miRNAs: Perspective Towards the Use for Body Fluid Identification

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ABSTRACT

Identification of body fluids provides an important lead for crime investigation by which it can give a clue about the nature of the case and assist crime reconstruction. In the last decade, miRNAs have emerged as promising markers for body fluid identification due to their cell- /tissue-specificities. miRNAs are a class of small noncoding RNAs with ~ 22 nucleotides in length and their small sizes enable them to be resistant to degradation. The possibility to adopt miRNA markers for body fluid identification has been studied in various forensically relevant body fluids. This review aims to give a comprehensive summary of proposed miRNA markers for identifying five body fluids (venous blood, menstrual blood, semen, vaginal secretion and saliva). Based on numerous evaluations of miRNA markers and the development of model analysis using a single panel of miRNAs to identify unknown samples, proposed panels and analysis strategies were gathered and discussed.

Keywords: miRNA; forensically relevant body fluids; body fluid identification; crime; forensic casework (Siriraj Med J 2020; 72: 512-526)

INTRODUCTION

miRNAs are a class of small non-coding RNAs with ~ 22 nucleotides in length that have been found in organisms ranging from viruses, plants, invertebrates, vertebrates to humans as demonstrated in the latest release of miRBase (v22), the online database of miRNA sequences which contains 48,860 miRNA sequences from 271 organisms.[1] It is known that miRNAs regulate gene expression within cells they are generated and they can be secreted into the extracellular space for regulating other cells or for cell-to-cell communication.[2,3] Following physiological activities of the cells and cell death, miRNAs are non-specifically released from cells.[4,5] These extracellular miRNAs have been found in vesicle-like molecules such as exosomes, microvesicles and apoptotic bodies[2,6], whereas some are associated with proteins, particularly AGO2.[4,6,7] This allows miRNAs to be shielded from RNAase degradation and to increase their stability in biological fluids.[4]

Studies on expression profiles of miRNAs revealed that they are differentially expressed in each cell type. In human cells, the Functional Annotation of the Mammalian Genome (FANTOM5) consortium has recently demonstrated that miRNAs are differentially expressed in 121 distinct cell types with the top five expressed miRNAs accounting for ~50% of the overall miRNA in each cell type.[8] These expression patterns of miRNAs suggest that miRNAs could be promising markers for cell type and tissue identification. For body fluid identification, miRNAs have been extensively studied in five forensically relevant body fluids: venous blood,

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menstrual blood, semen, vaginal secretion and saliva as previously reviewed in.[9-13] The first review by Courts C. *et al*. mentioned panels of miRNAs specific for the five body fluids from two publications that opened up the view of applying miRNAs for body fluid identification.[12] Subsequent reviews added more recent miRNA markers and covered issues of miRNA profiling methodologies, factors influencing miRNA expression[9], standardisation of analysis procedures (methods to isolate and quantify miRNAs)[10] and the potential use of miRNA in clinical conditions.[11] The present review aims to provide up-to-date work focusing on the five body fluids: venous blood, menstrual blood, semen, vaginal secretion and saliva that are relevant to violent and sexual assaults. The presence of venous blood is generally related to injuries and can be an indicator of offensive scenarios. Significant evidence in sexual assaults includes semen, vaginal secretion, menstrual blood and saliva. This review summarises and discusses model analyses consisting of proposed panels of miRNA to distinguish one kind of body fluids from other forensically relevant body fluids.

