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Suitability of miRNA assessment in postmortem interval estimation

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Abstract. – OBJECTIVE: The aim of this review was to explore recent pieces of evidence focused on the use of miRNAs for PMI estimation both in humans and animal experiments, with particular interest on the best miRNAs to use as reference/target markers in different tissues or biological fluids. MiRNAs are innovative biomarkers used in clinical and research field; they appear very attractive, being introduced in forensic research scenarios even for PMI estimation.

MATERIALS AND METHODS: Data from PubMed and Scopus were analyzed from January 2013 to August 2020. Based on inclusion/exclusion criteria, high-quality articles have been selected to become the subject of this review.

RESULTS: A total of 737 papers were found but, after titles/abstracts screening for inclusion criteria and a full-text careful selection, 33 papers were deeply studied. After the exclusion of 19 papers, 15 articles remained. Eight papers dealt with animals (mice/rats), two both with animals and humans (for method validation previously built), while 5 exclusively with humans. Myocardium (6/15) and brain (6/15) were the most studied tissues, respectively in mice/rats and humans. PMI considered was up to 7.5 days in mouse studies and less than 3 days in human models.

CONCLUSIONS: Because of their significant stability in both early and long PMI, miRNAs are the cleverest reference markers to be used. Temperature and environmental conditions influence mostly mRNA, while miRNAs are less susceptible to them. The best miRNA to choose depends on its tissue specificity, i.e., miR-9 and miR-125 in brain or miR-1 and miR-133 in skeletal muscle/heart.

Key Words:
Post-mortem interval (PMI), MicroRNA, Forensics.

Introduction

The estimation of the postmortem interval (PMI), the time-lapse occurred from the exact moment of death to the postmortem examination, represents one of the major challenges in forensic pathology and death investigations. While several forensic cases are characterized by known PMI, such as hospitalized patients or witnessed casualties, in the vast majority of cases the death occurs unexpectedly and without witnesses. The methods to evaluate the PMI used in forensic medicine range from the more traditional ones, livor mortis, rigor mortis and frigor, to those based on biochemical changes, e.g., in vitreous humour[1].

Each of the current methods presents several external and internal modifying factors and limitations, especially with advanced PMIs and, notwithstanding the integration of multiple data, it is only possible to locate the death within a wider or narrower interval of time.

Novel tools to accurately and reliably establish PMI are constantly under evaluation and, since several years, the degradation of DNA and RNA has been proposed as an innovative method for a more reproducible and reliable estimation of the PMI[2-7].

MiRNAs are a class of small, non-coding RNAs, 20-24 nucleotides in length, that regulate gene expression in multiple ways, such as by interacting post-transcriptionally with mRNA target and by promoting its degradation or disrupting its translation. The final effect is the repression of the mRNA transduction or its complete silencing. Each miRNA has the potential to regulate dozens or hundreds of target mRNAs

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creating an invisible and complex network to be studied and interpreted[8,9].

MiRNAs are innovative biomarkers that have been investigated and used both in clinical practice and in the research field, especially for the diagnosis, prognosis and treatment of neoplastic, cardiovascular and neurodegenerative diseases. While in cancer and cardiovascular research, biological fluids and pathological tissues are the most frequently analyzed matrices[10-12], in neurodegenerative diseases post-mortem brain tissue has been mostly studied[13,14]. Thus, as in a sort of contamination from each other research fields, post-mortem brain has been one of the most considered matrices in the forensic scenario.

Due to their long turnover in living systems and to their stability in postmortem tissues even in formalin-fixed paraffin-embedded samples[15-18], miRNAs appear very attractive for forensic pathology and have been introduced in several forensic research scenarios in the last 10 years, such as body fluid identification[19-21], determination of the cause of death[22] and recently for PMI estimation.

The present review aims to provide an overview of recent experimental pieces of evidence focused on the use of miRNAs for PMI estimation, with a particular interest on the best miRNAs to use as reference/target markers in different tissues or biological fluids.

Materials and Methods

In August 2020, a literature review was performed in two international databases (PubMed and Scopus) by using the following search terms: "*miRNA*" or "*microRNA*" coupled through the Boolean coordinator "AND" to either "*Postmortem interval*" or "*PMI*". After a manual exclusion of duplicates, a first paper selection was performed by reading titles and abstracts of the retrieved articles according to the following three inclusion criteria:

– English language or availability of English abstract;
– Range of publication time (from 2013 to 2020);
– Topic and content of clear forensic interest.

A second selection was performed by re-applying inclusion criteria 1-3 on the full-texts of the included papers. For this second selection, the following exclusion criteria were also used:

– Type of article, i.e., reviews and papers not presenting original/experimental data;
– Subject of the study, i.e., papers focused on PMI estimation but not involving miRNAs;
– Aims of the study, i.e., clinical purposes or forensic aims different from PMI estimation.

From selected papers the following data were extracted and used to build a database:

– Authors, title and year of publication;
– Aim of the study, classified in broad categories for simplification;
– Animal/human model or samples;
– Sample size and experimental design;
– Materials and methods employed;
– Analyzed tissues;
– Evaluated target and reference biomarkers;
– Evaluated PMI;
– Data analysis, inclusive of statistical evaluation;
– Study results.

Results

A total of 737 papers were found by launching the search and manually removing the duplicates. After titles and abstracts screening for inclusion criteria 1-3, 33 papers were completely read. After full-text careful selection, 19 papers were excluded for the following motivations: 2 papers consisted of reviews with no experimental data; 8 papers dealt with the estimation of PMI by RNA markers (mRNA, rRNA, RNA degradation) but did not include miRNAs; 9 studies analyzed miRNAs with clinical purpose or were related to trace deposition issues.

