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ORIGINAL ARTICLE

Medicine Science International Medical Journal

Medicine Science 2024;13(2):455-60

Determination of blood and saliva in mixture samples with miRNA

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Received 29 March 2024; Accepted 25 April 2024
Available online 01 June 2024 with doi: 10.5455/medscience.2024.03.029

Abstract

Sexual abuse and rape are among the crimes that are increasing day by day in our country. According to 2019 TUIK data, 13.1% of children brought to security units are victimized by sexual crimes. According to data published by the World Population Review website in 2020, 1071 people were raped in Türkiye that year. The provability of crimes is a very important factor for the perpetrator to be punished. The field of forensic sciences tries to serve justice by examining the evidence found at the crime scene. In cases where the possible body fluids of the perpetrator and the victim are mixed, it is very difficult to make an identification because there is no method for the separation of these mixed samples. In this study, we aimed to differentiate mixed body fluids (blood and saliva) by microRNA method. The samples collected from volunteers for this study were prepared as two-donor mixtures in four different groups in five different dilutions. A total of 36 mixtures were prepared from fluids collected from 4 female and 4 male individuals. The miR-451A biomarker was used to indicate the presence of blood and the miR-203A-3p marker was used to indicate the presence of saliva in the mixture samples. The samples were first centrifuged and then RNA isolation was performed using miRNeasy Mini Kit. The concentration and purity of the obtained RNAs were determined by spectrophotometer. Then cDNA (complementary DNA) was synthesized from RNAs with Taqman microRNA Reverse Transcription Kit.U6 gene region was preferred as the reference gene and analyzes were performed by RT-PCR. The presence of blood samples in the mixture was shown in all dilutions, the best level was seen in 1x1 ratio. When all groups were analyzed, it was seen that the mixtures with male donors were expressed at a higher rate than the other mixtures.

Keywords: Micro ribo nucleic acids, mix samples, blood, saliva, real time polymerase chain reaction

Introduction

Ribo nucleic acids (RNA) have been studied in the field of forensic science since 1984. Studies have focused on the role of gene expression and its application areas in forensic science, and interest in messenger RNA (mRNA) and micro RNAs (miRNA) has continued to increase. MiRNAs were first discovered in the nematode Caenorhabditis elegans in 1993. Later, it was found in large numbers in many living species, from arthropods to humans. The first researchers to study micro RNAs in the field of forensic sciences were Hanson et al. [1]. In their study, they examined different miRNAs in peripheral blood, saliva, semen, vaginal fluid and menstrual blood using Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) analysis. They stated that miRNAs, which show different expression levels in different body fluids, are markers that can be used in the determination of biological fluids. Although later studies conducted by different scientists yielded inconsistent results, many miRNA markers that can be used in obtaining different body fluids are known today. MiRNAs are small (22-24 nucleotides) non-coding RNAs that have potential applications in forensic medicine due to their degradation resistant properties and tissue specificity [2]. Due to their size and encapsulated structure within proteins and lipids, miRNAs are more difficult to degrade than other RNAs [3]. MiRNAs are not easily broken down by the ribo nuclease enzyme and are resistant to degradation against environmental

CITATION
Atalay R, Ercan B, Holumen N, et al. Determination of blood and saliva in mixture samples with miRNA. Med Science. 2024;13(2):455-60.

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DOI: 10.5455/medscience.2024.03.029

factors such as temperature, heat and potential hydrogen (pH). The presence of miRNAs has been detected even in degraded tissue samples. In addition to being used in the diagnosis, prognosis and treatment of many diseases for years, studies have also been carried out in the field of forensic sciences for the last decade. Currently, studies are being carried out to determine body fluids, distinguish monozygotic twins, distinguish between peripheral blood and menstrual blood, identify the perpetrator in degraded samples, and determine postmortem interval. Examples of understudied sample groups among forensic miRNA studies that enable justice to be provided and to shed light on forensic events are mixture samples in multiple-perpetrator sexual abuse or murder cases. Mixture samples are samples created by two or more participating donors. Its presence can be proven by DNA profiling analyzes in liquids that are invisible to the naked eye or whose source is unknown [1]. When interpreting the mixture samples that need to be analyzed, all loci may not be obtained as a result of Polymerase Chain Reaction (PCR) if the sample amount is too small to cause allele and locus loss or in case studies where spoiled samples are collected.

