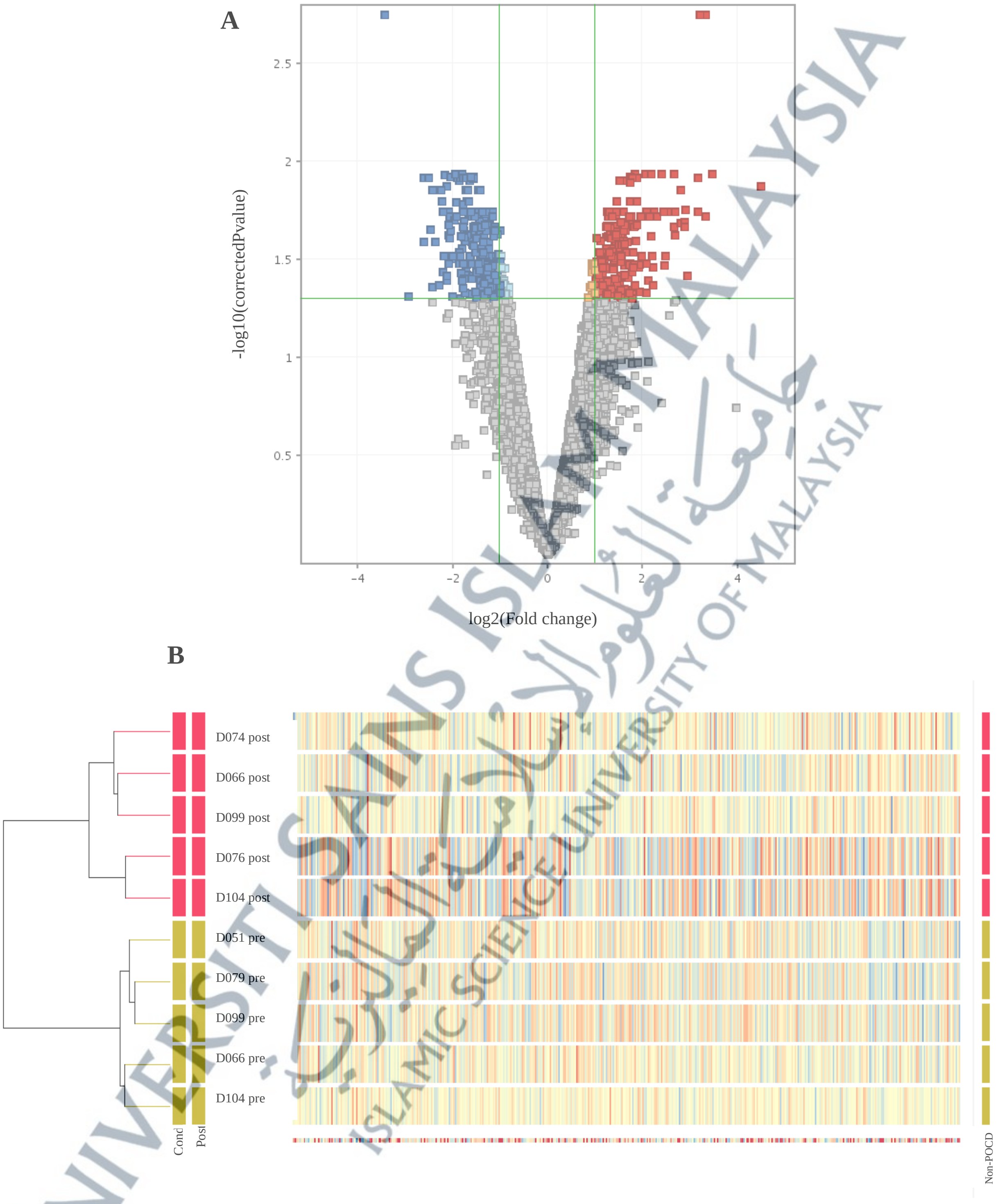


Figure 4.19: Volcano plot **(A)** and heatmap **(B)** for Comparison 4.

i) qPCR validation

We chose 2 genes of interest for validation for this comparison according to their function and possible association with POCD. The genes are ADAMTS2 and DCANP1. However, for gene ADAMTS2, it did not pass the primer efficiency test, hence, no validation for the gene. The figure below shows the amplification curve of the gene with their housekeeping gene, beta-actin (Figure 4.20 – Figure 4.21), CT value and fold change table (Table 4.13) and graphs (Figure 4.22). The fold change for DCANP1 shows downregulation in postoperative group that aligns with microarray results, even though it is not significant.