As a result, 15 articles evaluating the expression of miRNA in the attempt of estimating PMI in rats/mice or human models were retrieved between January 2013 and August 2020. Most of them were published between 2015 and 2016. The majority of papers (10 out of 15) included experimental studies on animals[23-32], among which 7 were exclusively focused on mice/rats (Table I), while 2 were performed firstly on rats and subsequently on humans for validation[25,26]. Five papers investigated human samples only[33-37] (Table II).

The majority of papers aimed at identifying the best target or reference miRNA biomarkers for PMI estimation across different variables, such as tissues, early/long PMI, different postmortem storage temperatures and causes of death. This goal arises from the evidence that a universal

Table I. MiRNAs expression in animal studies. Ref: reference. N: number. PMI: postmortem interval. D: days; H: hours.

Aim	Sample size and experimental design	Materials and methods	Analyzed tissues	Target markers (unstable)	Reference markers (stable)	PMI	Data analysis	Results	Ref.
Evaluation of stability/degradation of reference markers. Identification of tissue-specific best target markers. Building and validation of a mathematical model for PMI estimation in an advanced stage.	15 mice divided into control (PMI=0) and 4 experimental groups. N=3 per group + 15 for validation	Extraction of total RNAs and integrity analysis, cDNA synthesis, triplicates of assays, RT-qPCR and Ct determination (difference among Ct values < 1 acceptable). Mathematical model	Liver	GAPDH; RPS 18; U6; β-actine	miR-122; circ-AFF 1; LC-Ogdh	0-7.5 D	For candidate reference markers, geNorm and NormFInder algorithms and standard deviation analysis. As mathematical model, delta-Ct by normalization with 3 reference genes, delta-delta-Ct by reducing control groups and curve estimation by R^2. Model validation judged by error rate.	U6 - RPS 18 best target marker in liver and heart, U6 - β-actine best target biomarker in skeletal muscle	[32]
			Heart	GAPDH; RPS 18; U6; β-actine	miR-122; miR-133a; 18s r-RNA			confirmation of reference genes	
			Skeletal muscle	GAPDH; RPS 18; U6; β-actine	mir-133a; circ-AFF 1			High accuracy of the model for heart (-1-10%), error rate 80% and 41% in 1.5 D for liver and skeletal muscle	
Evaluation of stability/degradation of reference markers. Identification of tissue-specific best reference markers	45 mice divided into control (PMI=0) and 9 experimental groups. N=5 per group. Samples in RNA Later	Extraction of total RNAs and integrity analysis, cDNA synthesis, triplicates of assays, RT-qPCR and Ct determination (difference among Ct values < 1 acceptable)	Liver	–	β-actine; GAPDH; RPS 18; 5S; 18S; U6; miR-133a; miR-122; circ-AFF1, LC-Ogdh; LC-LRP6	0-8 D	Transformation of raw Ct values and analysis by geNorm and NormFInder algorithms to calculate average expression stability values and pairwise variation of candidate reference genes	miR-122; circ-AFF 1; LC-Ogdh showed prioritized stability in LIVER	[31]
			Heart					miR-122; miR-133a; 18S-rRNA showed prioritized stability in heart	
			Skeletal muscle					miR-133a; circ-AFF1; LC-LRP6 showed prioritized stability in skeletal muscle	
Correlation between early PMI and RNA markers at different temperatures	222 rats divided into control (PMI=0) and 4 experimental groups (5, 15, 25, 35°C), collected at 9 time points from 1 h to 24 h PMI + 6 rats used to validate the model	RNA extraction, RT-qPCR and ΔCt determination	Brain	β-actin, GAPDH, RPS29, 18S rRNA, 5S rRNA, U6 snRNA, miRNA-9 and miRNA-125b		1-24 h	Proper internal reference selected by geNorm software. Regression analysis of normalized RNA markers. Mathematical model for PMI estimation.	5S rRNA, miR-9 and miR-125b suitable as internal reference. β-actin and GAPDH degraded with prolongation of PMI	[24]
Evaluation of stability/degradation of reference and target markers. Identification of tissue-specific best target markers. Building and validation of a mathematical model for PMI estimation at different temperatures.	270 rats (study group) divided into control (PMI=0) and 4 experimental groups (4, 15, 25, 35°C), collected at 11 time points (1, 3, 6, 12, 24, 36, 48, 72, 96, 120, 144 hrs after death); + 36 rats for validation	RNase treatment for material, extraction of total RNAs and integrity analysis, cDNA synthesis, duplicates assays for each sample, RT-qPCR, Ct determination (threshold at 0.2) Mathematical model	Brain	β-actine; GAPDH; RPS29; 5SrRNA; 18RrRNA; U6; miR-9; miR-125b; let-7a		0-6 D	For candidate reference markers, geNorm algorithm and standard deviation analysis. For target markers, Ct normalization with 2 reference genes. Association with PMI by bivariate cubic curve fit analysis with R^2. Mathematical model by quadratic equation built with three unknows. Model validation judged by error rate.	miR-9 and miR-125b are suitable reference markers. β-actine has the best correlation with PMI	[28]
Evaluation of stability/degradation of reference and target markers at different conditions. Identification of best markers	16 rats divided in control (PMI=0) and 7 experimental groups. N=2 per sampling time. Two different temperature: room temperature and refrigeration. Samples fresh-frozen	Extraction of total RNAs and integrity analysis with RNA integrity number (RIN), cDNA synthesis, RT-qPCR in quintuplets.	Brain	histone modifications and proteins	miR-16; miR-34a; miR-124a; miR-134; RNU6B; U6; GAPDH	0-96 H	For mRNA, degradation, RIN and PMI and relative quantity and PMI relationships. For expression levels of miRNAs, mean of replicates and quantitation standard curve with ANOVA analysis.	GAPDH showed to be unstable across different brain area; histone acetylation is less stable than methylation. miRNAs were extremely stable [miR-16; miR-124a miR-134]	[29]

Continued

Table I *(Continued)*. MiRNAs expression in animal studies. Ref: reference. N: number. PMI: postmortem interval. D: days; H: hours.

