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Stability and Expression of Selected miRNAs, circRNAs, and rRNAs in Swiss Albino Mice Tissues to Preditc Port-Mortem Interval

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Abstract. In forensics, post-mortem interval (PMI) estimation is essential. Researchers have devised various approaches to accurately determine PMI, consequently, ribonucleic acid (RNA) molecules could be useful PMI estimation tool. This study aims to ascertain the stability and expression of some ribosomal RNAs, micro-RNAs, and circular-RNAs in rat post-mortem liver, heart and muscle tissues. Fifty healthy adult Swiss albino mice were divided into five groups, sacrificed and target tissues were harvested. These genes- *miR-122, 18S, miR-133a, RPS18, Circ-LRP6, 5S rRNA, Circ-AFF1, Circ-Ogdh, LC-Ogdh*, *U6* and *GAPDH*- were selected for the study. Extracted RNA was subjected to spectrophotometric analysis, complementary Deoxyribonucleic acid (cDNA) synthesis and amplified by Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using gene-specific primers. For liver samples, *miR-122* and *5S* had the highest and lowest Cq-values respectively. There was a significant difference between the expression of *GAPDH and Circ-Ogdh* and *5S* ($p \leq 0.05$). For the heart and muscle samples, *miR-122* and *Circ-LRP6* had the highest Cq-value respectively. In the heart, there was significant difference between the expression of *GAPDH and miR-122, miR-133a* and *RPS-18* ($p \leq 0.05$). In muscle tissue, there was significant difference between the expression of *GAPDH and U6,* Circ-*AFF1* and *Circ-LRP6* ($p \leq 0.05$). This study shows that *miR-122* and *LC-Ogdh* (liver tissues), *miR-122, miR-133a and Cir-LRP6* (heart *tissues), and miR-122* and *Circ-AFF1* (muscle tissues) are suitable reference genes for PMI estimation using *GAPDH* as a reference gene. In conclusion, a forensic method for PMI assessment may use a combination of tissue-specific miRNAs, ribosomal RNAs, SnRNAs, CircRNAs, and CircRNA+mRNA.

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1. Introduction

Determination of the time between physiological death and the analysis of the dead body is a major issue in Forensics, and researchers are continually looking for ways to estimate the time of death accurately. Molecular changes for instance protein, DNA, and RNA degradation are examined for a more accurate assessment of Post-mortem interval (PMI)[1]. Studies have found a correlation between RNA degradation and PMI[2,3]. Due to its high sensitivity and wide range of instruments and commercial assays, quantitative reverse transcriptase real-time polymerase chain reaction (RTqPCR) has been regarded as a useful technique for detecting RNA profiles[4]. Many forensic laboratories also monitor mRNA degradation to estimate PMI[5]. However, efforts to find a correlation between RNA degradation and PMI were not entirely successful because of the lack of a stable reference gene that is suitable for the degraded tissue[6].

Housekeeping genes and small nuclear RNA (snRNA) are commonly used as reference controls for fresh tissues. However, scientists have shown that some of them showed variability in their expression levels, both in terms of stability between different tissues and different post-mortem intervals[7]. MicroRNAs (miRNAs) and circular RNAs are being considered as alternatives. MicroRNAs, a small non-coding RNA class of 18-25 nucleotides in length, are also considered to be very stable[8]. A novel class of noncoding RNA - Circular RNAs (circRNAs) - are characterized by the presence of a covalent bond linking the 3′and 5′ ends[9,10,11]. CircRNAs are generated by backsplicing, which makes them much more stable than linear RNA[9,12,13]. Due to its high stability, abundance, and tissue-specific expression patterns, CircRNA is very attractive for studies investigating its potential as a molecular marker for disease diagnosis[14]. CircRNA is stable in cells and not easily degraded by nucleases[11,15] and is expected to be useful for estimating PMI. One limitation that restricts the application of CircRNAs is that CircRNAs may be barely detectable in some tissues and to address this limitation, the inclusion of both circular and linear RNAs (LC-RNAs) in mRNA profiling has been indicated to increase detection sensitivity[16].

Therefore, detecting CircRNAs or LC-RNAs when necessary, confers a promising strategy for PMI estimation.

The study aimed to assess the stability of some selected mRNA, rRNAs, miRNAs and the newly emerged CircRNAs in post-mortem tissues and their potential use in the estimation of PMI.

2. Materials and methods

2.1. Ethical approval

This project was conducted according to the rules and regulations of the University of Lagos Ethical Committee on the use of experimental animals. The ethical clearance was obtained from the Health Research Ethics Committee of the College of Medicine, University of Lagos, Nigeria (CMULHREC Number: CMUL/ACUREC/05/21/858).

2.2. Experimental animals, sample collection and storage

Fifty healthy adult Swiss albino mice each weighing 25–30 g were purchased from Biovaccines Nigeria Limited, Yaba, Lagos. The animals were housed in mice cages at normal room temperature with dark and light cycle (12/12 hr). They were fed with standard mouse pellet diet and tap water *ad libitum*. The animals were kept for two weeks in order to ensure proper acclimatization before commencing the experiment.

The mice were randomly divided into five groups: control group (PMI = Day 0) and experimental groups (PMI = 1, 2, 3, and 4 days, respectively) with 10 mice per group. All mice were sacrificed by cervical vertebra dislocation and placed in an iron cage. The experiment was carried out in the animal house in the Zoological Garden at University of Lagos, Nigeria. The average temperature and humidity of the days of sample collection were recorded. For the duration of the experiment, the average temperature (29.5 ^{o}C) humidity (71.7 %) and atmospheric pressure (29.94 inHg) was calculated. Liver, heart and muscle tissue samples were collected from all the mice at designated times in labelled Eppendorf tubes containing 1ml of TRIzol® solution each and stored at -80 °C until analysis.

2.3. Ribonucleic acid extraction

All the reagents, materials, and instruments were handled with care to ensure they were nuclease-free, to minimize the risk of RNA degradation during the experimental

process. Ribonucleic acid was extracted from each tissue sample using Trizol reagent[6]. Approximately 50 mg of each tissue sample was minced and homogenized with 1 mL of Trizol solvent and 0.2 mL chloroform, then centrifuged at 12,000 rpm for 15 minutes. The supernatant was decanted into new tubes and 0.5 mL of isopropanol was added to the supernatant. This was followed by precipitation at -80 °C for 30 minutes. The mixture was then centrifuged at 12,000 rpm for 10 min, after which the supernatant was discarded, and the precipitate was washed with 75 % ethanol. The samples were centrifuged, and the supernatant was discarded. Thereafter, total RNAs were adjusted to a concentration equivalent to 500 ng/ µL using nuclease-free water and stored at -80 °C until further use. The TRIzol® method of RNA extraction was chosen due to its several advantages which include high yield and quality, simplicity of workflow, and compatibility with downstream application.