miRNA markers in venous and menstrual blood

Discrimination between venous blood and menstrual blood could give a clue about the nature of the case and assist crime reconstruction whether it associates with violent and/or sexual assaults. The presence of venous blood is related to violent assaults causing injury or death. Menstrual blood could be involved in sexual assaults in a certain case. For instance, a woman who is sexually assaulted by a man during her menstruation can transfer the menstrual blood on the suspect's penis. Hanson E.K. *et al*. were the first to propose certain miRNA markers for identifying the forensically relevant body fluids including venous and menstrual blood. Using quantitative real-time polymerase chain reaction (qRT-PCR) as a miRNA profiling and validation method, they demonstrated that miR-451 and miR-16 were differentially expressed in venous blood compared to menstrual blood, semen, vaginal secretion and saliva. In line with this, several studies have shown that miR-451 and miR-16 were specific to venous blood and could be potential markers for venous blood identification[14-21] (Table 1). Based on a number of independent studies using qRT-PCR or microarray for miRNA profiling followed by validation of miRNA expression, an array of potential markers was proposed and repeatedly identified in several studies.[15,17,22-25] With the advance of technologies, more recent studies have conducted massively parallel sequencing (MPS) or next-generation sequencing (NGS) for screening miRNAs at the genome-wide level which allows determination of miRNA quantity and expression patterns. Hence, this enables the discovery of novel miRNAs in biological samples. Wang Z. *et al*. investigated the expression of miRNAs in venous blood using the Ion Personal Genome Machine™ (PGM™) System with the capacity to detect 2,588 annotated mature miRNAs in the human genome (miRBase v21).[18] The authors obtained a miRNA profile and ranked the most abundant miRNAs to search for potential miRNA markers. They showed the top six miRNAs in the blood samples to be miR-486-5p, miR-16-5p, miR-451a, miR-144-3p, miR-126-5p and miR-144-5p. The first four of which had been reported in previous studies as summarised in Table 1. In addition, a study used Illumina HiSeq 4000 to examine the miRNA expression in venous blood.[19] Following analyses of the data, the study identified the top 20 highly expressed miRNAs in which four of them were consistent with previous reports: miR-486-5p, miR-451a, miR-182-5p and miR-16-5p. More recently, a study on miRNA profiling in the venous blood using MPS and validation of miRNA expression via qRT-PCR has shown miR-451a, miR-486-5p, let-7i-5p, let-7b-5p and miR-92a-3p as the five most abundant miRNAs.[21] Strikingly, these miRNAs overlapped with the highly-expressed miRNAs reported by Wang Z. *et al*.[18] and El-Mogy M. *et al*.[19] Altogether, the accumulating data highlight a panel of miRNAs repeatedly found in venous blood and the analysis of such a panel, instead of individual miRNAs, could increase the specificity of venous blood identification. However, it is worth noting that some venous blood-specific miRNAs: miR-451, miR-185-3p and miR-144-3p have also been detected at an equivalent level in menstrual blood. This suggests that the application of miRNA markers for discrimination between venous blood and menstrual blood requires an analysis model in which a threshold or cutoff value is set up to assist the result interpretation.

miRNA profiling in menstrual blood is less extensively studied compared to studies on venous blood. Based on the relevant studies in Table 1, it was suggested that miR-144-3p could be a potential marker for menstrual blood; however, this miRNA was also highly expressed in venous blood.[22,23,25] Particularly, miRNA profiling in venous blood in healthy women during the menstrual cycle revealed that miR-144-3p was the second most abundant miRNA.[15] This underscores that miR-144-3p is commonly found in blood-type samples and its expression is independent of physiological changes during the menstrual cycle. Thus, the appropriate approach to differentiate menstrual blood from venous blood could be the establishment of a decision algorithm comprising miR-144-3p and additional miRNAs, yielding a score to

TABLE 2. Proposed panels of miRNA and model analyses to distinguish body fluids.

Groups	miRNAs in the panel	Types of model analysis	Model analyses	Accuracy/testing of the model	Experimental methods/reference RNA
1. Hanson E.K. *et al.*, 2009 [32]	- Venous blood: miR-451 and miR-16 - Menstrual blood: miR-451 and miR-412 - Semen: miR-135b and miR-10b - Vaginal secretion: miR-124a and miR-372 - Saliva: miR-658 and miR-205	2D scatter plots consisting of dCt of two markers in each type of body fluid	Identify unknown samples by positioning dCt on 2D scatter containing clusters of known samples	100% accuracy for venous blood, semen and saliva; ~90% accuracy for vaginal secretion; ~80% accuracy for menstrual blood	SYBR Green qRT-PCR/U6b
2. Hanson E.K. *et al.*, 2014 [23]	- Menstrual blood: miR-185-5p, miR-144-3p and miR-144-5p	Logistic model	Logit = -(0.005*dCt(miR-185-5p)*dCt (miR-144-3p)* dCt(miR-144-5p)) + (3.718*dCt(miR-185-5p)) – 32.017 Menstrual blood if p>0.5 (Chi-square)	100% accuracy (12 blinded samples tested)	SYBR Green qRT-PCR/miR-940
3. Wang Z. *et al.*, 2015 [34]	- Menstrual blood vaginal secretion and saliva: miR-205-5p - Menstrual blood: miR-214-3p - Saliva and menstrual blood: miR-203a - Semen: miR-891a	Stepwise strategy to identify saliva	Identify saliva using a combination of miRNAs to distinguish saliva from other body fluids	N/A	TaqMan qRT-PCR/ U6
4. Sauer E. *et al.*,2015 [33] 5. Sauer E. *et al.*,2016 [25]	- Semen: miR-891a - Venous and menstrual blood: miR-144-3p and miR-203a-3p - Vaginal secretion and saliva: miR-124-3p and miR-203a-3p	Decision algorithm	Started with quantification of miR-891a to differentiate semen. Non-semen samples were subsequently analysed using D = -1.504-0.127*dCt (miR-144-3p) +0.454*dCt (miR-203a-3p) Venous blood if D>0 and menstrual blood if D<0 D = -3.305-0.743*dCt (miR-203a-3p) +0.582*dCt (miR-124-3p) Saliva if D>0 and vaginal secretion if D<0	90% accuracy (9 out of 10 blinded samples were correctly identified.)	TaqMan qRT-PCR/ SNORD24, SNORD38B and SNORD43