Aim	Sample size and experimental design	Materials and methods	Analyzed tissues	Target markers (unstable)	Reference markers (stable)	PMI	Data analysis	Results	Ref.
Evaluation of tissue-specific expression of markers. Identification of circadian rhythm. Evaluation of stability/degradation of target markers	5-8 mice	Extraction of total RNAs, cDNA synthesis, assays in triplicates, RT-qPCR (U6 and β-actine as invariant control) and Ct determination.	Liver; lungs; pancreas; kidney; heart; brain	mir-2909 and AATF		0-72 H	Delta-Ct analysis. For tissue-specificity expression and circadian pattern, mean, standard deviation and ANOVA	AATF mRNA and miR-2909 derived from BLOOD and BRAIN / HEART tissues exhibit circadian rhythm characteristic of biological clock: mir-2909 expression was stable ut to 48 h post-death at 8 PM and 12 h post-death at 12 noon	[30]
	For identification of circadian rhythm, divided into 3 groups of nocturnal (4 AM), diurnal (n⌊on) and evening (8 PM) deaths. N=3 per group		brain heart						
	For evaluation of stability of markers, mice divided into control (PMI=0) and 5 experimental groups, each composed of two sets of diurnal (noon) and evening (8 PM) deaths. N=6 per group								
Evaluation of stability/degradation of reference and target markers at different temperatures. Identification of best markers. Building and validation of a mathematical model for PMI estimation	12 rats divided in 2 experimental groups for temperature. N=6 per group. Collection of samples at t=0 and 11-15 time points + 6 rats for validation. Samples in RNA Later	RNase treatment for material, extraction of total RNAs and integrity analysis, cDNA synthesis with gDNA eraser, duplicates for each sample for each of 3 assays, RT-qPCR and Ct determination. gDNA contamination control with Ct>38=not expressing miRNA. Mathematical model in single-blinded fashion	Spleen	GAPDH 1 and 2; β-actine 1 and 2; U6; 18S-rRNA; miR-125b; miR-143;		0-6 D	For stability evaluation, Ct determination, average among replicates, mean and standard deviation at different time points and Genorm analysis. To build the mathematical model, delta-Ct calculation with respect to the stable marker, delta-delta-Ct calculation with respect to control, normalization of relative transcripts levels to 2 reference markers and exploration of 6 mathematical models. Curve fit analysis with R2. Model validation judged by error rate.	GAPDH, β-actine degraded rapidly after death making them suitable for estimating early PMI; 18S-rRNA showed various degradation patterns in relation to PMI and temperature so it is more suitable for longer PMI estimation	[27]
Evaluation of the usability of 18S-rRNA in heart tissue as PMI biomarker. Evaluation of stability/degradation of reference and target markers	Rats at controlled temperature divided into control (PMI=0) and 15 experimental groups. N=6 per group. Samples in RNA Later	RNase treatment for material, extraction of total RNAs and integrity analysis (28S and 5S-RNA as controls), cDNA synthesis, RT-qPCR in replicates samples. Ct determination.	Heart	18S-rRNA	miR-1; miR-133, miR-143; miR-206, miR-208	1-7 D	Analysis of standard deviation between replicates for reference markers. For target markers, linear regression between Ct value and delta-Ct, after normalization with one reference) and PMI.	miR-1 was chosen as the best endogenous marker (it was stable within 7 days after death); 18SrRNA/mir-1 is useful to PMI determination within seven days	[23]

Table II. MiRNAs expression in different human tissues, including mixed animal/human models. Ref: reference. N: number. PMI: postmortem interval. D: days; H: hours; Y: years.