2.4. Spectrophotometric analysis

The quality and quantity of the RNA extracts were determined spectrophotometrically by analysing absorption ratios: A260/230 and A260/280 using a Nanodrop 1000 spectrophotometer (6305 JENWAY spectrometer).

2.5. Housekeeping gene and target cenes selection

The genes used in this experiment were chosen based on the results of earlier studies that showed their stability. The gene, GAPDH was used as the housekeeping gene. The sequences of the primers used in this study are shown in Table 1.

2.6. Real time quantitative PCR (RT-qPCR)

The qPCR was carried out using the Chelex Connect Real-time Machine (Bio-Rad) in 96 well plates with a reaction volume of 20 µL and runs up to 40 cycles. The components of the qPCR reaction mix need included 4 µL of 5X HOT FIREPol EvaGreen qPCR Supermix, 0.5 µL each of the forward and reverse primers, 5 µL of DNA template and 10 µL H_2O PCR grade. The qPCR cycles were carried out in detailed fashion: the first stage was the activation of the polymerase which included an incubation step at 95 °C and was carried out for 12 minutes and it went for 1 cycle, this was followed by the denaturation step which occurred at a temperature of 95 °C for 15 seconds and was repeated for 40 cycles. After denaturation step, the annealing step was the next step, it was carried out at 60-65 °C for 20 seconds and it

was also repeated for 40 cycles. Lastly, the elongation step was carried out for 20 seconds and the process was also repeated for 40 cycles.

Table 1. Selected genes and their forward and reverse primers[10].

Gene (category)	Organ	Forward Primer	Reverse Primer
GAPDH **(mRNA)**	Reference Gene	TGGCAAAGTGGAGATTGTTG	CATTCTCGGCCTTGACTGTG
18S **(rRNA)**	Heart	AGAAACGGCTACCACATCCA	CCCTCCAATGGATCCTCGTT
miR-122 **(miRNA)**	Heart, Liver, Muscle	GGCTGTGGAGTGTGACAATG	GAGGTATTCGCACCAGAGGA
miR-133a **(miRNA)**	Heart, Muscle	AAGCTAGCGAATTCCATGTGACCCCTCACACACA	TTCTCGAGACAAGGGGAGCCTG
RPS18 **(mRNA)**	Heart	AAGAGGGCTGGAGAACTCAC	GCAGCTTGTTGTCTAGACCG
Circ-LRP6 **(CirRNA)**	Heart	TAAATTCTGTTCGTTTAATGGCTTC	AGCTATTGCCTTAGATCCTTCAAGT
5S rRNA **(rRNA)**	Liver	CATACCACCCTGAACGCG	CTACAGCACCCGGTATTCCC
Circ-AFF1 **(CirRNA)**	Liver, Muscle	CAGCAAAGCTTTGAGAAACCAGA	CATGACCTAAGGGATATCTGGGC
Circ-Ogdh **(CirRNA)**	Liver	ATACGAGGGCACCATGTAG	AGCTTCGACTCAGGGAAAGG
LC-Ogdh **(CirRNA+ mRNA)**	Liver, Muscle	GGTGGAAGCACAACCTAACG	TGAGATAATGTCAGCGGGCA
U6 **(SnRNA)**	Muscle	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

2.7. Statistical analysis

All numerical data from this study was subjected to statistical analysis using the statistical package IBM SPSS version 26. Mean values ± Standard Error of Mean (SEM) were calculated. P-values equal to or less than 0.05 and or 0.01 were considered as statistically significant. Correlations between variables were done using Pearson correlation coefficients. The bar chart was constructed using Microsoft Excel (2019).

3. Results

3.1. Descriptive statistics of spectrophotometry analysis

The spectrophotometry analysis of RNA samples obtained from the heart, liver and muscle tissues were subjected to statistical analysis using IBM SPSS (v. 26). Of the three organs, the highest purity yield was obtained from heart tissue (1.98 ± 0.04),

while the lowest was observed in the muscle tissues (0.75 ± 0.26) on days 1 and 3 respectively.

Table 2. Descriptive statistics of spectrophotometric values. Values are represented as Mean ± Standard Error of Mean (S.E.M).

		Day 1	Day 2	Day 3	Day 4
Heart	**Purity** (260/280)	1.98 ± 0.04	1.8 ± 0.01	1.73 ± 0.07	1.58 ± 0.07
	Concentration (ng/uL)	1997.07 ± 568.22	1773. ± 196.23	781.6 ± 80.46	148.9 ± 0.63
Liver	**Purity** (260/280)	1.41 ± 0.99	1.49 ± 0.06	1.24 ± 0.56	1.97 ± 0.02
	Concentration (ng/uL)	3354.17 ± 103.56	3747.30 ± 34.66	2341.13 ± 472.25	1020.50 ± 2.00
Muscle	**Purity** (260/280)	1.68 ± 0.04	1.69 ± 0.04	0.75 ± 0.26	1.79 ± 0.02
	Concentration (ng/uL)	1709.13 ± 423.80	774.83 ± 187.76	282.80 ± 2.00	230.87 ± 50.78

Within the muscle tissues groups, the highest purity value (1.79 ± 0.02) was on the 4th day, while the lowest purity value (0.75 ± 0.26) was on day 3. The highest (1709.13 ± 423.80 ng/µL) and lowest (230.87 ± 50.78 ng/µL) concentration values were from day 2 and 3 in that order.

Spectrophotometric values of the heart tissues showed that day 1 and day 4 exhibited the highest (1.98 ± 0.04) and lowest (1.58 ± 0.06) purity values respectively. The highest concentration value (1997.07 ± 568.22 ng/µL) was obtained from day 1, while the lowest value (148.90 ± 2.00 ng/µL) was obtained from day 4.

The highest (1.97 ± 0.02) and lowest (1.41 ± 0.99) purity values of the liver tissues were from days 4 and 1 respectively. The highest concentration value (3354.17 ± 103.56 ng/uL) was obtained from day 2 while the lowest concentration value (1020.50 ± 2.00 ng/uL) was obtained from day 4. The descriptive statistics is represented in Table 2.

3.2. Pearson Correlation Analysis of the Spectrophotometric Values

Pearson correlation analysis was carried out using the spectrophotometric values as shown in Tables 3, 4 and 5. Within the heart tissue samples, there was positive correlation ($P \leq 0.05$) between total RNA concentration of days 1 and 2, days 1 and 4, and days 2 and 4 respectively. A negative correlation ($P \leq 0.05$) was observed between the total RNA purity of days 1 and day 4 while there was positive correlation ($P \leq 0.05$) between total RNA purity of days 2 and 4 and days 3 and 4 respectively as shown in Table 3.