TABLE 1. Summary of reported miRNAs in forensically relevant body fluids, analysed by miRNA profiling and/or validation. (continue)

Groups	Venous blood	Menstrual blood	Semen	Vaginal secretion	Saliva	Screening platforms	Validation methods/reference RNA	Evaluation of miRNA stability in stain samples
6. Rekker K. *et al.*, 2013 [15]	miR-451a* miR-144-3p* miR-16-5p* miR-15a-5p* miR-19b-3p miR-142-3p miR-486-5p miR-92a-3p* miR-20a-5p miR-223-3p miR-103a-3p miR-93-5p miR-106a-5p let-7g-5p* let-7b-5p*	N/A	N/A	N/A	N/A	qRT-PCR (Exiqon miRCURY LNA microRNA) Human panel I)	N/A	N/A
7. Wang Z. *et al.*, 2013 [17]	miR-486* miR-16*	miR-214	miR-888 miR-891a*	N/A	N/A	Microarray	TaqMan qRT-PCR/U6	- Bloodstains stored at RT, 1 month: No significant change in miR-486 and miR-16 expression compared to fresh samples - Menstrual bloodstains stored at RT, 1 month: No significant change in miR-214 expression compared to fresh samples - Semen stains stored at RT, 1 month: No significant change in miR-888 and miR-891 expression compared to fresh samples

TABLE 1. Summary of reported miRNAs in forensically relevant body fluids, analysed by miRNA profiling and/or validation. (continue)

Groups	Venous blood	Menstrual blood	Semen	Vaginal secretion	Saliva	Screening platforms	Validation methods/reference RNA	Evaluation of miRNA stability in stain samples
8. Hanson E.K. *et al.*, 2014 [23]	miR-185-5p* miR-144-3p*	miR-185-5p* miR-144-3p* miR-144-5p*	N/A	N/A	N/A	qRT-PCR	SYBR Green qRT-PCR/miR-940	N/A
9. Park J.L. *et al.*, 2014 [24]	miR-484 miR-182*	N/A	miR-2392 miR-3197	miR-1260b miR-654-5p	miR-223 miR-145	Microarray	SYBR Green qRT-PCR/U6	N/A
10. Sauer E. *et al.*, 2015 [33]	miR-144-3p*	miR-203a-3p*	miR-891a*	miR-124-3p	miR-203a*	N/A	TaqMan qRT-PCR/SNORD24, SNORD38B and SNORD43	N/A
11. Tong D. *et al.*, 2015[38]	N/A	N/A	miR-10b miR-135b	N/A	N/A	N/A	TaqMan qRT-PCR/U6b	- Semen stains stored at 25°C, 1 year: No significant change in miR-10b and miR-135b expression compared to stains at day 1
12. Wang Z. *et al.*, 2015 [34]	N/A	N/A	N/A	N/A	miR-200c-3p* miR-203a* miR-205-5p*	N/A	TaqMan qRT-PCR/U6	N/A
13. Sauer E. *et al.*, 2016 [25]	miR-144-3p*	miR-144-3p*	miR-891a* miR-10a miR-10b miR-135b	N/A	N/A	Microarray	TaqMan qRT-PCR/ SNORD24, SNORD38B and SNORD43	- Bloodstains stored at RT, 1 to 36 years: miR-144-3p was detectable at the level that yielded the correct fluid identification using the decision algorithm. - Semen stains stored at RT, 1 year: miR-891a was detectable at the level that yielded the correct fluid identification using the decision algorithm.