Aim	Sample source	Sample size and experimental design	Materials and methods	Analyzed tissues	Target markers (unstable)	Reference markers (stable)	PMI	Data analysis	Results	Ref.
Evaluation of stability/degradation of reference and target markers. Identification of tissue-specific best markers.	Human	71 human bones divided into four experimental groups. N=37, 18, 11, 5 per group	Extraction of total RNAs and integrity analysis, cDNA synthesis, duplicates RT-qPCR (automatic Ct threshold) and Ct determination	Bones (patella)	let-7e, miR-16	Ce-miR-39_1	1 D-2 Y	For target markers, Ct normalization with 1 reference genes. Pearson correlation between target miRNAs and PMI. Unpaired t-test among groups	let-7e and miR-16 decreased with increasing PMI and was statistically different among groups of PMI <1 and >1 month	[35]
Evaluation of stability/degradation of reference and target markers. Identification of tissue-specific best markers. Building and validation of a mathematical model for PMI estimation at different temperatures.	Rat	270 rats (study group) divided into control (PMI=0) and 4 experimental groups (4, 15, 25, 35°C), collected at 11 PMI. N=6 per group + 36 for validation	Extraction of total RNAs and integrity analysis, cDNA synthesis, duplicates assays for each sample, RT-qPCR. Mathematical model	Myocardium (apex cordis); liver (right lobe)	β-actine; GAPDH: RPS29	18SrRNA; 5S-RNA; miR-1; miR-133a; miR-122; miR-9 and miR-125b	6-71 H	For candidate reference markers, geNorm algorithm and standard deviation analysis. For target markers, Ct normalization with 2 reference genes. Association with PMI by bivariate cubic curve fit analysis with R^2. Mathematical model by quadratic equation built with three unknows. Model validation judged by error rate.	5S-sRNA; miR-1; miR-133a are fairly stable in the myocardium over 5 days when exposed to a range of temperatures (chosen as reference marker); miR-122 degrade with PMI (particularly at 35°C); **5S-rRNA** chosen as the only reference marker for liver tissues. Target genes: β-actine (heart and liver)	[25]
	Human	13 individuals, 7 males 6 females not kept in cold storage. Causes of death: 5 hemorrhagic shocks, 4 brain trauma, 4 mechanical asphyxia. Diseases excluded. Samples preserved in RNA Later solvent		Myocardium (apex cordis); liver (right lobe); brain (frontal lobe)						
Evaluation of stability/degradation of reference and target markers. Identification of tissue-specific best markers.Analysis of relationship between expression levels of markers and relevant factors (age, gender, cause of death). Building and validation of a mathematical model for PMI estimation.	Human	12 individuals with known PMI	Not available in abstract	Brain	β-actine; GAPDH; RPS 29; 18S-rRNA; 5S-rRNA; U6; miR-9; miR-125b		4.3-22.5 H	Not available in abstract	5SrRNA; miR-9; miR-125b showed quite stable expression (useful as reference markers in early PMI). The expression of β-actine correlates with PMI	[34]
Evaluation of stability/degradation of reference/target markers at different temperatures. Identification of tissue-specific best markers. Identification of suitability of markers in human samples. Building and validation of a mathematical model for PMI estimation	Rat	216 rats divided into 3 groups for temperature and 11 experimental groups for sacrifice. N=6 per group + 15 for validation	RNase treatment for material, extraction of total RNAs and integrity analysis, cDNA synthesis with gDNA eraser, duplicates for each sample for each of 3 assays, RT-qPCR and Ct determination. Mathematical model in single-blinded fashion	Lung; skeletal muscle	GAPDH; β-actine	RPS 29; let-7a; miR-195; miR-200; miR-1; miR-206; 5S; U6	0-6 D	Transformation of raw Ct values and analysis by geNorm and NormFInder algorithms to calculate average expression stability values and pairwise variation of candidate reference genes. As mathematical model, exploration by linear, quadratic and cubic function and curve estimation by R^2 and residual standard deviation. Model validation judged by error rate	β-actine and GAPDH were useful for estimate PMI both rat and human (target biomarker). 5S, U6, RPS29 and miR-195, miR-200c as the best reference marker for lung; 5S, RPS29 and mir-1, miR-206 the best reference marker for skeletal muscle. All of these did not show any variation in relation to age, gender, t°, cause of death and PMI (human)	[26]
	Human	12 individuals		Lung; skeletal muscle	GAPDH; β-actine	β-actine; GAPDH;18S; RPS 29; let-7a; miR-195; miR-200; miR-1; miR-206; 5S; U6	7-73 H			

Continued

Table II *(Continued).* MiRNAs expression in different human tissues, including mixed animal/human models. Ref: reference. N: number. PMI: postmortem interval. D: days; H: hours; Y: years.

Aim	Sample source	Sample size and experimental design	Materials and methods	Analyzed tissues	Target markers (unstable)	Reference markers (stable)	PMI	Data analysis	Results	Ref.
Evaluation of feasibility of tissue-specific miRNA analysis. Evaluation of stability/degradation of target markers. Evaluation of tissue-specific expression of markers. Identification of circadian rhythm.	Human	18 individuals, divided in 2 groups of nocturnal and diurnal deaths. N=4-8 per group. Age 24-93 with precise assessment of cause and time of death. N sample=12 whole blood; 12 vitreous humour.	Extraction of total RNAs and quantification, cDNA synthesis, duplicates of RT-qPCR for 40 cycles and Ct determination (threshold at 0.1; Ct>34=not expressing miRNA). Mean and standard deviation considered.	Blood; vitreous humour	miR-15a-5p; miR-541-3p; miR-142-3p; miR-26a-5p; miR-182-5p; miR-219a-5p; miR-194-5p; miR-142-5p; miR-132-3p; miR-106b-5p; miR-96-5p	SNORD-61; SNORD-95; RNU6-2; miR-15a	<48 H for blood; < 24 H for vitreous humor	GeNorm to evaluate the best reference markers. Delta Ct analysis (reference vs target markers), expression level estimated as relative expression or fold change =2^deltaCt by Genex software. T-test to evaluate circadian rhythm between diurnal and nocturnal deaths.	SNORD-95 was the best reference marker in vitreous and blood; mir-15a can be a positive control in blood analysis because of its high expression but it is not the same in vitreous humour; two miRNA showed a circadian pattern in humour vitreous: miR-106b and miR-96; two miRNA showed circadian pattern in blood: miR-142-5p and mir-219	[33]
Evaluation of feasibility of tissue-specific miRNA analysis. Evaluation of tissue-specific expression of markers. Identification of circadian rhythm.	Human	7 individuals, divided in 2 groups of nocturnal and diurnal deaths. N=3-4 per group. 7 humor vitreous samples. Sample frozen.	Extraction of total RNAs and integrity analysis, cDNA synthesis, duplicates of RT-qPCR and Ct determination (threshold at 0.2); gDNA contamination control with Ct>32=not expressing miRNA	Vitreous humour	multiple miRNAs including: miR-34c; miR-541; miR-888; miR-484: miR-142-5p; miR-222; let-7b; miR-186; miR-532-5p		< 24 H	GenNorm to evaluate the best reference markers. Delta Ct analysis (reference vs target markers), expression level estimated as relative expression or fold change =2^deltaCt by Genex software. T-test to evaluate circadian rhythm between diurnal and nocturnal deaths.	No correlation was found between miRNA expression and PMI; no significant difference in miRNA expression related to sex or age; miR-142-5p and miR-541 showed a circadian behaviour; miR-222 best reference marker	[36]
Evaluation of stability/degradation of target markers		7 individuals. 34 humor vitreous samples. Sample frozen.			miR-222; miR-34c: miR-541; miR-888; miR-484; miR-142-5p	miR-222; miR-let-7b; miR-186; miR-532-5p		Correlation between miRNAs expression and PMI, R2 evaluation.		
Evaluation of stability/degradation of reference markers. Identification of tissue-specific best target markers.Evaluation of feasibility of tissue-specific miRNA analysis. Evaluation of influence of other factors (cause of death)	Human	40 individuals. Causes of death: hemorrhagic shocks, brain trauma, mechanical asphyxia, sudden cardiac death. Samples preserved in RNA Later solvent	Extraction of total RNAs and integrity analysis with RNA integrity number (RIN), cDNA synthesis, duplicates of RT-qPCR and Ct determination	heart; brain; kidney; skin	not evaluated	β-actine, GAPDH, B2M, 18SrRNA, U6; miR-1; miR-9; miR-194-1; miR-203	1-71 H	Classification of samples in 17 groups based on PMI and cause of death and GeNorm algorithm analysis (stability measure M and pairwise variations). Bonferroni test for RNA and RIN differences with storage time	Reference markers differ in different tissues, in relation to various cause of death and various PMI. U6 is the best reference marker in the whole tissues studied. miRNAs should not be chosen for endogenous control genes.	[37]