As a result, a reliable identification may not be possible. Different methods have been tried to eliminate such difficulties. These studies include multiple Single Nucleotide Polymorphisms (SNPs), compound markers (i.e., DIP-STRs, SNP-STRs, and DIP-SNPs), lineage markers (Y chromosome STRs), and traditional Short Tandem Repeat Regions (STR) [4]. However, there is no fully effective and definitive method. The aim of this study is to determine to what extent these biological samples can be detected using miRNA biomarkers if the biological material of more than one person is mixed at the crime scene.

MiRNAs are non-coding RNA molecules that are 20-25 nucleotides long [5]. It is considered more worthy of study than DNA and other RNA types, as it is resistant to adverse environmental conditions and can be analyzed from small amounts of samples [1,6,7]. Studies show that miRNAs are tissue specific. miRNAs, which were first said to be biomarkers for body fluids by Hanson et al. were found to be compatible markers for blood and semen in the studies conducted by Zubakov et al. [8]. Studies conducted in subsequent years have found miRNA markers specific to different body fluids. The existence of different miRNA biomarkers has been proven even in identical twins with identical DNA [9]. Micro RNAs are named according to the naming system, with the abbreviation 'miR' followed by the number indicating the order in which they are found [2]. As a result of years of research, various miRNA regions designated for body fluids have been identified. In our study, we used the miR 451A marker, which is the most commonly used in blood fluid studies, and the miR 203A-3p marker in saliva fluid (Table 1).

Table 1. Candidate miRNAs used in the identification of blood fluid [10]

Body Fluid	miRNA Marker
Venous Blood	miR-451A, miR-144-3p, miR-16, miR-185, miR-126, miR-150, miR-486, miR-943, miR-200b, miR-142-3p
Saliva	miR-205-5p, miR-203A, miR-658, miR-142-3p, miR-200c, miR-26b, miR-223

Material and Methods

Working Group and Sample Preparation

For the research, samples were taken from 4 men and 4 women over the age of 18 who signed the voluntary consent form. In this study, blood and saliva samples were collected from people with a total of 36 mixed samples. A blood sample of 5 ml was taken venously, and a saliva sample was collected by spitting into a selected appropriate container and collected separately in suitable sterile containers. The samples were kept at +4°C until working time. Afterwards, mixture samples that could be encountered at a possible crime scene were prepared from the collected body fluids. The mixtures were prepared by modeling as two-donor mixtures. Samples of each mixture were prepared in proportions of 1/1, 1/10, 1/20, 1/50, 1/100. The mixture samples were adjusted to be in the combination of Female Blood-Female Saliva, Female Saliva-Male Blood, Male Saliva-Female Blood, Male Blood-Male Saliva.

It was stated that our study was found ethically appropriate by the İstanbul University-Cerrahpaşa Clinical Research Ethics Committee, with the letter dated 12.02.2021 and numbered E-86669574-302.14.06-30503, provided that Scientific Research Projects (BAP) support was received.

RNA Isolation in Blood and Saliva Samples

The prepared 500 µl mixture solutions were poured into containers containing ceramic beads, respectively. 1 ml purezol was added to the tubes and centrifuged for 45 seconds at maximum speed. After the mixture turned pink, it was left at room temperature for 5 minutes. 200 µl chloroform was added to each tube and left for 5 minutes. In this way, a mixture of RNA, DNA and protein was obtained. By centrifuging at 12,000 g, 20 minutes, 4°C, the mixture was ensured to form 3 phases at the end of the centrifugation.