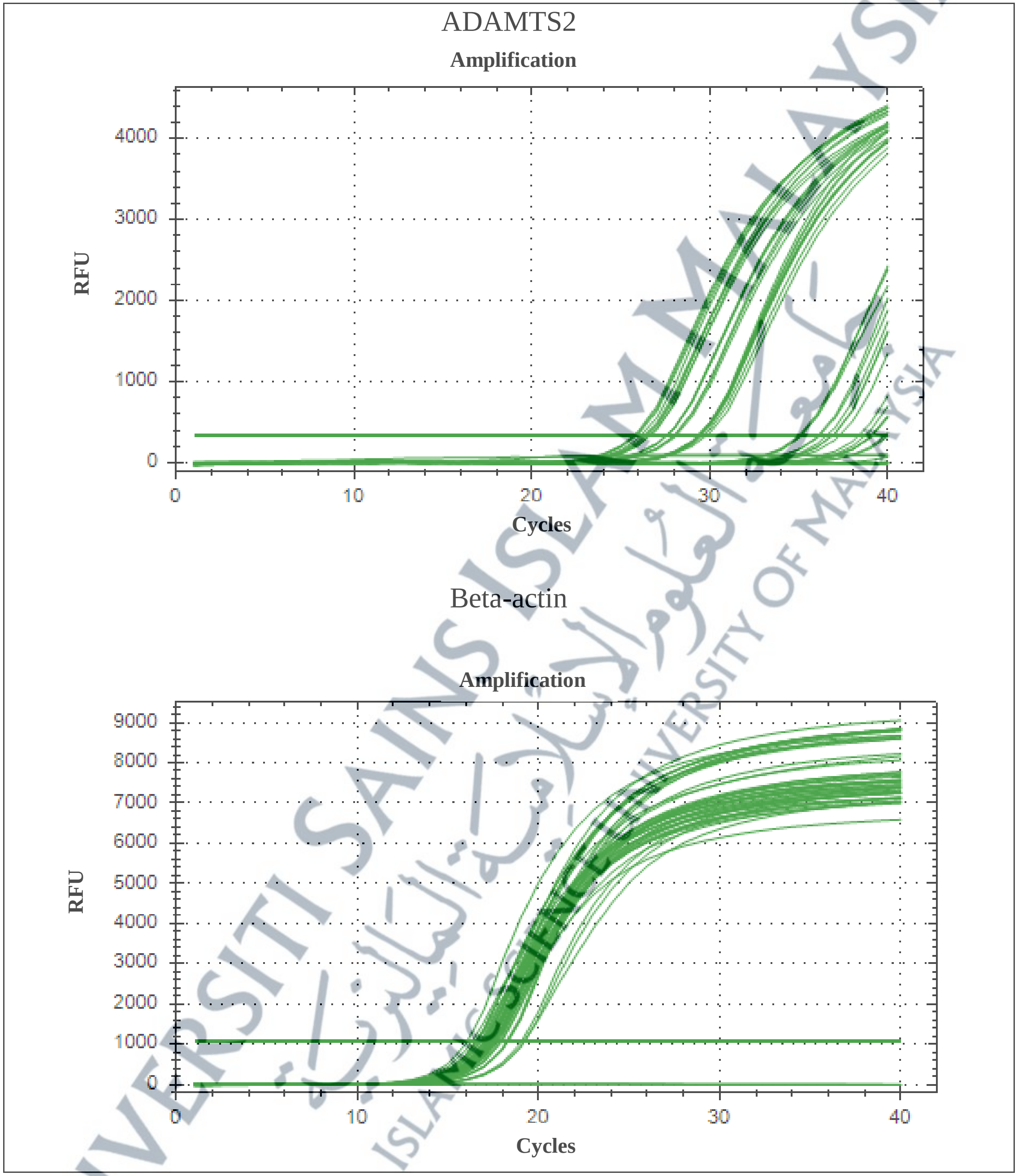
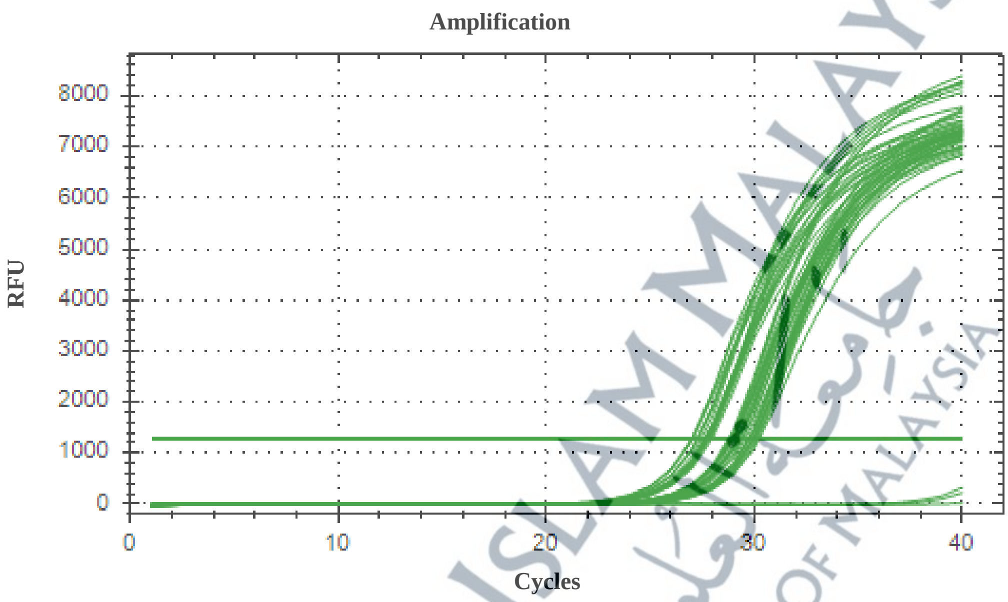