TABLE 1. Summary of reported miRNAs in forensically relevant body fluids, analysed by miRNA profiling and/or validation. (continue)

Groups	Venous blood	Menstrual blood	Semen	Vaginal secretion	Saliva	Screening platforms	Validation methods/reference RNA	Evaluation of miRNA stability in stain samples
14. Seashols-Williams S. *et al.*,2016 [28]	N/A	N/A	miR-891a*	N/A	miR-26b	Massively parallel sequencing (Illumina Hiseq)	SYBR Green qRT-PCR/Let7i and Let-7g	N/A
15. Wang Z.*et al.*, 2016 [18]	miR-486-5p* miR-16-5p* miR-451a* miR-144-3p* miR-126-5p miR-144-5p	N/A	N/A	N/A	miR-203a-3p* miR-205-5p* miR-223-3p miR-200c-3p* miR-141-3p miR-375* miR-34a-5p let-7c-5p miR-27b-3p* miR-125b-5p miR-23b-3p miR-99a-5p* miR-29a-3p miR-23a-3p miR-27a-3p miR-210-3p miR-24-3p* miR-29b-3p miR-22-3p	Massively parallel sequencing (Ion Personal Genome Machine 1 system)	N/A	N/A
16. Li Z. *et al.*, 2017 [39]	N/A	miR-141-3p miR-497-5p miR-143-5p	N/A	N/A	N/A	Microarray	SYBR Green and TaqMan qRT-PCR/U6	N/A

TABLE 1. Summary of reported miRNAs in forensically relevant body fluids, analysed by miRNA profiling and/or validation. (continue)

Groups	Venous blood	Menstrual blood	Semen	Vaginal secretion	Saliva	Screening platforms	Validation methods/reference RNA	Evaluation of miRNA stability in stain samples
17. Wang Z. *et al.*, 2017 [40]	N/A	N/A	miR-891a* miR-888* miR-429 miR-449a miR-34b miR-2392 miR-3197 miR-30a miR-196b	N/A	N/A	Massively parallel sequencing	N/A	N/A
18. El-Mogy M. *et al.*, 2018 [19]	miR-486-5p* let-7f-5p miR-451a* miR-92a-3p* miR-191-5p let-7a-5p let-7i-5p* let-7g-5p miR-182-5p* let-7b-5p* miR-185-5p* miR-16-5p* miR-26a-5p miR-25-3p miR-183-5p miR-181a-5p miR-151a-5p miR-151b miR-101-3p miR-30d-5p miR-30e-5p	N/A	N/A	N/A	miR-143-3p miR-203a-3p* miR191-5p miR-26a-5p let-7f-5p miR-486-5p miR-378a-3p miR-27b-3p* let-7g-5p miR-24-3p* let-7a-5p miR-375* miR-148a-3p miR-21-5p miR-205-5p* miR-320a miR-99a-5p* let-7i-5p miR-92a-3p let-7b-5p	Massively parallel sequencing	N/A	N/A

TABLE 1. Summary of reported miRNAs in forensically relevant body fluids, analysed by miRNA profiling and/or validation. (continue)

Groups	Venous blood	Menstrual blood	Semen	Vaginal secretion	Saliva	Screening platforms	Validation methods/reference RNA	Evaluation of miRNA stability in stain samples
19. O Leary K.R. *et al.*, 2018 [20]	miR-451*	N/A	miR-891a*	N/A	N/A	N/A	TaqMan qRT-PCR/miR-16	N/A
20. Tian H. *et al.*, [0] 2018 [3]	N/A	N/A	miR-891a* miR-888* miR-10a miR-10b miR-135b	N/A	N/A	N/A	TaqMan qRT-PCR/U6	N/A
21. Fang C. *et al.*, 2019 [21]	miR-451a* miR-486-5p* let-7i-5p* let-7b-5p* miR-92a-3p*	N/A	N/A	N/A	N/A	Massively parallel sequencing	SYBR Green qRT-PCR/ Cel-miR-39	- Bloodstains stored at RT, 3 weeks and 37°C, 1 day: No significant change in miR-451a and miR-486-5p expression compared to fresh samples

N/A: Not applicable
RT: Room temperature
Asterisks indicate miRNAs that were repeatedly found in more than one study.
miR-451 is also known as miR-451a.
miR-16 is also known as miR-16-5p.
miR-182 is also known as miR-182-5p.
miR-205 is also known as miR-205-5p.
miR-124a is also known as miR-124-3p.

classify the type of the blood. Indeed, Hanson E.K. *et al.* have generated a probability algorithm using miR-144-3p, miR-144-5p and miR-185-5p and the validation of this model showed 100% accuracy in a set of test samples, which are independent of samples used in the model development.[23] To allow the use of the model in practical work, the authors determined the expression of the miRNAs throughout 28 days of the menstrual cycle. The results from two donors showed that the developed model successfully identified menstrual blood between day 1 through 4 of menstruations. Sauer E. *et al.* also proposed an algorithm using miR-144-3p and miR-203a-3p to distinguish menstrual blood from venous blood with 100% accuracy.[25] Analysis of miRNA stability in aged samples, approximately one to four years old, demonstrated that the menstrual blood samples were successfully classified from venous blood, suggesting the flexible implementation of the algorithm in aged menstrual blood. Because both algorithms were validated in a small sample size: 12 and 2 samples, respectively, further studies may be conducted in a larger sample size prior to application for casework.