Table 3. Pearson correlation output of spectrophotometric values of RNA extracts from heart tissue samples. Correlation values represented with an asterisk (*) were found to be significant at the 0.05 level. Correlation significant at the 0.01 level is represented with two asterisks (**).

		Day 1		Day 2		Day 3		Day 4	
Day	Parameter	Purity	Conc (ng/µL)	Purity	Conc (ng/µL)	Purity	Conc (ng/µL)	Purity	Conc (ng/µL)
Day 1	Purity	1	-0.45 (0.37)	0.75 (0.09)	-0.04 (0.94)	-0.04 (0.94)	-0.97** (0.00)	-1.00**	-1.00**
	Conc (ng/µL)	-0.45 (0.37)	1	-0.89* (0.02)	0.90** (0.01)	0.90** (0.01)	0.36 (0.48)	1.00**	1.00**
Day 2	Purity	0.75 (0.09)	-0.89* (0.02)	1	-0.61 (0.20)	-0.62 (0.21)	-0.73 (0.10)	1.00**	1.00**
	Conc (ng/µL)	-0.04 (0.94)	0.90** (0.01)	-0.61 (0.20)	1	0.99** (0.00)	-0.07 (0.90)	1.00**	1.00**
Day 3	Purity	-0.04 (0.94)	0.90** (0.01)	-0.62 (0.19)	0.99** (0.00)	1	-0.06 (0.91)	1.00**	1.00**
	Conc (ng/µL)	-0.97 ** (0.00)	0.36 (0.49)	-0.73 (0.10)	-0.07 (0.90)	-0.06 (0.91)	1	1.00**	1.00**
Day 4	Purity	-1.00 **	1.00**	1.00**	1.00**	1.00**	1.00**	1	1.00**
	Conc (ng/µL)	-1.00**	1.00**	1.00**	1.00**	1.00**	1.00**	1.00**	1

For the purity value of the liver tissue samples (Table 4), there was correlation ($P \leq 0.01$) between days 1 and 3, and days 1, 2 and 3 and day 4 respectively. Compared to day 3, days 1 and 2 concentration values showed significant correlation ($P \leq 0.05$). There was also a correlation between the concentration values on days 1, 2 and 3 compared to day 4.

Table 4. Pearson correlation analysis output of Spectrophotometric values of RNA extracts from liver tissue samples. Correlation values represented with an asterisk (*) were found to be significant at the 0.05 level. Correlation significant at the 0.01 level is represented with two asterisks (**).

		Day 1		Day 2		Day 3		Day 4	
Day	Parameter	Purity	Conc.	Purity	Conc.	Purity	Conc.	Purity	Conc.
Day 1	Purity	1	-0.99** (0.00)	-0.42 (0.40)	0.49 (0.31)	-0.97** (0.01)	0.90** (0.01)	1.00**	1.00**
	Conc. (ng/µL)	-0.99** (0.00)	1	0.44 (0.38)	-0.50 (0.31)	0.99** (0.00)	-0.90** (0.01)	1.00**	1.00**
Day 2	Purity	-0.42 (0.41)	0.44 (0.39)	1	-0.99** (0.00)	0.45 (0.37)	-0.77 (0.07)	1.00**	1.00**
	Conc. (ng/µL)	0.49 (0.31)	-0.50 (0.31)	-0.99** (0.00)	1	-0.49 (0.32)	0.08* (0.05)	1.00**	1.00**
Day 3	Purity	-0.97** (0.01)	0.99** (0.00)	0.45 (0.37)	-0.49 (0.32)	1	-0.89* (0.02)	1.00**	1.00**
	Conc. (ng/µL)	0.90** (0.01)	-0.90** (0.01)	-0.77 (0.07)	0.82* (0.05)	-0.89* (0.02)	1	1.00**	1.00**
Day 4	Purity	1.00**	1.00**	1.00**	1.00**	1.00**	1.00**	1	1.00**
	Conc. (ng/µL)	1.00**	1.00**	1.00**	1.00**	1.00**	1.00**	1.00**	1

For the muscle tissues samples, there was positive correlation (P ≤ 0.05) between total RNA purity of days 1, 2 and 3 and day 4 respectively. A positive correlation (P ≤ 0.05) was observed between the total RNA concentration of days 1 and 3 and days 3 and 4 while days 2 and 4 showed negative correlation (P ≤ 0.05) for total RNA concentration as shown in Table 5.

Table 5. Pearson correlation analysis output of Spectrophotometric values of RNA extracts from muscle tissue samples. Correlation values represented with an asterisk (*) were found to be significant at the 0.05 level. Correlation significant at the 0.01 level is represented with two asterisks (**).

Day	Parameter	Day 1		Day 2		Day 3		Day 4	
		Purity	Conc.	Purity	Conc.	Purity	Conc.	Purity	Conc.
Day 1	Purity	1	-0.887* (0.018)	0.359 (0.484)	0.742 (0.091)	-0.196 (0.709)	-0.350 (0.497)	1.000** .	1.000** .
	Conc. (ng/µL)	-0.887* (0.018)	1	0.038 (0.943)	-0.449 (0.372)	0.596 (0.211)	0.705 (0.118)	1.000** .	1.000** .
Day 2	Purity	0.359 (0.484)	0.038 (0.943)	1	0.855* (0.030)	0.762 (0.078)	0.720 (0.107)	1.000** .	1.000** .
	Conc. (ng/µL)	0.742 (0.091)	-0.449 (0.372)	0.855* (0.030)	1	0.428 (0.397)	0.317 (0.540)	1.000** .	1.000** .
Day 3	Purity	-0.196 (0.709)	0.596 (0.211)	0.762 (0.078)	0.428 (0.397)	1	0.974** (0.001)	1.000** .	1.000** .
	Conc. (ng/µL)	-0.350 (0.497)	0.705 (0.118)	0.720 (0.107)	0.317 (0.540)	0.974** (0.001)	1	1.000** .	1.000** .
Day 4	Purity	1.000** .	1.000** .	1.000** .	1.000** .	1.000** .	1.000** .	1	1.000** .
	Conc. (ng/µL)	1.000** .	1.000** .	1.000** .	1.000** .	1.000** .	1.000** .	1.000** .	1

3.3. Descriptive statistics of RT-qPCR values of heart genes

The Cq values obtained from the real-time quantitative PCR of the isolated total RNA of heart tissues were compared against the days of study as shown in Table 6. Of all the genes investigated, *miR-133a* on the 3rd day had the highest Cq value. There was a downward trend in the Cq values of *miR-122* from day 2 to day 4. Similarly, *18S* and *RPS-18* had a downward trend from day 1 to day 4. However, *Circ-LRP6* Cq values did not exhibit a pattern, while *mir-133a* had a decrease after day 3.