Figure 4.20: Amplification curve for ADAMTS2 gene and beta-actin.

DCANP1



Beta-actin

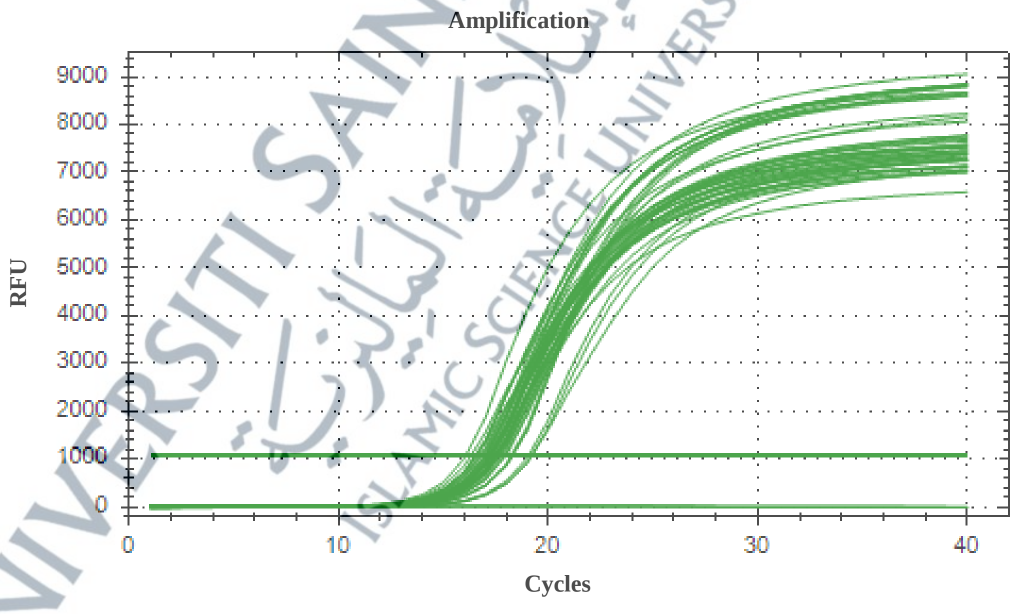


Figure 4.21: Amplification curve for DCANP1 gene and beta-actin.

Table 4.13: CT value and fold change for DCANP1 gene.

Group	Sample no	Avg GOI CT	Avg HG CT	ΔCT	ΔΔCT	2^(-ΔΔCt)	log 2^(-ΔΔCt)	SEM	*p*-value
Pre	9	27.90	17.67	10.23	-0.29	1.22	0.09	0.187	0.18
	11	28.94	17.24	11.70	1.18	0.44	-0.35		
	13	27.04	17.57	9.47	-1.05	2.08	0.32		
	7	29.25	17.04	12.21	1.69	0.31	-0.51		
	15	27.28	18.28	9.00	-1.52	2.86	0.46		
Post	10	28.84	16.55	12.29	1.77	0.29	-0.53	0.128	
	12	27.78	17.29	10.49	-0.03	1.02	0.01		
	14	27.59	16.33	11.26	0.74	0.60	-0.22		
	8	29.81	17.22	12.58	2.06	0.24	-0.62		
	16	30.09	17.43	12.66	2.14	0.23	-0.64		
Average pre				10.52					

CT, cycle threshold; GOI, gene of interest; HG, housekeeping gene; SEM, standard error mean.