To be able to adopt miRNAs as markers for body fluid identification, their stability within the samples is one of the most important concerns. In bloodstains, it was shown that potential markers for venous blood including miR-16, miR-144-3p, miR-486, miR-451a were relatively stable in samples stored at room temperature.[17,21,25] Particularly, miR-144-3p abundance was not significantly altered in bloodstains kept for one year[22] and 36 years.[25] Quantification of miR-451a in bloodstains exposed to natural heat (8.9-61°C), humidity (10-99%) and sunlight for 180 days revealed that miR-451a was detected, though its level was significantly decreased as compared to the control samples kept at room temperature, low humidity and darkness.[26] In line with this, bloodstains treated with 1% sodium hypochlorite solutions showed a significant downregulation of six blood markers (miR-16, miR-486, miR-451a, miR-20a, miR-151a and miR-148a) compared to untreated controls.[21] Based on the studies of miRNA stability in various circumstances, it seems that miRNAs tend to be degraded in the samples under environmental challenges and harsh chemical exposure. Taken together, this suggests that samples for miRNA analysis should be stored in an appropriate condition and be protected from exposure to chemicals. In menstrual bloodstains, expression of miR-214 in samples stored at room temperature for one month was not significantly altered compared to the fresh sample.[17] In contrast, miRNAs in liquid whole blood appeared to be decreased following sample storage at room temperature for 72 hours and at -80°C for nine months.[27] This suggests that sample storage in dehydrated form is preferable to liquid form to protect miRNAs from degradation, and the specimen collection using a dry swab and stains of biological fluids gathered at a crime scene are applicable for miRNA analyses.

miRNA markers in semen

Semen is crucial evidence in sexual assault cases that can be present in the form of stains on objects or fabrics and it can be recovered from a victim's body, e.g. skin and vagina. Semen comprises spermatozoa and fluids from the seminal vesicles, the prostate and the bulbourethral glands. Although spermatozoa are ultimate markers of semen, they do not always exist especially in sterile seminal fluid. To search for alternative markers for identifying semen, miRNA profiling was conducted by the abovementioned studies (Table 1). Among the proposed miRNAs, it was clear that miR-891a was highly and exclusively expressed in semen compared to other forensically relevant body fluids: venous blood, menstrual blood, vaginal secretion and saliva.[17,20,22,25,28] Due to its distinctive expression in semen, miR-891a was suggested to be solely used for semen identification without the necessity of a statistical algorithm.

Evaluation of time-wise miRNA stability of miR-891a using qRT-PCR revealed that semen stains stored at room temperature for one month[17], one year[22] and three years[25] had a similar miRNA expression compared to fresh samples. Furthermore, a study has determined the effects of heat, humidity and sunlight on the stability of miR-891a by which semen stains were kept in an environmental chamber mimicking dry 24 hours in a Virginia summer with a temperature between 45-52°C and humidity of 50% for 14 days.[29] Throughout the experiment, it was observed that expression of miR-891a was not significantly changed in the stain kept in the chamber compared to the control samples kept at room temperature.[29] Whereas, samples treated with dish detergents showed amplification failures of let-7g, a highly expressed reference miRNA that could be because the detergent disrupts the cell membrane lipid composition of miRNA-containing microvesicles.[29] Another study has treated semen stains at a higher temperature and humidity (a temperature between 8.9-61.0°C and humidity ranged from 10-99%) for 180 days. It was revealed that expression of miR-891a was dramatically decreased from day 120, compared to control samples kept at room temperature, low humidity and darkness (a temperature between 19.0-23.7 °C and humidity ranged from 38-52%).[26] This provides examples of environmental

factors and chemical exposure affecting the analysis of miRNA expression in semen. Further studies on other factors are required to understand the limitation of miRNA analysis which would be a great consideration for implementation into casework.