PHASE 1 (Aqueous phase): Contains RNA, colorless.

PHASE 2: Contains DNA, is white in color.

PHASE 3 (Organic phase): Contains protein, is red in color.

Since RNA isolation was performed, it was transferred to the first tube and 500 µl isopropanol was added to a different tube and

DOI: 10.5455/medscience.2024.03.029

incubated for 10 minutes. Then, it was centrifuged at 12,000 g, 10 min, 4°C and the supernatants were discarded. 1 ml 75% EtOH was added to the observed precipitate formation and centrifuged at 7,500 x g, 5 min, 4°C. The supernatant was discarded and EtOH was evaporated at 57°C. 50-100 µl RNAse-free water was added to the precipitate and pipetted.

Obtaining cDNA

A cDNA synthesis reaction was prepared for each RNA sample we wanted to analyze by qPCR. To obtain cDNA from dilutions prepared from a known concentration of synthetic miRNA, RNA, mRQ buffer, and mRQ enzyme were added to a total volume of 10 in an RNAse-free 0.2 ml tube. The tube was incubated for 1 hour at 37°C, incubated at 85°C for 5 hours, and terminated. 90 µl ddH2O was added to make the total volume 100 µl and cDNA was obtained. In order to identify the mixtures, body fluid-specific miRNA biomarkers were determined using the miRBase database. Reverse primers for our marker miR-451-A are universal and included in the kit. Forward primer sequences are given in Table 2.

Table 2. Primers used for miRNAs for gene expression analysis

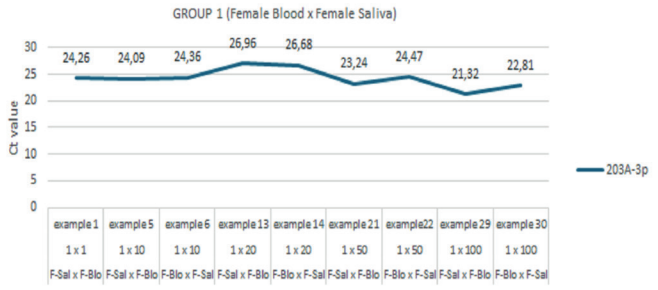
miRNA	Primer sequences	miRbase code
hsa-miR-451A	5' AAACCGUUACCAUUACUGAGUU 3'	MIMAT0001631
hsa-miR-203A-3p	5' GUGAAAUGUUUAGGACCACUAG 3'	MIMAT0000264

Normalization was performed using the U6 reference gene with the 2–ΔΔCt method. All statistical analyzes were performed using the T test with the SPSS v 26.01 program

Results

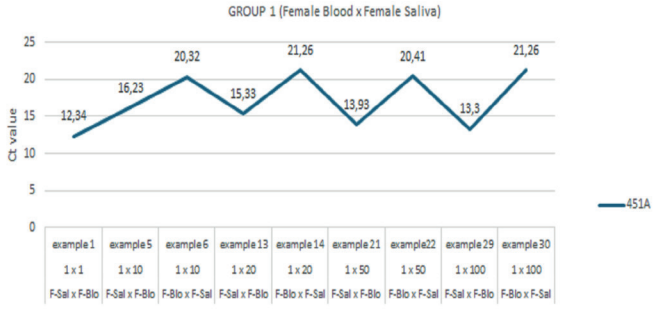
Mixtures were made as 250x250 for 1/1 ratio, 500x50 for 1/10 ratio, 500x25 for 1/20 ratio, 500x10 for 1/50 ratio, 500x5 ul for 1/100 ratio. In order to analyze the suitability of using hsa-miR-203A-3p for Saliva and hsa-miR-451A for Peripheral Blood as biomarkers, all mixtures were prepared as mixtures containing blood and saliva. In the samples, the expression levels of the markers hsa-miR-203A-3p and hsa-miR-451A were measured.