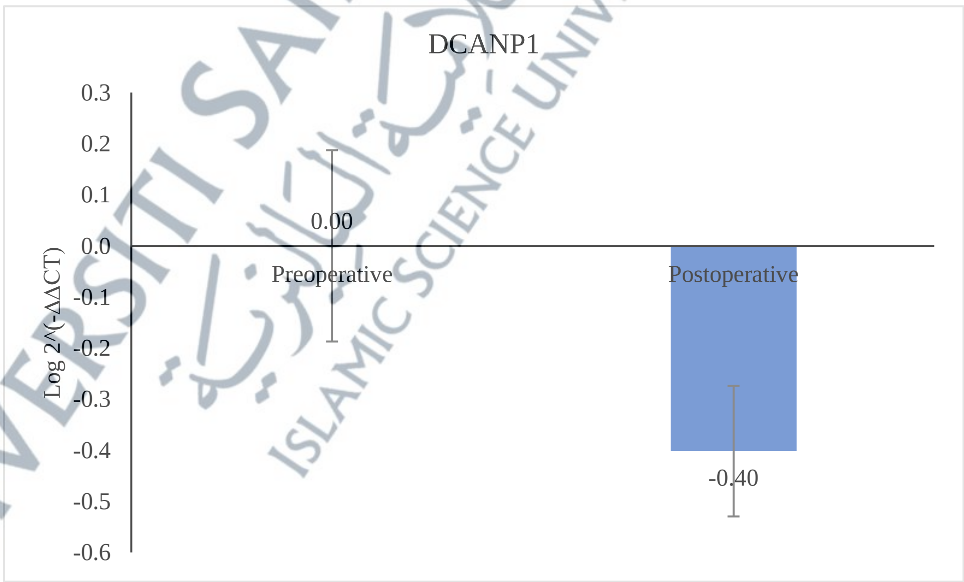


Figure 4.22: Bar chart showing fold change of DCANP1 gene.

4.4 GO and Pathway Analysis

GO analysis of the differentially expressed genes from all comparisons was performed using DAVID. Functional annotation tools were provided in the database to help researchers understand the biological meaning of the gene list. GO terms were analysed for each comparison. Table 4.14 lists the GO terms on cellular components in POCD groups. Functional pathway analysis of the listed genes was performed using KEGG pathway. Two pathways involved our genes in Comparison 1, preoperative versus postoperative POCD groups. The pathways involved are antigen processing and presentation, and natural killer cell-mediated toxicity. The pathways are shown in Figure 4.23 and Figure 4.24 respectively.

Table 4.14: GO Analysis on cellular components and molecular function of targeted genes in POCD and non-POCD groups.

Category	GO Term	GO ID	Gene Count	*p*-value
Cellular components	**POCD**			
	Plasma membrane	GO:0005886	3	0.026
	Membrane	GO:0016020	4	0.055
	Non-POCD			
	Extracellular region	GO:0005576	4	0.042
	Membrane	GO:0016020	5	0.013
	Plasma membrane	GO:0005886	3	0.065
Molecular function	Protein binding	GO:0005515	7	0.047