It is likely that miR-891a can be used to identify infertile semen. Although miR-891a was significantly down-regulated in four types of infertile semen (asthenospermia, oligospermia, azoospermia and oligospermia combined with asthenospermia) compared to normal semen, its expression was still higher in the infertile semen when compared to non-semen body fluids (venous blood, menstrual blood, vaginal secretion and saliva).[30] This suggests that miR-891a could be used to discriminate semen from other forensic-related body fluids. Conversely, a subsequent study showed that the expression of miR-891a in azoospermia and asthenospermia was comparable to that in normal semen.[31] The discrepancy between the two studies can be derived from differences in reference RNAs used in the miRNA quantification in which the former study used SNORD24 and SNORD38B, whereas the latter study used 5s-rRNA. Taken together, the findings point out the potential of miR-891a for identifying both normal and infertile semen from other forensically relevant body fluids.

miRNA markers in vaginal secretion and saliva

Vaginal secretion is one of the most important forensically relevant body fluids, particularly in sexual assault cases. It can be present on penile swabs, male underpants and objects with alleged vaginal penetration. Among several studies, it has been shown by two groups that miR-124a (also known as miR-124-3p) was strongly associated with vaginal secretion.[32,33] A miRNA assay combining miR-124a with miR-372 revealed that the assay correctly differentiated ten of a total of eleven vaginal secretion samples from venous blood, menstrual blood, semen and saliva, suggesting that these miRNA markers could be used for an initial screening of unknown samples.[32] Likewise, miR-124a was selected together with miR-203a-3p to develop a decision tree facilitating the determination of vaginal secretion from venous blood, menstrual blood, semen and saliva. Through the validation of the decision tree using blind samples, it was demonstrated that nine out of a total of ten samples were correctly identified.[33] The overlap of miR-124a in these studies suggests that miR-124a could be a promising marker for vaginal secretion.

The identification of saliva can be proof that an assault occurred, especially in sexual assault cases. For example, saliva may be deposited on female underpants or bodies in a disputed licking event. Several independent lines of evidence suggested that the three miRNAs: miR-200c-3p, miR-203a and miR-205-5p were potential markers for saliva.[14,18,19,33,34] Quantification of the three miRNAs in saliva, vaginal secretion, semen, venous blood and menstrual blood revealed that they were also detected in vaginal secretion and menstrual blood at a comparable level to that in saliva, whereas their expression was low in semen and venous blood.[34] This is possible due to the fact that both saliva and vaginal secretion contain mucous membranes and menstrual blood consists of vaginal epithelial cells.[35] Thus, these body fluids share a similar pattern of miRNA expression. Although miR-200c-3p, miR-203a and miR-205-5p were not exclusively expressed in saliva, they tended to be potential markers for saliva identification when they were combined with other body fluid-specific miRNAs to establish a stepwise approach for body fluid identification.[33,34]

Proposed miRNA panels and analysis strategies to identify forensically relevant body fluids

To effectively identify forensically relevant body fluids, an ideal strategy would be the analysis of a panel, which consists of differentially expressed miRNAs. As shown in Table 2, the first panel was proposed by Hanson E.K. *et al.* who quantified two specific markers for each type of body fluid and plotted dCt values in two-dimensional (2D) scatter plot.[32] In this model, the unknown samples could be categorised against the cluster of dCt values on the 2D scatter plot of known samples, allowing identification of the body fluid origin. The authors tested the specificity of the model and showed that the accuracy was in the range of 80-100% for the five body fluids. Although the accuracy was close to 100%, the model may not be able to identify body fluids of which do not clearly belong to a particular cluster. This suggests the requirement of statistical approaches to establish a threshold for the probability of identification. Subsequent studies have addressed this issue and developed statistical models to determine the source of body fluids.[23,25,30,33,41] A significant model was reported by the group of Sauer E. *et al.* who suggested to initially distinguish semen from other body fluids using miR-891a, a truly semen-specific marker.[25,33] If the sample was non-semen, further statistical analysis was applied to differentiate venous blood from menstrual blood and vaginal secretion from saliva. The expression of semen-specific markers (miR-891a, miR-135a and miR-10a) was extended in infertile semen and it was shown that their expression was significantly down-regulated in infertile semen compared to normal semen.[30] However, the reduction in miRNA expression did not affect the

TABLE 2. Proposed panels of miRNA and model analyses to distinguish body fluids.