The Cq values obtained from the real-time quantitative PCR of the isolated total RNA of liver tissues were compared to the days of study as shown in Table 7. The highest Cq value (36.42 ± 1.15) was obtained from *miR-122* on day 4. However, *5S* on day 4 had the lowest Cq value (18.08 ± 1.15). *5S* had a downward trend from day 1 to day 4. It was observed that *LC-Ogdh* had a downward trend from day 1 to day 3, with an increase in day 4.

Table 6. Descriptive Statistics of RT-qPCR values of Heart Genes. Values are represented as Mean ± Standard Error of Mean (S.E.M).

	Day 1	Day 2	Day 3	Day 4
miR-122	37.31 ± 0.82	39.27 ± 1.15	33.48 ± 1.15	32.93 ± 1.15
mir-133a	35.01 ± 1.45	34.13 ± 1.19	39.37 ± 1.15	23.61 ± 0.96
18S	36.57 ± 0.88	35.68 ± 0.73	33.53 ± 1.18	26.32 ± 1.15
Circ-LRP6	39.07 ± 0.78	35.80 ± 1.15	37.31 ± 0.74	35.89 ± 1.15
RPS-18	32.99 ± 3.84	33.98 ± 1.93	23.97 ± 1.49	20.16 ± 1.15
GAPDH	33.85 ± 0.88	32.36 ± 0.90	33.01 ± 0.22	34.01 ± 2.86

Table 7. Descriptive Statistics of RT-qPCR values of Liver Genes. Values are represented as Mean ± Standard Error of Mean (S.E.M).

	Day 1	Day 2	Day 3	Day 4
miR-122	33.40 ± 0.66	36.74 ± 1.21	34.43 ± 1.15	36.42 ± 1.15
Circ-AFF1	32.42 ± 1.15	29.37 ± 0.96	32.02 ± 1.42	29.85 ± 1.15
LC-Ogdh	35.02 ± 1.93	31.14 ± 1.78	30.67 ± 0.88	36.28 ± 1.15
Circ-Ogdh	32.92 ± 0.59	28.41 ± 0.84	30.45 ± 1.15	32.89 ± 2.40
5S	36.90 ± 0.97	35.38 ± 1.16	26.08 ± 1.15	18.08 ± 1.15
GAPDH	33.85 ± 0.88	32.36 ± 0.90	33.01 ± 0.22	34.01 ± 2.86

The Cq values obtained from the real-time quantitative PCR of the isolated total RNA of muscle tissues were compared against the days of study as shown in Table 8. The highest Cq value (40.91 ± 1.15) was obtained from *Circ-LRP6* on day 1. However, *U6* on day 4 had the lowest Cq value (18.17 ± 1.15). A downward trend was observed in *U6* and *Circ-LRP6* from day 1 to day 4.

Table 8. Descriptive Statistics of RT-qPCR values of Muscle Genes. Values are represented as Mean ± Standard Error of Mean (S.E.M).

	Day 1	Day 2	Day 3	Day 4
U6	34.35 ± 1.24	29.92 ± 1.59	25.56 ± 2.12	18.17 ± 1.15
miR-122	34.25 ± 1.01	35.59 ± 1.15	31.67 ± 0.88	34.03 ± 2.00
LC-Ogdh	31.30 ± 0.73	33.95 ± 0.61	32.47 ± 1.46	35.04 ± 1.15
Circ-AFF1	32.64 ± 1.15	23.02 ± 1.15	25.69 ± 2.68	35.94 ± 1.15
Circ-LRP6	40.91 ± 1.15	36.13 ± 1.15	32.37 ± 0.76	33.47 ± 1.15
GAPDH	33.85 ± 0.88	32.36 ± 0.90	33.01 ± 0.22	34.01 ± 2.86

3.4. Pearson correlation coefficient analysis of studied genes for the heart, liver and muscle tissues with the reference gene (*GAPDH*)

Pearson correlation analysis of the genes of interest for the heart tissues against the reference gene (GAPDH) revealed that there was a positive correlation between *GAPDH* (day 1) and *miR-133a* (day 2) ($r = -1.00$, $p \leq 0.05$) and *RPS-18* (day 2) ($r = -1.00$, $p \leq 0.05$) respectively as shown in Table 9. This suggests a significant relationship between these biomarkers. This also implies that they may serve as valuable biomarkers in understanding changes in gene expression over time in heart tissues, potentially contributing to more accurate PMI estimations. There was no correlation between *miR-122* and *GAPDH* on any day. Similarly, there was no correlation between *GAPDH* and *18S, and GAPDH and Circ-LRP6.*

Table 9. Pearson correlation output of studied genes for the heart against the reference gene (*GAPDH*). Correlation values represented with an asterisk (*) were found to be significant at the 0.05 level. Correlation significant at the 0.01 level is represented with two asterisks (**).

		GAPDH			
Parameter	Days	Day 1	Day 2	Day 3	Day 4
miR-122	Day 1	-0.92 (0.26)	0.74 (0.47)	0.75 (0.46)	0.64 (0.56)
	Day 2	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 3	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 4	-0.71 (0.50)	0.90 (0.28)	-0.34 (0.78)	0.95 (0.19)
miR-133a	Day 1	0.98 (0.12)	-0.87 (0.34)	-0.59 (0.60)	-0.79 (0.42)
	Day 2	-1.00* (0.02)	0.93 (0.24)	0.46 (0.70)	0.87 (0.33)
	Day 3	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 4	-0.55 (0.51)	-0.24 (0.84)	-0.99 (0.09)	-0.11 (0.93)
18S	Day 1	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 2	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 3	-0.02 (0.99)	0.35 (0.77)	-0.90 (0.29)	0.48 (0.68)
	Day 4	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
Circ-LRP6	Day 1	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 2	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 3	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 4	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
RPS-18	Day 1	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 2	-1.00* (0.01)	0.95 (0.20)	0.41 (0.73)	0.90 (0.29)
	Day 3	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 4	-0.71 (0.50)	0.90 (0.28)	-0.34 (0.78)	0.95 (0.19)

Pearson correlation analysis of the genes of interest for the liver tissues against the reference gene (GAPDH) revealed that there was a positive correlation between *GAPDH* (day 2) and *miR-122* (day 1) (r = -1.00, p ≤ 0.05), and *GAPDH* (day 1) and *Circ-Ogdh* (day 4) (r = -1.00, p ≤ 0.05) as shown in Table 10. This result suggests that *miR-122* may be a reliable biomarker for assessing gene expression changes in liver tissues, potentially aiding in the estimation of PMI. Additionally, this correlation highlights the potential of *Circ-Ogdh* gene as a useful biomarker in tracking gene expression changes over a longer period in liver tissues. The relationship of *miR-122* and *Circ-Ogdh* with GAPDH may provide valuable insights into PMI estimation when used in combination with other markers. There was no correlation between *GAPDH and Circ-AFF1, LC-Ogdh,* and *5S* respectively.