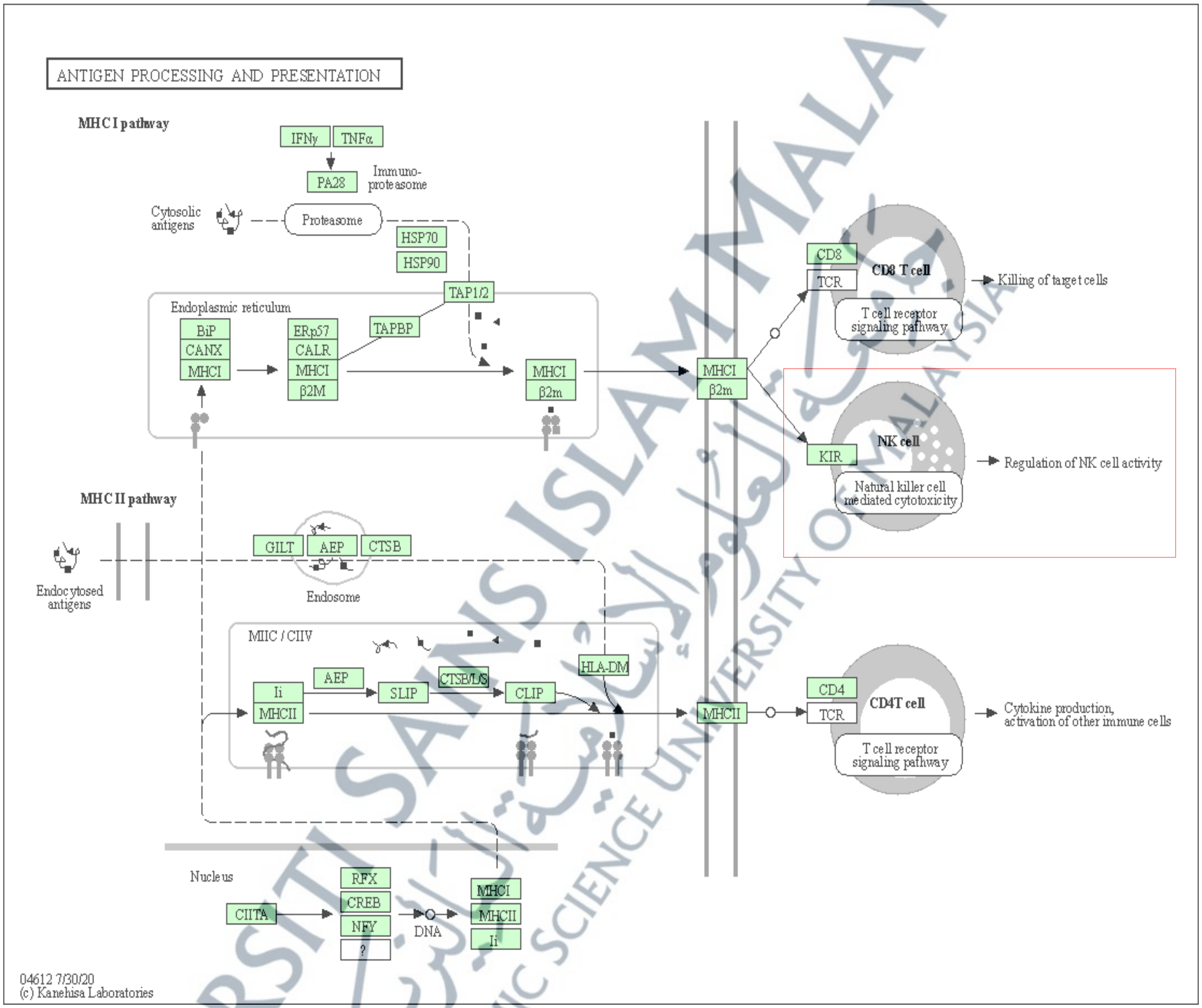


Figure 4.23: Antigen processing and presentation pathway generated from gene list in POCD group. Gene KIR that differentially expressed in POCD group was involved in this pathway. Red box shows the location of the gene in the pathway.