Groups	miRNAs in the panel	Types of model analysis	Model analyses	Accuracy/testing of the model	Experimental methods/reference RNA
1. Hanson E.K. *et al.*, 2009 [32]	- Venous blood: miR-451 and miR-16 - Menstrual blood: miR-451 and miR-412 - Semen: miR-135b and miR-10b - Vaginal secretion: miR-124a and miR-372 - Saliva: miR-658 and miR-205	2D scatter plots consisting of dCt of two markers in each type of body fluid	Identify unknown samples by positioning dCt on 2D scatter containing clusters of known samples	100% accuracy for venous blood, semen and saliva; ~90% accuracy for vaginal secretion; ~80% accuracy for menstrual blood	SYBR Green qRT-PCR/U6b
2. Hanson E.K. *et al.*, 2014 [23]	- Menstrual blood: miR-185-5p, miR-144-3p and miR-144-5p	Logistic model	Logit = -(0.005*dCt(miR-185-5p)*dCt (miR-144-3p)* dCt(miR-144-5p)) + (3.718*dCt(miR-185-5p)) – 32.017 Menstrual blood if p>0.5 (Chi-square)	100% accuracy (12 blinded samples tested)	SYBR Green qRT-PCR/miR-940
3. Wang Z. *et al.*, 2015 [34]	- Menstrual blood vaginal secretion and saliva: miR-205-5p - Menstrual blood: miR-214-3p - Saliva and menstrual blood: miR-203a - Semen: miR-891a	Stepwise strategy to identify saliva	Identify saliva using a combination of miRNAs to distinguish saliva from other body fluids	N/A	TaqMan qRT-PCR/ U6
4. Sauer E. *et al.*,2015 [33] 5. Sauer E. *et al.*,2016 [25]	- Semen: miR-891a - Venous and menstrual blood: miR-144-3p and miR-203a-3p - Vaginal secretion and saliva: miR-124-3p and miR-203a-3p	Decision algorithm	Started with quantification of miR-891a to differentiate semen. Non-semen samples were subsequently analysed using D = -1.504-0.127*dCt (miR-144-3p) +0.454*dCt (miR-203a-3p) Venous blood if D>0 and menstrual blood if D<0 D = -3.305-0.743*dCt (miR-203a-3p) +0.582*dCt (miR-124-3p) Saliva if D>0 and vaginal secretion if D<0	90% accuracy (9 out of 10 blinded samples were correctly identified.)	TaqMan qRT-PCR/ SNORD24, SNORD38B and SNORD43

TABLE 2. Proposed panels of miRNA and model analyses to distinguish body fluids. (continue)

Groups	miRNAs in the panel	Types of model analysis	Model analyses	Accuracy/testing of the model	Experimental methods/reference RNA
6. Sirker M. *et al.*,2017 [42]	- Venous and menstrual blood: miR-451 - Semen: miR-10b and miR-374 - Vaginal secretion: miR-203 - Saliva: miR-943	Receiver operator characteristic (ROC)	Calculating the area under the ROC curve (AUC) to discriminate between pairs of body fluids	N/A	TaqMan qRT-PCR/ miR-26b, miR-92, miR-484, miR-144
7. Tian H. *et al.,* 2018 [30]	- Semen: miR-891a, miR-135a and miR-10a	Discrimination function analysis	Y2 = -1.152*dCt(miR-10a) + 0.910*dCt(miR-135a) + 1.092*dCt (miR-891a) - 0.639 Semen if Y2 < 0 and non-semen if Y2 > 0.	97.5% accuracy	TaqMan qRT-PCR/ U6
8. Dorum G. *et al.*, 2019 [41]	- Venous blood: miR-126-3p, miR-486-3p and miR-486-5p - Menstrual blood: miR-200a-3p and miR-451a - Semen: miR-375 - Vaginal secretion: miR-203b-5p and miR-205-5p - Saliva and skin: miR-1246	Partial least squares (PLS) and linear discriminant analysis (LDA)	-	65% accuracy (13 out of 20 blinded samples were correctly identified.)	Massively parallel sequencing
9. Fujimoto S. *et al.*,2019 [31]	- Venous blood: miR-144-3p and miR-451a-5p - Semen: miR-888-5p and miR-891a-5p - Vaginal secretion: miR-1260b - Saliva: miR-203a-3p and miR-223-3p	Partial least squares-discriminant analysis (PLS-DA)	-	>90 % posterior probability (13 out of 14 blinded samples were correctly identified.)	SYBR Green qRT-PCR/miR-484, miR-92a-3p and 5S-rRNA

2D: two-dimensional
N/A: Not applicable

discrimination function analysis to differentiate semen from non-semen as follows: Y2 = -1.152*dCt (miR-10a) + 0.910*dCt (miR-135a) +1.092*dCt (miR-891a)-0.639, semen if Y2 < 0 and non-semen if Y2 > 0. Thus, this underscores the potential of miR-891a for identifying semen and suggests possible implementation in casework.