target or reference marker which can be used in every tissue and for the whole autoptic cases has not been identified yet. According to the results of the present review, such factors have a deep impact and influence on miRNA expression, so that it is highly likely that a single biomarker would never be enough. Additionally, 6 studies aimed at building and validating a mathematical model for PMI estimation[25-28,32,34] and 2 analyzed the relationships among markers and other factors besides PMI[34,37]. The effects of temperature were evaluated by 5 studies[25-29].

Three out of 15 papers (2 on human samples and 1 on mice) were focused on the identification of a circadian activity for some specific miRNAs, able eventually to be related to the PMI[30,33,36].

In all the 15 studies included in the present review, an evaluation of the stability/degradation of reference or target analyzed biomarkers overtime was performed.

The applied qRT-PCR methodology adhered and specifically cited the guidelines for Quantitative Real-Time PCR Experiments (MIQE)[38] in only 3 cases[25,26,28]. Statistical evaluations of the stability/degradation of markers ranged from the identification of mean and SD across different experimental groups and *t*-test or ANOVA to more complex analysis, e.g., correlations with variable curves. Experimental designs, materials and methods, as well as evaluated markers, data analysis and summarized results, are shown in Tables I and II. The most studied tissues were myocardium (6/15) and brain (6/15), respectively the most popular in mice/rats and humans. Spleen was not analyzed in humans, but only in rats[27]. Biological fluids were studied essentially in human samples, with a predominance of vitreous humour compared to blood; no studies were performed on rats/mice fluids. Only one study focused on human bones[35].

The PMI considered was longer in mouse model studies, up to 7.5 days after animal sacrifice, while in humans the range was 4.3-71 hours (less than 3 days) after death. The only exception was the study involving human bones (patella), with a PMI up to 2 years[35].

Mathematical models and validations were applied in five animal studies[25-28,32], two of which validated results of animal experiments on human samples[25,26].

Among the ten papers dealing with animals (rats/mice), 5 reported the approval of Ethical Committee[23,25,26,28,31]. In 2/10 Principles for the Care and the Use of Laboratory Animal Committee were followed[27,32]. In the last 3 papers no mention of any ethical committee activity was reported in the text[24,29,30].

In 4 out of 7 papers dealing with human samples, no mention about any Ethical Committee approval was reported[33,34,36,38]; in 2/7 an Ethical Committee approved the experiment especially in human samples[25,26] while in the last one, the Institutional Review Board of Pusan National University Yangsan Hospital was asked for approval[35].

Discussion

Animal-Human Models and Methodological Issues

The number of papers dealing with human and animal tissues samples was quite similar. Animal models are convenient since they allow greater sample size, more experimental groups, controlled conditions, longer and certain PMI and high standardization. On the other hand, the amount of analyzed tissue is significantly lower in animals than in humans and the effects of the volume on conservation and PMI is unknown. Moreover, some antemortem conditions, which could deeply affect postmortem changes and miRNAs, such as agony, hypoxia or coma, are hardly reproducible in animal experimental settings[29].

The evaluated PMI is certainly one of the main limitations of the experimental studies on humans since it is difficult to obtain samples from the same individual over a long time, due to ethical and juridical reason. A prolonged PMI was evaluated just in a single study focusing on skeletal remains in real forensic cases. Moreover, even though these results added significant value to the topic, a relatively low R^2 with PMI was found[35]. In real forensic cases, environmental conditions cannot be controlled representing significant variables[39].

The lack of human data is one of the biggest limitations for the forensic application of PMI estimation models based on miRNAs analysis. The present review highlighted a few studies in which models developed on animals were validated on human samples, sometimes with satisfactory or even good results in terms of error rates (for instance, mean estimation error of 5.06 h[25]).

Human studies and real forensic cases can add value to animal models on PMI estimation by miRNAs analysis, by confirming the stabil-

ity/degradation of reference and target markers, which might differ from species to species, and by validating previously developed mathematical models. However, the interpretation of such studies and the transfer of markers and models from animal to humans is under evaluation because of some critical issues, such as potential differences in species-specificity of some markers, in the sampling site, in the sample storage, in the cause of death, etc.[25,35]. E.g. it is unknown whether antemortem factors, as the cause of death, affected the results of Lv et al[25] and how this influence was accounted for.

Since real-life situations pose several challenges, the need for standardized analytical protocols from sample collection to data analysis, and guidelines is even more pressing[23,27].

In the present review, complete information regarding gDNA removal or complete PCR reaction conditions was not declared in all the studies; this can clearly affect studies reliability and influence comparisons among papers.

RNAse treatment of materials was unevenly reported; total RNA integrity and level of degradation were assessed by either agarose gel electrophoresis or RNA integrity number (RIN), but, as evaluated by one of the included paper, RIN gives important information on the integrity of mRNA, it does not represent all transcripts[29]. To assess the reliability of target markers, the included studies evaluated the threshold cycle (Ct), as an indicator of the original relative expression level of the gene of interest and searched for changes in Ct over time and experimental conditions. Ct was either investigated as a raw indicator, as delta-Ct or delta-delta-Ct with respect to reference markers, by standard deviation or by algorithms (geNorm and NormFinder). Different Ct values were chosen to take into account the gDNA contamination. Within studies focused on circadian-miRNAs, for example, some considered the relative expression or fold change by Genex software, while others directly the Ct variation.