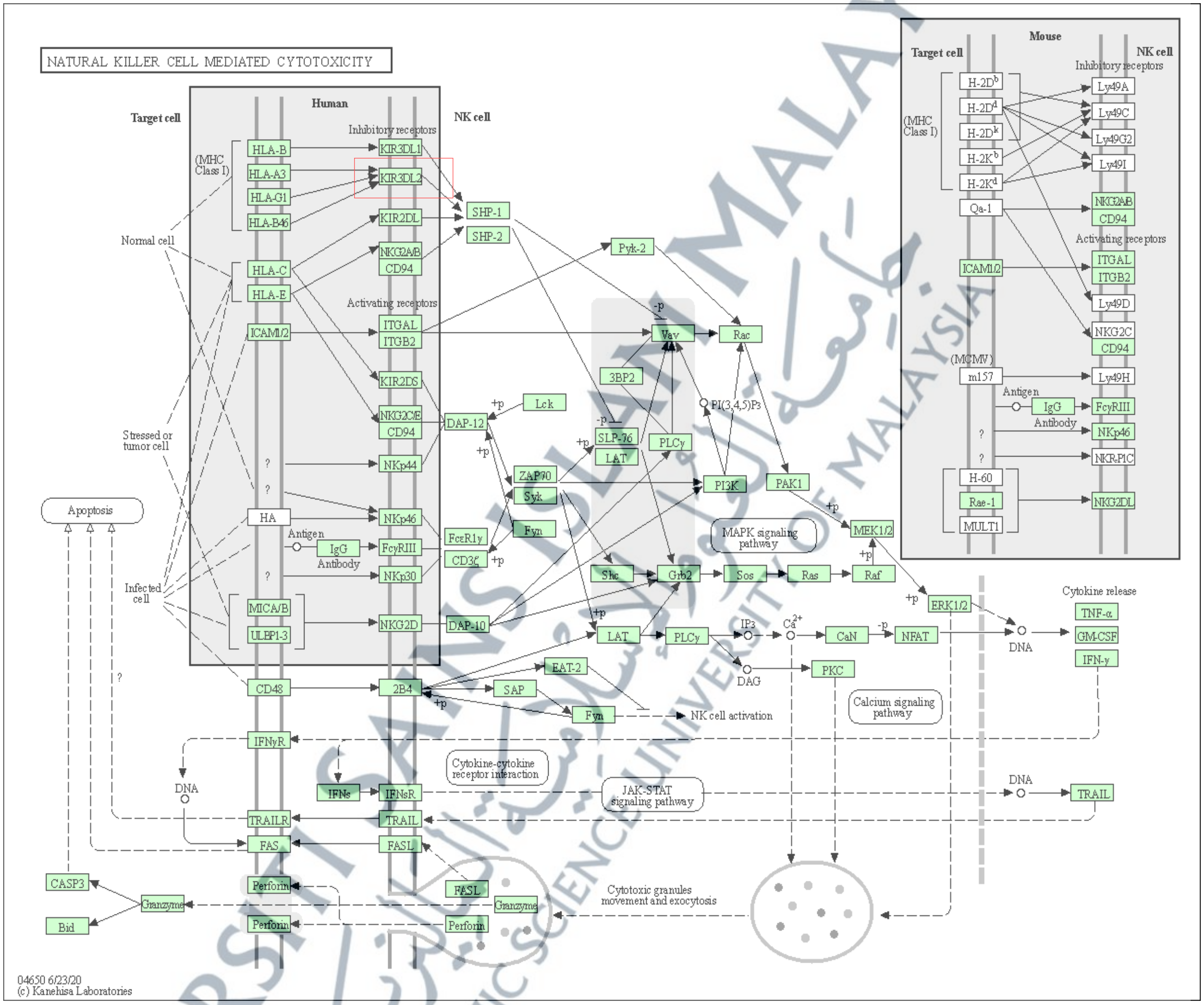


Figure 4.24: Natural killer cell mediated cytotoxicity pathway generated from gene list in POCD group. Gene KIR3DL2 that downregulated in the POCD group was involved in this pathway. Red box shows the location of the gene in the pathway.

4.5 Immunoassay

From the differentially expressed genes in POCD groups, one gene, erythroferrone, ERFE was selected further to analyse its protein concentration in patients' blood samples. ERFE was selected due to its possible association with the development of POCD. Protein concentration analysis was done using enzyme-linked immunosorbent assay (ELISA). There were two groups, POCD and non-POCD group, with a total of 88 samples. Twenty-five samples were in group POCD, while the rest 63 were in non-POCD groups. The calculation of sample size was explained in Chapter 3. All samples were done in duplicate.

Quantitative analysis of samples was performed using four parameters logistic curve (4PL). 4PL curve is a sigmoidal shape curve that commonly used in bioassay. 4PL curve starts as a constant value (asymptote) at low concentration, gradually increases to S shaped, before it gets asymptote again at high concentration. Hence, it is better curve compare to standard linear when analysing immunoassay. Calculation of the standard using 4PL was done using the MyCurveFit add-on in Microsoft Excel. The formula for the standard curve is shown below:

$$\boldsymbol{y = d + \frac{a - d}{1 + \left(\frac{x}{c}\right)^{b}}} \tag{4.1}$$

Where:

a = minimum value that can be obtained
d = maximum value that can be obtained
c = the point of inflection
b = Hill's slope of the curve

The results showed that our standard curve has R^2 value of 0.9999. The standard curve is shown in Figure 4.25. Sample analysis revealed that there is a significant difference in erythroferrone concentration in the POCD group compared to the non-POCD group ($p<0.05$) (Table 4.15). The POCD group has a higher concentration of ERFE (1.072 ± 0.465 ng/ml). This is consistent with our microarray findings that the ERFE gene was upregulated in the POCD group for both groups. The bar graph is shown in Figure 4.26.