Table 10. Pearson correlation output of studied genes for the liver against the reference gene (GAPDH). Correlation values represented with an asterisk (*) were found to be significant at 0.05 level. Correlation significant at 0.01 level is represented with two asterisks (**).

		GAPDH			
	Days	Day 1	Day 2	Day 3	Day 4
miR-122	Day 1	0.92 (0.26)	-1.00* (0.04)	-0.04 (0.97)	-1.00 (0.05)
	Day 2	0.69 (0.52)	-0.89 (0.30)	0.36 (0.76)	-0.95 (0.21)
	Day 3	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 4	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
Circ – AFF1	Day 1	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 2	-0.65 (0.55)	0.87 (0.33)	-0.41 (0.73)	0.93 (0.24)
	Day 3	0.90 (0.28)	-0.71 (0.50)	-0.78 (0.43)	-0.60 (0.59)
	Day 4	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
LC – Ogdh	Day 1	-0.57 (0.61)	0.27 (0.83)	0.99 (0.11)	0.13 (0.92)
	Day 2	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 3	-0.56 (0.62)	0.81 (0.41)	-0.51 (0.66)	0.88 (0.32)
	Day 4	-0.71 (0.50)	0.90 (0.28)	-0.34 (0.78)	0.95 (0.19)
Circ – Ogdh	Day 1	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 2	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 3	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 4	-1.00* (0.01)	0.95 (0.20)	0.41 (0.73)	0.90 (0.19)
5S	Day 1	0.02 (0.99)	0.32 (0.80)	-0.91 (0.27)	0.45 (0.71)
	Day 2	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 3	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 4	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)

Pearson correlation analysis of the genes of interest for the muscle tissues against the reference gene (*GAPDH*) revealed that there was a positive correlation between *GAPDH* (day 1) and *U6* (day 2) (r = -1.00, p ≤ 0.05), *GAPDH* (day 2) and *miR-122* (day 4) (r = -1.00, p ≤ 0.05), and *GAPDH* (day 2) and *LC-Ogdh* (day 3) (r = -0.99, ≤ 0.05) as shown in Table 11. This implies that there is a strong correlation between these biomarkers. This suggests that they might be useful biomarkers for comprehending how gene expression changes over time in muscle tissues, which could lead to more precise PMI estimations. There was no correlation between *GAPDH and Circ-AFF1,* and *Circ-LRP6* respectively.

Table 11. Pearson correlation output of studied genes for the muscle against the reference gene (*GAPDH*).

		GAPDH			
Parameter	Days	Day 1	Day 2	Day 3	Day 4
U6	Day 1	0.71 (0.50)	-0.90 (0.28)	0.38 (0.78)	-0.95 (0.19)
	Day 2	-1.00* (0.01)	0.95 (0.20)	0.41 (0.73)	0.90 (0.29)
	Day 3	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 4	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
miR-122	Day 1	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 2	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 3	-0.90 (0.29)	0.70 (0.50)	0.78 (0.43)	0.60 (0.59)
	Day 4	-0.97 (0.17)	1.00* (0.05)	0.18 (0.89)	0.98 (0.14)
LC-Ogdh	Day 1	0.48 (0.68)	-0.75 (0.46)	0.59 (0.60)	-0.83 (0.37)
	Day 2	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 3	0.92 (0.25)	-0.99* (0.03)	-0.05 (0.97)	-0.99 (0.06)
	Day 4	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
Circ-AFF1	Day 1	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 2	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 3	-0.71 (0.50)	0.90 (0.28)	-0.34 (0.78)	0.95 (0.19)
	Day 4	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
Circ-LRP6	Day 1	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 2	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 3	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)
	Day 4	0.71 (0.50)	-0.90 (0.28)	0.34 (0.78)	-0.95 (0.19)

3.5. Analysis of variance (ANOVA) test

ANOVA test was used to compare between the genes of interest of the heart, liver and muscle tissues as shown in Tables 12, 13 and 14 respectively. *miR-122, miR-133a, and RPS18* showed a significance at $p \leq 0.05$ as shown in Table 12. There was no significant difference with the other genes in the heart tissue.

Table 12. ANOVA test of genes of interest of heart tissue.

		Sum of Squares	Df	Mean Square	F	Sig.
miR-122	Between Groups	83.82	3	27.94	7.98	0.01*
	Within Groups	28.00	8	3.50		
miR-133a	Between Groups	402.00	3	134.00	31.02	0.00*
	Within Groups	34.56	8	4.32		
18s	Between Groups	802.60	3	267.50	1.627	0.21
	Within Groups	3781.00	23	164.40		
Circ-LRP6	Between Groups	31.30	3	10.43	2.87	0.07
	Within Groups	50.89	14	3.64		
RPS-18	Between Groups	674.70	3	224.90	3.60	0.03*
	Within Groups	1250	20	62.51		

The ANOVA test for the genes of interest for the liver tissue is shown in Table 13. The test result showed that *Circ-Ogdh* and *5S* were significant at $p \leq 0.05$ level of significance.

Table 13. ANOVA test of some genes in the liver tissue.

		Sum of Squares	Df	Mean Square	F	Sig.
miR-122	Between Groups	23.06	3	7.69	2.24	0.16
	Within Groups	27.40	8	3.43		
Circ-AFF1	Between Groups	26.78	3	8.93	1.76	0.21
	Within Groups	55.94	11	5.09		
LC-Ogdh	Between Groups	81.41	3	27.14	2.30	0.13
	Within Groups	129.80	11	11.80		
Circ-Ogdh	Between Groups	83.58	3	27.86	5.293	0.009*
	Within Groups	89.48	17	5.26		
5S	Between Groups	970.00	3	323.30	44.28	0.0001*
	Within Groups	124.20	17	7.30		

The ANOVA test analysis for muscle tissue and the targeted genes are represented in Table 14. Genes *U6, Circ-AFF1, and Circ-LRP6* showed an acceptable level of significance ($p \leq 0.05$).

Table 14. Analysis of variance output of target genes of interest in muscle tissue.

		Sum of Squares	Df	Mean Square	F	Sig.
U6	Between Groups	687.60	3	229.20	12.12	<0.0001*
	Within Groups	434.90	23	18.91		
miR-122	Between Groups	24.25	3	8.08	1.32	0.31
	Within Groups	67.17	11	6.11		
LC-Ogdh	Between Groups	48.26	3	16.09	1.84	0.16
	Within Groups	227.10	26	8.74		
Circ-AFF1	Between Groups	354.70	3	118.20	5.43	0.02*
	Within Groups	239.5	11	21.77		
Circ-LRP6	Between Groups	156.9	3	52.31	13.93	0.0005*
	Within Groups	41.30	11	3.75		

3.6 Decay gene expression

3.6.1 Heart tissue

Decay gene expression of the heart tissue revealed that all genes were highly expressed on the first day compared to other days as shown in Figure 1. *RPS-18* had the highest expression on the first day among all genes, while *18S* had the least expression. Every gene showed a downward trend except for *18S* which showed a stable gene level on the four days. *RPS-18*'s high expression suggests that it is one of the most preserved transcripts immediately after death, making it a potentially reliable marker for very early PMI estimation. Although *18S* is also a component of the ribosomal RNA complex, its lower expression compared to *RPS-18* may be attributed to differences in the degradation rates of specific rRNA subunits or possibly due to varying starting quantities in the tissue samples.