As for mathematical models for PMI estimation, several "curve fittings" were attempted and, up to now, a clear superiority of one compared to others has not emerged, though R^2 and error rate are a shared methodology to judge the robustness of the correlation and the model. As reported by Ma et al[28] a linear assumption between markers expression and PMI lacks verification. Indeed, three-unknown model was proposed in the study.

Single-blind validation was proposed as particularly critical for model verification and its use should be encouraged in similar analyses[27].

Finally, even though the involvement of an Ethical Committee was not a rule among the included studies, the approval by an external and independent organ (generally the Ethical Committee) represents an indispensable step for animal and human study nowadays. This review let us remind that only papers published before 2016 were accepted by journals without the Ethical Committee approval, after that year Ethical Committee approval seems to become mandatory.

Tissues and Tissue-Specificity

In the PMI investigation by miRNA analysis, there is no consensus regarding which tissue or biological fluid is the ideal one. Heart and brain tissues were the most investigated in the present review, probably because both of them are protected: one in the thoracic cavity, the other into the skull and both of them are less affected by environmental factors[23]. Whichever is the available or chosen matrix, the evaluated tissue strongly affects the feasibility of miRNA analysis, the pre-analytical or analytical steps to be performed, as well as the reference and target miRNA markers to be analyzed. E.g., in the vitreous humour, the expression of miRNAs was very low and required a preamplification step[36].

The literature and the present review accordingly allowed to confirm miRNAs tissue-specificity, even inter-species. This is not surprising as some authors, in an attempt to construct an atlas of miRNAs belonging to different human tissues, demonstrated tissue-specific characteristics of some miRNAs[40,41]. Tissue specificity was almost confirmed from rats to human tissues[41]. According to our review, miR-133a and miR-1 have high specificity and stability in heart tissues, miR-9 and miR-125b in the brain, and miR-133a and miR-1 for skeletal muscle (moreover miR-206 in rats and miR-200 in humans) (Table III). Our review shows MiR-195 and miR-200 to behave as good reference markers in mice and human lung tissue but just in one study[26], being this evidence not confirmed by Landgraf et al[40] and Ludwig et al[41]. MiR-200 was found specific for epididymis, skin, kidney and thyroid but not for lungs[41], while in our review it was a good and stable reference marker in lung and human skeletal muscle. MiR-15a, which had been previously considered ubiquitous, was found at very low expression levels in humour vitreous[33].-

Table III. MiRNAs and tissues.

Tissue	miRNAs used as reference marker	Species	Results on miRNA	Ref.
Brain	miR-9; miR-125b	Human	Stable expression up to 144 hrs and among 10°-35°C	[28]
	miR-16; miR-34a; miR-124a; miR-134	Rats	Slight decrease after 24 hrs[miR-34a]; stable up to 96 hrs [miR-16; miR-124a; miR-134]	[29]
	miR-9; miR-125b	Rat	Stable up to 24 hrs and up to 35°C	[24]
	miR-9	Human	Stable: in head injury with PMI 10-24 hrs; in haemorrhage shock with PMI 10-24 hrs; mechanical asphyxia with PMI > 20 hrs	[37]
	miR-9; miR-125b	Human	Stable up to 25 hrs	[34]
	miR-2909	Mice	Stability over 36 hrs in brain	[30]
Heart	miR-122; miR-133a	Mice	Stable up to 8 days	[32]
	miR-122; miR-133a	Mice	Stable up to 8 days, especially for miR-133a	[31]
	miR-2909	Mice	Stability over 12 hrs in heart	[30]
	miR 1	Rat	Stable up to 7 days	[23]
	miR-1; miR-133a	Human and rat	Stable over 5 days and even at 35°C	[25]
	miR-1	Human	Stable: head injury with PMI < 10 hrs	[37]
Skeletal muscle	miR-133a	Mice	Stable up to 8 days	[32]
	miR-133a	Mice	Stable uo to 8 days	[31]
	miR-1; miR-206; miR-200	Human and rat	Stable both up to 30°C [mir-1 and miR-206 in RAT] [mir-200 in Human]	[26]
Liver	miR-122	Mice	Stable with fluctuation related to PMI	[32]
	miR-122	Human and rat	Tend to degrade after 4 days particularly at 35°C	[25]
	miR-122	Mice	Highest expression influenced by PMI	[31]
Spleen	miR-125b; miR-143	Rats	Fluctuated slightly within 36 hrs and then increased at 25°C; the same trend observed within 144 hrs and then increased at 4°C	[27]
Lung	miR-195; miR-200	Human and rat	High stability at 20°C and lower temperature; miR-200 higher stability up to 30°C	[26]
Kidney	miR-194	Human	Stable in hemorrhagic shock with PMI < 10 hrs and 10-20 hrs	[37]
Skin	miR-203	Human	Stable in head injury with PMI < 10 hrs; in mechanical asphyxia with PMI > 20 hrs	[37]

Reference Markers

RT-qPCR shows high quantitative accuracy so that minimal RNA levels modification by sample processing might alter the target gene expression deeply. Thus, it is always necessary to use some normalizer (or reference) markers[39]. However, no consensus exists about the best ones in human or mouse/rat models. As shown in Tables I and II, several papers evaluated the issues related to reference markers. Changes in expression of the reference markers could falsely point towards a change in the expression of target biomarkers, thus affecting the reliability of experimental studies[42]. It is fundamental to verify the expressional stability of a hypothetic reference marker in each tissue investigated and condition analyzed (e.g., cause of death, individual parameters, etc., PMI[26,37]). One of the main concerns emerged, indeed, is represented by autolysis, which could affect miRNAs stability over time. The proposed best reference markers, for each tissue studied both in human and in mice/rats, as emerged by the present review, are shown in Tables I and II.