a	0.065887
b	0.874767
c	17.07733
d	8.339583
R^2	0.999983

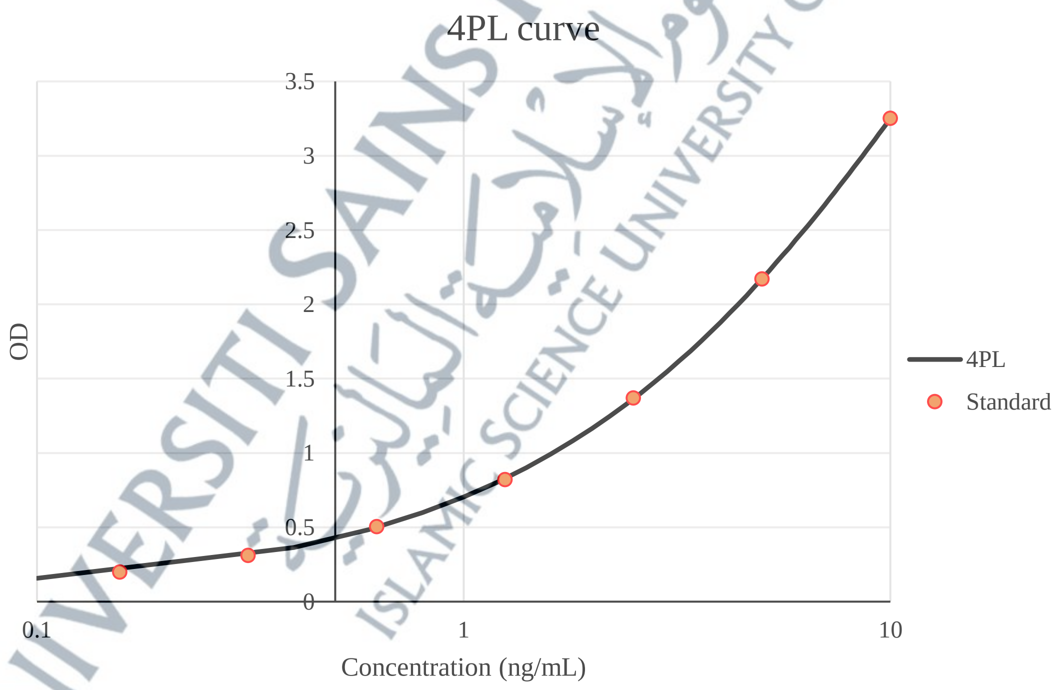


Figure 4.25: Standard curve of protein erythroferrone (ERFE).

Table 4.15: Concentration of ERFE in plasma for POCD and non-POCD group.

	Concentration (ng/ml)		***p*-value**
	Non-POCD (n=63)	**POCD (n=25)**	
Mean	0.764	1.072	<0.05*
Standard Error Mean (SEM)	0.0616	0.0693	
Maximum	2.258	2.258	
Minimum	0.033	0.212	

**p*-value significant at *p*<0.05 using independent sample t-test.

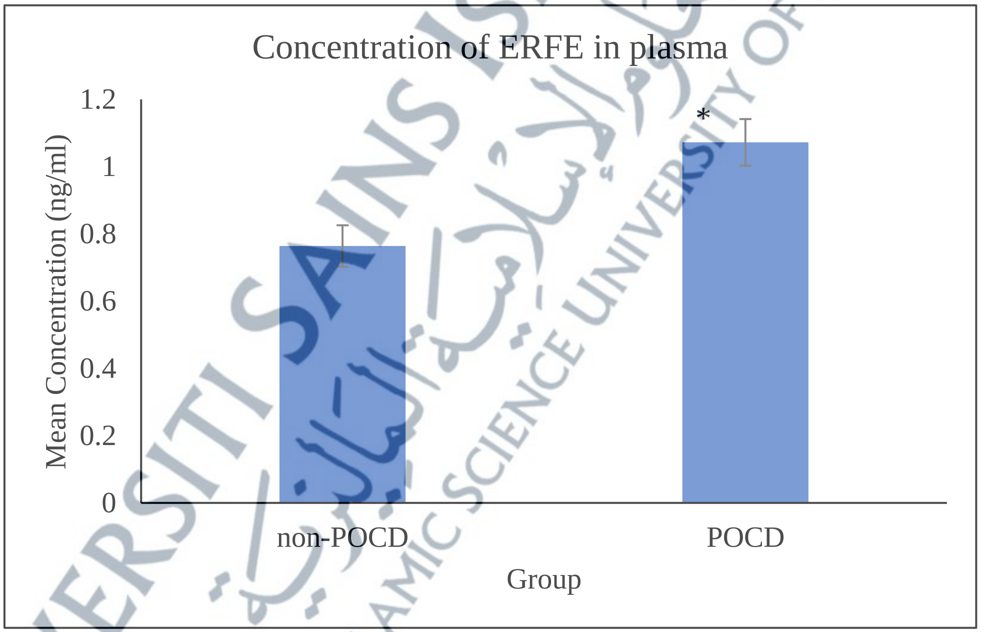


Figure 4.26: Bar chart showing concentration of ERFE in POCD and non-POCD group. *p-value significant at p<0.05 using independent t-test.