3.6.2 Liver tissue

Decay gene expression of the liver tissue revealed that all genes were highly expressed on the first day compared to other days as shown in Figure 2. *Circ-Ogdh* levels decreased on the first three days before increasing on the fourth day. This non-linear trend is intriguing and suggests that circRNAs may have distinct degradation kinetics compared to other types of RNA. The fluctuating levels of *Circ-Ogdh* suggest that circRNAs might not follow the same degradation patterns as other

RNAs, potentially complicating their use as straightforward PMI markers. However, *LC-Ogdh* showed a consistent decrease in gene levels from day 1 to day 4. Similarly, *miR-122* showed a reduction in levels from day 1 to day 4. It had the lowest gene level on the fourth day of the study. This suggests that they might be useful biomarkers for comprehending how gene expression changes over time in muscle tissues, which could lead to more precise PMI estimations.

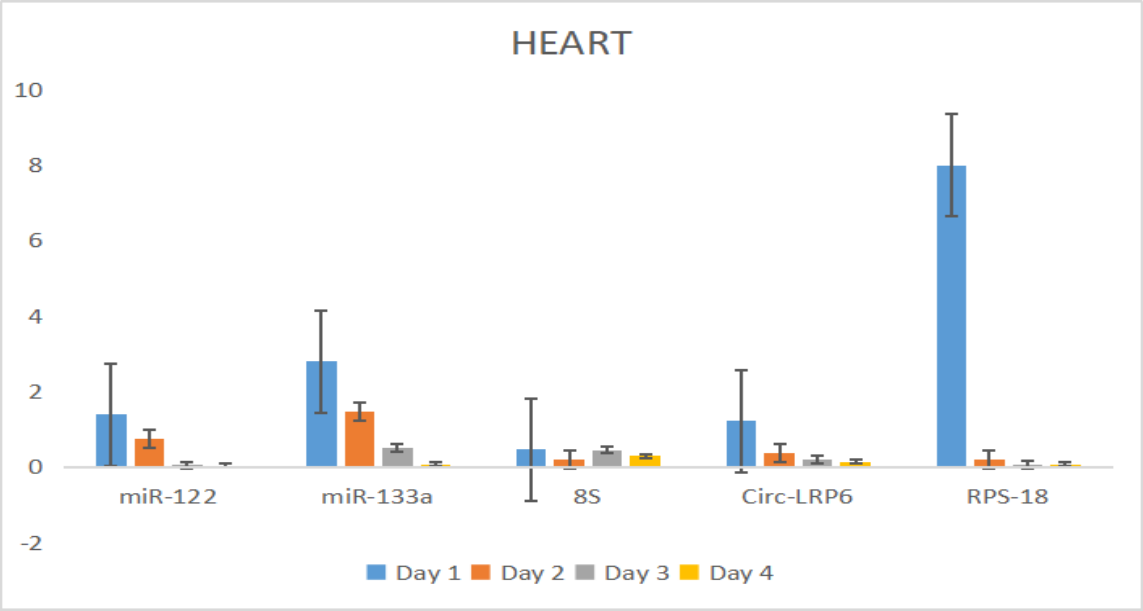

Figure 1. Image showing decay gene expression in the heart tissue.

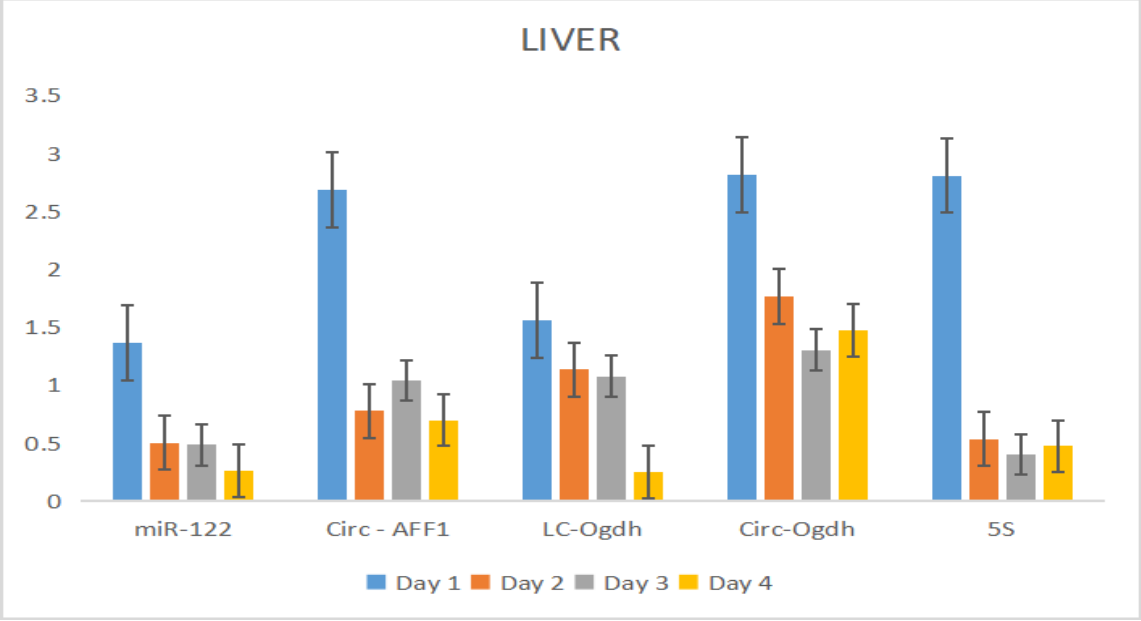

Figure 2. Image showing decay gene expression in the liver tissue.

3.6.3 Muscle tissue

Decay gene expression of the muscle tissue revealed that all genes were highly expressed on the first day compared to other days as shown in Figure 3. U6 gene had higher levels on the first day than any other gene. *miR-122* and *Circ-AFF1* experienced a consistent downward trend from the first day to the fourth day respectively. This indicates a gradual degradation of these RNA molecules over time. The consistent decline in *miR-122* and *Circ-AFF1* levels suggests that these RNA types could be reliable biomarkers for estimating PMI over a few days post-mortem. Their gradual degradation makes them suitable for tracking changes in RNA integrity over time. *LC-Ogdh* levels reduced on the second day, and subsequently increased on the third day of study. By the fourth day, genes *U6* and *Circ-AFF1* had little to no expressible genes.