Graph 1. miR-203A-3p expression levels for Group 1



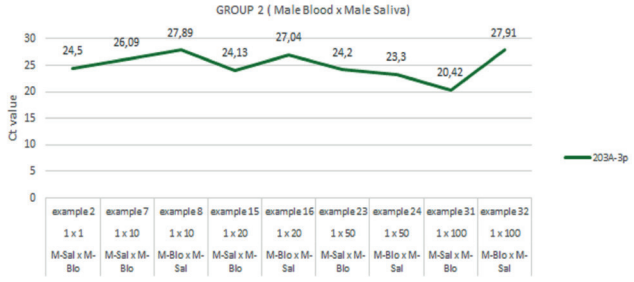
203A-3p expression levels observed in Group 1 samples were not significant

Graph 2. miR-451A expression levels for Group 1



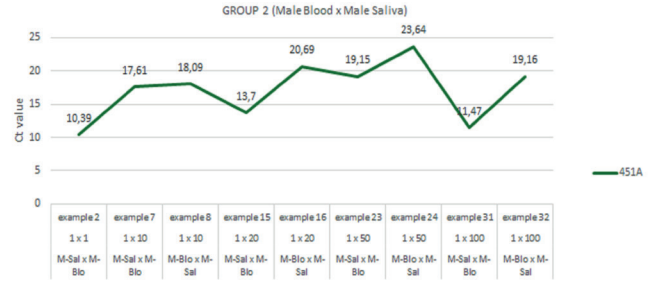
451A expression levels measured in Group 1 samples were found significant

Graph 3. miR-203A-3p expression levels for Group 2



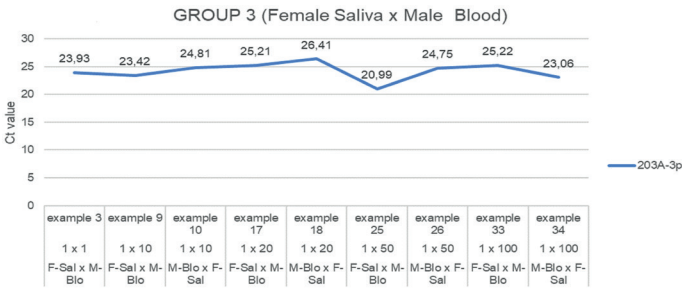
203A-3p expression levels measured in Group 2 samples were not significant

Graph 4. miR-403A expression levels for Group 2



403A expression levels measured in Group 2 samples were found significant

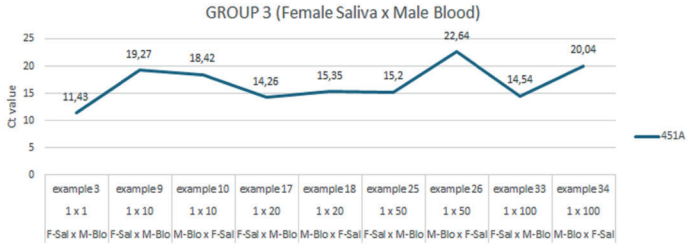
Graph 5. miR-203A-3p expression levels for Group 3