In the study of Zhang et al[37], traditionally mRNAs used as normalizers (Gapdh, β-actin), 18s-rRNA and U6 (snRNA) appeared to be reliable reference markers in human myocardium, brain, kidney and skin, while miRNAs (MiR-1, miR-9, miR-194-1, and miR-203) were not suitable as endogenous control genes. However, these results were contradicted by other upcoming papers, which provided evidence that some of the most used reference genes, such as Gapdh, β-actin, U6, etc are not suitable for this role in brain, liver, spleen, heart, and skeletal muscles, due to degradation over time or low amplification efficacy[27-29,31].

In rats heart, a study evaluating miRNAs as reference biomarkers showed relatively stable Ct values for miR-1 (stable over 7 days after death) and selected it as endogenous control for normalization of 18S-rRNA, used as target biomarker[23]. MiR-1 has demonstrated a high tissue specificity both in heart and muscle, as its tissue specificity index (TSI) approaches 100 in a study performed on humans[41].

MiR-122, miR-133a, and 18s-rRNA in mice heart and skeletal muscle tissues appeared satisfactory as reference biomarkers[31,32]. Human heart studies confirmed the animal models, by showing miR-133a and miR-1, as fairly stable over 5 days after death and different temperatures range[25]. Even miRNA 133-a – as miRNA 1 – showed high tissue specificity in heart and muscle in a study performed on humans. MiR-122 was also selected as reference marker in a study on vitreous humour focused on the evaluation of a circadian rhythm[26]. In mice skeletal muscle miR-133a, circ-AFF 1 and LC-LRC6 were good reference markers[31,32]. These markers were not evaluated in a study which analyzed both animals and human samples and selected miR-1, miR-206, 5S and RPS29 for skeletal muscles by algorithm analysis[26]. Approximately the same markers, and particularly miR-195, miR-200c, along with 5S, U6, RPS-29, were considered the best reference markers, by the same approach from animals to humans trough algorithms[26].

Animal brain analyses mostly confirmed this data, showing that miR-16, miR-34a, miR-124a, and miR-134 remained stable up to 96 h postmortem in rats, long after primary transcripts are silenced[29]. This high stability over time, despite a period of conservation at 4°C degrees, might be related to brain tissue characteristics. Another study on rat brains, not analyzing the abovementioned miRNAs, showed that miR-9 and 125b were the best reference markers with high stability over 6 days even at higher temperatures[28], while in humans both miR-9[37] and miR-9 and miR-125 showed high stability patterns[34] allowing them to be considered the best reference markers. The stability of one of the two (miRNA 9) in brain was confirmed later on in a study dealing with tissue specificity miRNAs: miRNA 9 presented the highest specificity for brain[41].

In mice liver, the best reference markers were mir-122 and other RNAs (circRNAs, circ-AFF 1 and LC-Ogdh)[31], while in another study 5SrRNA appeared to be more stable than mir-122, so it was chosen as the best reference markers in both rats and human liver[25]. Despite high liver specificity had been shown for mir-122[39,41], it tended to degrade in human liver experiments at 35°C and with PMI[25]. In rat and human lungs, the best reference markers were mir-195, mir-200c along with other RNAs (5S, U6, RPS-29)[26]. Two reference miRNAs, miR-125b, and miR-143, showed high stability even in rat spleen, although spleen high degradability. Compared to mRNAs (β-actine, Gadph) and snRNA (U6) and 18s-rRNA, miRNAs appeared less susceptible to degradation, likely due to their small size[27].

While the study conducted on spleen remains of exclusive non-human relevance, heart, lung, skeletal muscle and liver tissues were used as samples in more papers, both in humans and animals. Usage of the same matrix in human and not-human experiments and the finding of the same reference miRNAs in that tissue independently from the species allowed to hypothesize a strong correlation among certain miRNAs and tissues. Some miRNAs seem to replicate their behaviour (Table III) inside the same organ regardless of whether that organ belongs to a man or a mice/rat.

Most of the papers accordingly highlighted the need of analyzing more than one reference biomarker[23,39]. A suitable number of reference genes to be analyzed could be obtained by pairwise variation V, offered by algorithms as geNorm, as proposed by several articles[26,31,37]. Zhang et al[37] demonstrated that the number of endogenous references could be reduced by considering some critical parameters, such as the cause of death beside tissue and PMI.

Beside PMI, cause of death, gender, age at death and diseases might influence the expression of miRNAs and the impact of these factors is still to be elucidated[39].

Target Markers

Many papers aimed at identifying the best target markers in relation to PMI in human/mice/rats samples collected from different tissues or biological fluids. Contrarily to the reference marker, the ideal target marker should present a sensible expression alteration in relation to the variable under investigation. Thus, for PMI estimation, the ideal target marker should modify its expression progressively and in a measurable manner over time since death. The most significant and measurable the expression variability of the target marker, the more the target become a good PMI estimator.

In most of these studies, miRNAs have been only evaluated or proved useful as reference and not as target markers, due to their high and maintained stability across different tissues, conditions and PMIs. MiRNAs were evaluated as target markers in some human studies with early and medium PMI[33-36] but only in bones they showed a statistically significant difference between PMI < and > 1 month. However, a negative linear regression, by plotting miRNA *vs*. PMI, only provided[35] a low R^2. This result is in accordance with their stability in early and medium PMI; it takes a longer time for miRNAs to degrade, so they could become target markers for longer PMI evaluation. In the majority of the studies other RNAs, mostly mRNAs, were evaluated as target markers to determine the PMI.