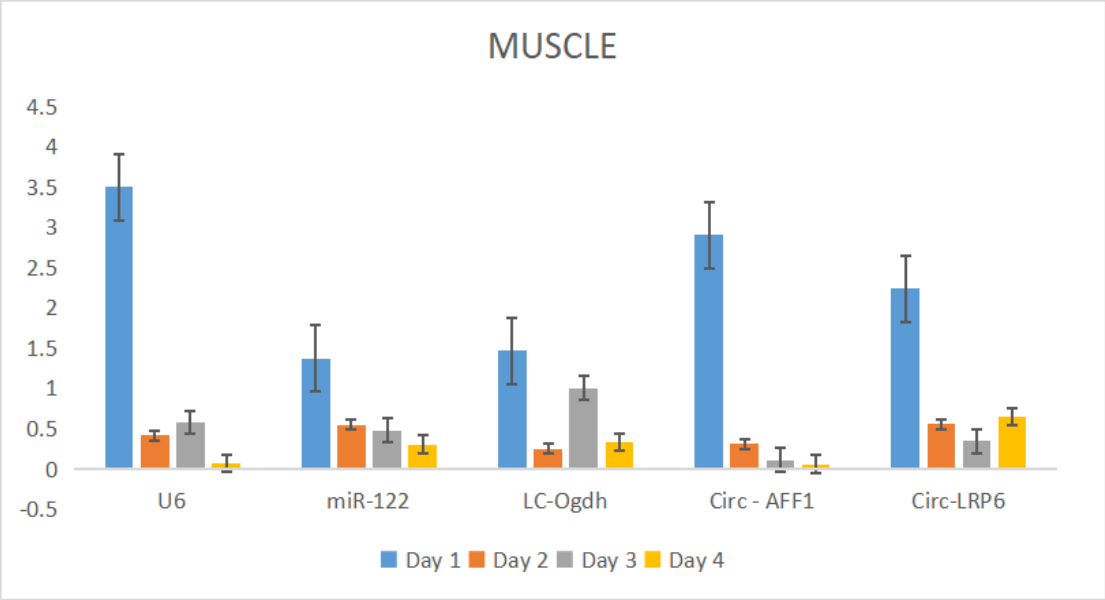

Figure 3. Image showing decay gene expression in the muscle tissue.

4. Discussion

The time between the examination of the corpse and the physiological death is known as post-mortem interval (PMI). PMI is a very important and frequently asked questions in forensic practice because of its medico-legal and investigative importance. A number of intrinsic (e.g. age, gender, genetics, pathological and physiological states of the corpse) and extrinsic (e.g. soil type, humidity, clothing or

coverings, temperature, concrete encasement, solar radiation, and insect activity) factors can influence the determination of PMI.

In this study, tissue samples could only be obtained for up to 4 days. This does not agree with the studies carried out by Tu *et al*.,[3, 6] that reported a PMI study of more than 5 days at an ambient temperature of 25 °C. Additionally, Elghamry *et al*.[17] reported a PMI study of more than 5 days at an air temperature of 30 °C and 6 °C. This may be due to the environmental conditions with the temperature and humidity varying from day to day and the microbial load of the environment.

The intrinsic characteristics of miRNA, and subsequently, their resistance to degradation, make them suitable as endogenous markers in PMI determination[18-20]. Some researchers showed that miRNAs present good stability in the first 24 h of PMI, and some of them begin to decrease after 24 h[21]. A study has discovered a potential correlation between RNA degradation in a corpse and PMI and some mRNA markers *GAPDH, Beta-actin* and *Rps18* can be used as endogenous control markers[3]. Studies have shown that the *GAPDH* mRNA level in rat heart and skin[17] and brain[22] could be a useful marker for estimation of PMI under various environmental conditions. Some Researchers also concluded that as reference genes, microRNAs (miRNAs) and circular (CircRNAs) were more stable in dead bodies than other types of RNAs[3].

The RNA yield gotten from liver samples was high as at the time of death and there was a slight decrease in the quantity of RNA as PMI increased. There were also changes in the purity values as the time of death increased. This change in the degree of purity and concentration of the RNA might be due to the endogenous and exogenous ribonucleases, which are found to be responsible for the RNA degradation. The storage chemical condition may also influence RNA purity and concentration[23].

The purity of the heart tissues was assessed, and the highest purity was observed on the first day with a mean value of 1.98 ± 0.04, while the lowest was observed on the fourth day with a value of 1.58 ± 0.06. There was consistency in decrease of RNA purity in the heart. However, there was a strong negative correlation observed between day 1 (0 hours) and day 4 (72 hours) and this agrees with Birdsill *et al*.[27], who reported that there was a negative correlation between RNA purity and PMI for up to 50 hours. Additionally, Sampaio-Silva *et al*., also reported a negative correlation between RNA purity and PMI for up to 11 hours[28]. There was

consistency in decrease of RNA purity. However, there was a strong negative correlation observed between day 1 (0 hour) and day 4 (72 hours) and this agrees with Birdsill *et al*., who reported that there was negative correlation between RNA purity and PMI for up to 50 hours[27].

MiR-122 and *miR-133a* were expressed daily in heart tissues and this is in accordance with the study by Tu *et al.,* which showed that *miR-122, miR-133a* and *18S* showed prioritized stability and are suitable reference genes in heart tissues[6]. *mir-133a* was stable in the first three days of study before declining on the fourth day of study. This partially agrees with the report of Tu *et al*., which concluded that *mir-133a* was only suitably expressed in muscle tissues and repressed in other types of tissue[6]. The result showed that 18S Cq values were stable in the first four days. This disagrees with the results of a study that shows a characteristic parabola relationship between post-mortem period and Ct values for *18S-rRNA* in dead rat hearts[29]. *18S* did not have a positive correlation with *GAPDH* and this disagrees with Li *et al*., who worked on PMI studies using *18S* rRNA and miRNA on dead rat heart tissues, and they concluded that *18S* rRNA was suitable for PMI studies on heart samples[29]. *18S* was expressed daily and this agrees with the report of Li *et al.,* that the Ct value of *18S* rRNA and delta Ct value present a good correlation with PMI, and can be markers for approximately calculating early PMI[29]. The discrepancy between these findings and those of Li *et al*. highlights the complexity and variability inherent in biomarker efficacy across different studies. While Li *et al*. supported the use of 18S rRNA as a stable marker for PMI in heart tissues, this study suggests that *18S* rRNA may not be as effective in the context of the tissue examined. This divergence underscores the need for careful selection and validation of biomarkers based on tissue type and experimental conditions. *18S* levels were stable for the first four days. This agrees with Li *et al*., who evaluated the expression levels of two RNA markers (*miR-1-2* and *18S rRNA*) in rats' cardiac tissues at different PMI in a controlled temperature system (25 °C) and they observed that *18S* levels increased within 96 h after death and then declined gradually and can be used for the estimation of PMI[29]. Among reference genes, *18S* had one of the lowest cq values on the first day of the study, which agrees with the study of Zhang et al.[34].