The receiver operating characteristics (ROC) curve was generated to predict the performance of ERFE in the development of POCD. The results in Figure 4.27 shows that the area under the curve for ERFE level was 0.683 (95% CI: 0.564 to 0.802, p<0.05). This suggests that postoperative ERFE levels in our samples could moderately predict the occurrence of POCD. The threshold for POCD was set at 0.761 with specificity and sensitivity of 50% and 80%.

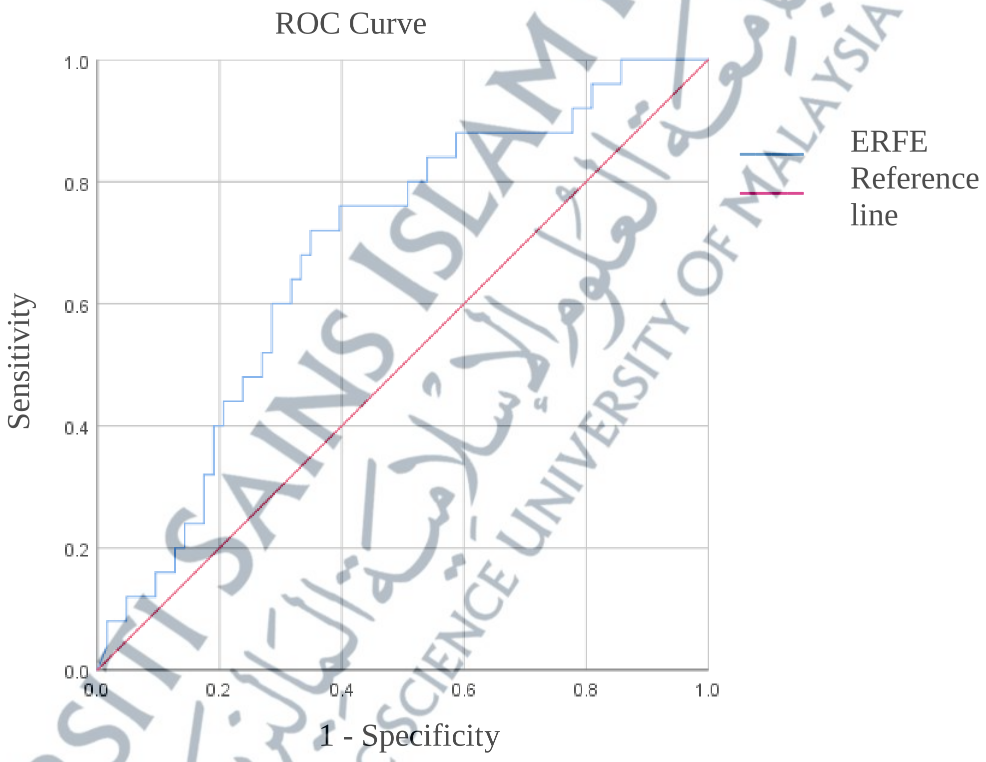


Figure 4.27: ROC curve for postoperative ERFE level.

4.6 Summary

In short, some genes such as KIR2DS3, KIR3DL2, and DCANP1 shows downregulation during microarray and qPCR validation. ERFE on the other hand, did show upregulation in qPCR that concurrent with microarray. However, the high SEM value in preoperative group make it failed to reach the significance. Other genes such as BTNL3, MORN4 and CCL23 show contradicting qPCR results from microarray. Since the samples used for both tests were the same samples, RNA degradation through multiple freeze thaw cycle become the main concern that affected the overall results. Nevertheless, the involvement of all genes found in microarray shows importance in POCD development and will be discussed further in next chapter. The microarray and qPCR validation were summarized into the table 4.16 below.

Table 4.16: Summary of microarray, qPCR validation and immunoassay results.

Comparison	Genes	Microarray regulation	qPCR validation	Significant	Protein significant
1	ERFE	Up	Up	No	Yes
	KIR2DS3	Down	Down	Yes	-
	KIR3DL2	Down	Down	Yes	-
	KIR2DS2	Down	Not passed	-	-
2	BTNL3	Down	Up	No	-
3	MORN4	Down	Up	No	-
	CCL23	Down	Up	No	-
	GPR37L1	Down	Not passed	-	-
4	ADAMTS2	Up	Not passed	-	-
	DCANP1	Down	Down	No	-