Based on miRNA expression using MPS, a recent study proposed a panel of nine miRNAs for identification of six body fluids/tissues: venous blood, menstrual blood, semen, vaginal secretion, saliva and skin.[41] Notably, three miRNAs (miR-126-3p, miR-486-3p and miR-486-5p) were selected as venous blood-specific markers which is in accordance with previous studies.[14,15,17-19,21] While the panel included miR-205-5p as a vaginal secretion marker, the miRNA has been also shown to be highly expressed in saliva.[14,18,19,32,34] Possibly, both vaginal secretion and saliva are generated from mucosa-like environments, allowing them to share a similar pattern of miRNA expression. The overlap pattern of miRNA expression adds an extra layer of complexity to the development of a miRNA panel. This problem could be overcome by exploring more specific miRNAs and/or generating a statistical algorithm to analyse a differential expression that would permit the prediction of body fluid origins. To date, the existing data provide a good starting point for further work, showing the potential of a miRNA panel to simultaneously analyse several forensically important body fluids. Prior to the implementation of a miRNA panel in forensic casework, it is necessary to validate such a panel in a larger sample size. Besides, the analysis of miRNA via qRT-PCR requires the standardisation of the procedure using robust reference genes for normalisation that would decrease inter-laboratory variations and take miRNAs a step closer from research to casework for body fluid identification.

Conclusions and future directions towards applications of miRNA markers in forensic casework

During the last decade, miRNAs have emerged as promising markers for body fluid identification and several independent lines of evidence have reported a range of potential miRNAs specific to venous blood, menstrual blood, semen, vaginal secretion and saliva, which are commonly significant evidence for crime reconstruction. A great effort has been made to transfer these miRNAs from laboratory benches to applications in forensic casework through an evaluation of the markers in fluid stains and development of model analysis to accurately differentiate a certain body fluid from the others. An ideal strategy involves the establishment of a miRNA panel consisting of differentially expressed miRNAs in forensically relevant body fluids that will allow identification of unknown stains. To achieve this, several studies have developed various model analyses and some of them used overlapped miRNA markers from previous studies. Based on the previous studies, it appears that all panels allow distinct identification of blood in general and semen, whereas the candidate miRNAs showed less association with vaginal secretion and saliva. This suggests that the initial implementation of miRNA markers for routine casework could be applicable for the identification of blood and semen.

To apply a miRNA analysis into routine procedures, it is necessary to establish standardised protocols starting from miRNA extraction, determination of miRNA expression, validation of reference RNAs for qRT-PCR normalisation, result analysis and result interpretation. At the step of miRNA quantification using qRT-PCR, the protocol largely requires a solid normalisation of reference genes, which are non-biological variances to deliver reliable and reproducible results. To address this point, the MIQE-guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) is recommended to be adopted in forensic genetics for method standardization.[43] The MIQE-guidelines suggest a qRT-PCR checklist covering details of samples and sample processing, assay optimisation/validation, experiment setup and data analysis that should be included into the submitted manuscript along with the full disclosure of oligonucleotide sequences, aiming to increase experimental transparency and reliability of the result.[44,45] This would also help researchers to reproduce the published protocol and unequivocally interpret the result. Besides, the standardised protocols can be conducted in workgroup trials at which a selected miRNA panel is validated in a large sample size. The accumulation of the result from laboratory members would also allow determination of inter-laboratory variances that would be useful for protocol optimisation or development of the assay guideline. At present, identification of forensically relevant body fluids usually relies on mRNA-based analysis. This is likely due to the existence of several collaborative exercises on DNA/mRNA co-analysis which provides guidelines for DNA/mRNA co-extraction, mRNA profiling, data interpretation as well as awareness throughout the technique.[46-49] If inter-laboratory trials of miRNA markers were established, it would be worthwhile to include miRNA/mRNA co-analysis. This would maximise the capacity of the approach in body fluid identification. At the step of the extraction, several studies have demonstrated that miRNAs in forensically relevant body fluids can be co-extracted with DNA using standard DNA extraction

methods.[50-52] More recently, it has been shown that a co-extraction kit, the AllPrep DNA/RNA/miRNA Universal Kit (APU), had comparable efficiency to standard kits for mRNA/DNA or miRNA/DNA co-extraction.[53] The successful co-extraction provides good support for the development of the protocol to analyse miRNAs, mRNAs and DNA, especially when amounts of sample are limited and DNA typing is more preferable. This would allow the method to be flexible and effective in sample consumption.

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