Mir-122 had high expression levels in liver tissues, and this agrees with the findings of Tu *et al.*[6] and Lv *et al.*[2], which reported that *mir-122* expression was stable

in liver tissues of rats for extended PMI stages. This high *miR-122* expression value in the liver, agrees with the study of Chang *et al*.[24], who reported on *miR-122* being a liver specific micro-RNA and the study of Sharapova *et al*.[25], who demonstrated that *miR-122* is highly expressed in liver tissues and concluded that *miR-122* is a liver-specific micro-RNA. These consistent results underline the utility of *miR-122* as a valuable biomarker for PMI estimation in forensic investigations. The stability of *miR-122* in liver tissues over time supports its potential as a reliable indicator for determining the time of death, particularly in cases involving liver samples. This stability is crucial because it allows forensic scientists to make more accurate PMI estimations, which can be pivotal in criminal investigations and legal proceedings. Given that *miR-122* is highly specific to liver tissues, its use can help refine PMI prediction models by providing a more targeted approach to assessing postmortem intervals. This specificity enhances the accuracy of PMI estimations, reducing the potential for error associated with less specific biomarkers. Consequently, integrating *miR-122* into forensic practices could improve the precision of time-of-death determinations, making it a promising candidate for inclusion in routine forensic analyses of liver tissues. A study by Clarke *et al.* shows that microRNA-122 (*miR-122*) is increasingly being measured in pre-clinical and clinical settings as a result of greater sensitivity and hepatic specificity compared to the gold standard liver injury biomarker alanine aminotransferase (ALT)[26]. The *miR-122* level in the heart showed a downward decrease from the first day to the fourth day. This agrees with the study which revealed that three miRNAs (*miR-122, miR-150, miR-195*) are stable in the first 24 h of post-mortem Interval, declining after that time[21]. The *mir-122* level in the heart was expressed from the second day to the fourth day but not on the first day.

For circular RNAs the results showed that *Circ-AFF1*, *LC-Ogdh* and *Circ-Ogdh* were stable enough to be expressed in the liver tissues suggesting that circular RNAs should also be prioritized when selecting reference genes for PMI estimation using liver tissues. This also agrees with the findings of Tu *et al.*[6] who reported that *Circ-AFF1* and *LC-Ogdh* were the most stable genes in liver tissues. Ribosomal RNA, *5S* had the lowest expression value and this agrees with the findings of Lv *et al.*[2], who reported that *5S* had the lowest expression in post-mortem tissues. The *5S* levels in the liver experienced a decline from day 1 to day 4 suggesting that PMI can be determined using the gene. This doesn't agree with a study by Lv *et al.*[33], which claimed *5S* gene is suitable as control markers for muscle tissues.

In the muscle tissue, there was a strong positive correlation observed between day 1 and day 4, day 2 and day 4, day 3 and 4 and this does not agree with Birdsill *et al*.[27], who reported that there was negative correlation between RNA purity and PMI for up to 50 hours. *Circ-LRP6* was expressed daily in the muscle tissue. This might be attributed to the report by Hall *et al.*[30] that *Circ-LRP6* is an intracellular modulator and a natural sponge for *miR-145*, counterbalancing the functions of the miRNA in Vascular Smooth Muscle Cells (VSMCs). *Circ-LRP6* showed a consistent decrease in muscle tissue. Gene *U6* also showed a consistent downward trend which suggests that it can be a suitable gene of study for muscle tissue. This agrees with a study using *U6* gene for estimation of PMI by Lv *et al.*[31], which reported that *U6* transcript level exhibited exponential decay at 25 °C. *miR-122* was expressed daily in the muscle tissue, this does not agree with the study of Chang *et al*.[24], who reported on *miR-122* being a liver specific micro-RNA. Gene *Circ-AFF1* was expressed every day in the skeletal muscle tissue. It showed a consistent downward trend suggesting that PMI can be observed with Circ-AFF1 in the skeletal muscle tissue. This agrees with the study of Tu *et al*.[6], which showed *circ-AFF1* being a suitable gene for PMI estimation in muscle tissues.

RPS-18 and *U6* were expressed daily in the heart and muscle respectively and displayed a downward decrease as the days went by. This agrees with the study by Burke *et al*. [32], which found that the target biomarkers in heart and liver tissues are *U6* and *RPS-18* while that in skeletal muscle tissues are *U6* and *beta-actin*. They also observed that *RPS-18* degraded during late PMI and thus is considered appropriate as a biomarker in the heart tissues of a corpse[32]. The findings that *RPS-18* and *U6* exhibit daily expression in heart and muscle tissues, respectively, and show a downward trend over time, have significant implications for PMI (postmortem interval) prediction in forensic science. This degradation pattern indicates that *RPS-18* could serve as an effective marker for distinguishing between early and late PMI stages in heart tissues. Similarly, the daily expression of *U6* in muscle tissues and its consistent patterns further supports its role as a reliable marker for PMI prediction in skeletal muscles. These findings underscore the value of incorporating *RPS-18* and *U6* into forensic investigations to improve the accuracy of PMI estimations. The ability to track the degradation of *RPS-18* in heart tissues and the stable expression of *U6* in muscle tissues provides forensic scientists with additional tools to refine PMI

predictions. By understanding how these biomarkers behave over time, forensic experts can enhance their ability to estimate the time of death more precisely, which is crucial for legal and investigative purposes. In the muscle tissue, Gene *U6* showed an acceptable level of significance ($p \leq 0.05$). This can be attributed to the findings that *U6* is protected from external factors through the ribonucleoproteins, but this eventually vanishes with delay in death time.

5. Conclusion

This study highlights the critical role of biomarker expression levels in forensic science, particularly in the estimation of the postmortem interval (PMI). The findings of this study reveal that varying expression levels of biomarkers across different tissue samples can significantly enhance PMI estimation. The use of a combination of biomarkers, including MicroRNAs (miRNAs), ribosomal RNAs (rRNA), small nuclear RNAs (snRNA), circular RNAs (circRNA), and circRNA + messenger RNA (mRNA), has been shown to provide more accurate PMI estimates. Given that most biomarkers exhibit tissue-specific properties, integrating samples from different human tissues, collected at various times and under diverse environmental and storage conditions, is essential for reliable PMI determination. Therefore, employing a panel of tissue-specific biomarkers, such as miRNAs, rRNAs, snRNAs, circRNA, and circRNA + mRNA, holds significant promise for advancing forensic practices and potentially establishing a new standard in routine forensic PMI assessments.

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