The importance of a multiple evaluation, i.e., of the analysis of different target and reference markers in several tissues was especially highlighted by reviewed papers, as well as the need for a confirmation of the usefulness of the markers in longer PMI intervals[35].

Temperature

Temperature has a relevant impact on mRNA degradation process[2]. On the contrary, Lv et al[25-27] demonstrated that the degradation rate of the reference miRNAs was quite low under experimental conditions: delta-Ct values increased with temperature increasing, meaning that the degradation rate of mRNA markers was higher than that of miRNA reference markers.

However, while this increase was slight across different PMI at lower temperatures, when considering higher temperatures, e.g., 30°C, the relationship between delta-Ct values and PMI changed from linear to curvilinear, thus delta-Ct values increased in early PMI and then decreased in later PMI when the degradation rate of reference miRNAs exceeded the degradation rate of target mRNAs[26].

These findings were further confirmed by applying an animal-based mathematical model on human samples, it was shown that some reference markers, such as mir-122, tended to degrade after 4 days at 30°C[25]. These findings suggest that miRNAs are less susceptible to degradation induced by PMI and environmental conditions because of their short length.

Considering that temperature has a big impact on the degradation of mRNAs, which were demonstrated to be useful target markers for PMI evaluation, knowing and taking into account this parameter and its variations is crucial in every experiment.

Most of the animal studies are performed in controlled conditions, while temperature and environment might significantly change across the interval of the PMI for real corpses. Bodies are susceptible to day/night temperature variations, humidity and different kind of storage, among other confounding factors. Moreover, information regarding the temperature history is not always available in humans experiments.

Since the mathematical model to be applied changes across different environmental temperatures, to transfer the estimation in real forensic cases, it is fundamental to obtain as much information as possible regarding the death scene, the temperature history of the corpse and its storage[26]. The validation on real forensic cases is strongly suggested[35].

Circadian MiRNAs

A biological 24 h clock allows all organisms to adapt and coordinate to daily rhythms (e.g., environmental light) and miRNAs might have a role in the regulation of the human circadian clock by post-transcriptional regulations[43]. The analysis of markers expressing a circadian rhythm, including miRNAs, has been used to determine the time of deposition of human traces[44]. Similarly, in forensic pathology, the characteristics of "biological clock" of miRNAs have been evaluated to determine time since death. In the present review, the expression of a circadian pattern by miRNAs was investigated both in animals (brain and heart)[30], and in humans (blood and vitreous humour)[33,36]. The only animal study dealing with miRNA circadian pattern was performed in mice; it analyzed miR-2909 and AATF mRNA (apoptosis antagonizing transcription factor involved in cell cycle control and DNA damage responses)[30]. AATF mRNA and miR-2909 were predominantly expressed in heart and brain among a wide panel of tissues and exhibited a circadian rhythm so that animal sacrificed at daytime and in the nighttime displayed a different time-dependent expression. Quantitative values were not reported by the paper, though the attached figures demonstrated that miR-2909 expression was more stable in brain than in heart. AATF expression dropped earlier than miR-2909 in mice sacrificed at 8 pm, while both targets markers rapidly decreased in expression in mice sacrificed at noon[30]. Given the type of article, i.e., rapid communication, and

the low amount of studies in this field, further research and more quantitative data are necessary to confirm the hypothesis regarding AATF as a useful biomarker for PMI estimation.

In humans, no solid tissues have been studied for the identification of circadian-miRNAs, so far. The feasibility of miRNAs analysis from human vitreous humour has been investigated by two studies, which differed in the number of profiled miRNAs: 754 miRNAs were analyzed in vitreous humour by Odriazola et al[36], while just 10 miRNAs in vitreous and blood by Corradini et al[33].

Even though the methodologies and data analyses (Table II) were similar, different reference markers were used in the two experiments: the former used mir-222 while the latter used SNORD95 (a small nucleolar RNA).

The first study on human vitreous humour (explorative analysis on 7 samples, in-depth analysis of selected marker on 34 samples), showed a significant difference in expression for several miRNAs, particularly miR-142-5p and miR-541, between diurnal and nocturnal deaths[36]. Later on, this evidence was confuted by the study of Corradini et al[33], who recognized a circadian pattern for miR-142-5p and miR-541 only in blood, but not in the vitreous humour. Conversely, miR-106b and miR-96 were proposed as circadian markers in vitreous humour by Corradini et al[33].

Besides differences in the applied methodologies, this contradictory result could be related to the low number of samples studied in both experiments.

No significant correlation was demonstrated between the level of miRNA expression and PMI[36]. Thus, the evaluation of these markers might be useful in establishing if death occurred during day-time or night-time but not in the estimation of the interval since death. Further studies, applied on a wider casuistry, are needed.

Conclusions

Determining elapsed time since death or post-mortem interval (PMI) is one of the most important and frequently asked questions in forensics, but can sometimes be tricky to answer due to many factors involved. The need for identifying new biomarkers related to chronological changes after death, along with the technological progress, has been propelling the scientific research in this field leading to the publication of many relevant studies. It is of pivotal importance to identify, evaluate and summarize these findings, thereby making them more accessible to the scientific community.

This review highlights two important issues. First, no miRNA has been identified as a good target marker to evaluate PMI, but they are excellent reference markers because of their stability. Secondly, tissue-specific miRNAs should be selected as endogenous controls for each tissue because of their stability, their tissue-specificity and their similar behaviour both in rats and humans.

A multiple parameter analysis on several tissues is fundamental to reduce the error rate. Future research will fill the gap for those tissues in which specific miRNAs have not been identified, yet (i.e., lung, kidney, spleen) and should be focused on the evaluation of interfering factors, such as environmental and storage conditions. Moreover, the validation of animal mathematical models on human tissue is recommended.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Authors' Contribution

EM conceived and designed the study and wrote the first draft; AG revised and wrote the final draft; AF, AT, FPB, RG critically revised and contributed to the final written manuscript.

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