The 203A-3p expression levels measured in Group 3 samples were not significant

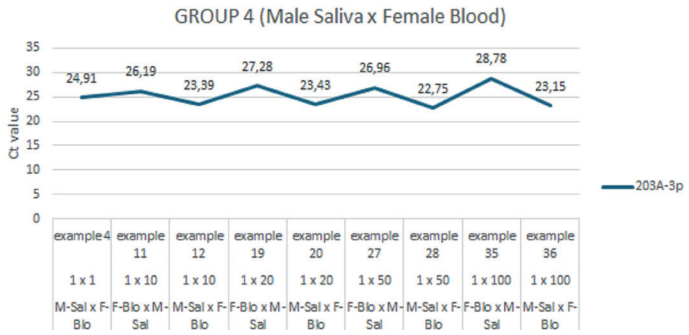
DOI: 10.5455/medscience.2024.03.029

Graph 6. miR-451A expression levels for Group 3



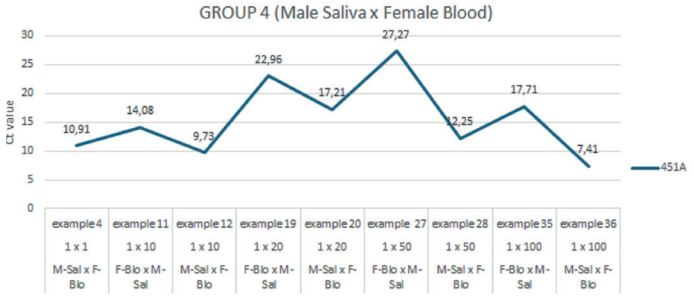
The 451A expression levels measured for Group 3 were found to be significant

Graph 7. miR-203A-3p expression levels for Group 4



The 203A-3p expression levels measured for Group 4 were not significant

Graph 8. miR-451A expression levels for Group 4



451A expression levels measured for Group 4 were found to be significant

Discussion

In forensic cases, biological samples collected from the scene serve as evidence to elucidate the incident. For this reason, they must be collected from the scene, stored under appropriate conditions, analyzed and interpreted using correct methods. Although each crime scene is unique, the body fluids (sweat, urine, semen, saliva, blood, etc.) left behind by the perpetrator and/or victim are limited. In studies, various genetic markers are used to make reliable interpretations of these body fluids. In the last decade, miRNAs have begun to be used for forensic purposes, unlike other methods. In miRNA studies, which enable justice to be ensured and to elucidate judicial events, samples of mixtures in multi-perpetrator sexual abuse, murder and similar cases have not yet been studied sufficiently. This study aimed to demonstrate the presence of body fluids in the mixture samples by using miRNA biomarkers.

Blood and saliva samples that were likely to be seen at a possible crime scene were selected for the study. miR-203A-3p [10-12] was used to detect the presence of saliva in the mixture, and miR-451A biomarker was used to detect the presence of peripheral blood [13-15]. For analysis, the Real Time PCR technique, which is not affected by non-specific amplifications and can perform analysis during the reaction, was used [8]. Expression levels obtained by RT-PCR give us a Ct value. This value shows how many cycles it takes to obtain the marker we selected. When interpreting the analysis, samples with a Ct value of 25 and above are not considered applicable, considering primary dimer formation (Not Applicable:NA).

The U6 (housekeeping) gene was taken as a reference for the study. U6 gene expression level was calculated for each sample as a control group. Ct values of the hsa-miR-451A marker, which we chose to distinguish blood fluid, showed that it is an accurate marker for this body fluid. Early expression levels occurred in all samples. Many studies have shown that it is a suitable biomarker for blood fluid [13-15]. In our study, a result consistent with the studies in the literature was obtained. According to the T test result for the density difference expressions in blood and saliva samples, $p<0.005$ was found; It has been shown that a significant difference exists between expression levels.

In our study, when interpreting the hsa-miR-451A expression levels obtained according to the ratios in the groups, the amount of saliva and blood in the mixtures was examined. High expression rates indicate that the marker appeared late and was difficult to detect. For each of the four groups in the study, examples of different intensities that could be encountered at a possible crime scene were tried to be created. Comparison of expression levels in different body fluids was calculated by the 2^-delta delta Ct method [16].

In our study, we tried to determine the expression level of miRNA 451A biomarker in the mixture of female blood-female saliva samples in GROUP 1. At every ratio, earlier expression was achieved in the mixture with higher blood density than in the mixture with higher saliva density. As the dilution rate increased, the difference in expression between blood and saliva also increased.

Male blood-male saliva samples in GROUP 2 were mixed in different ratios and the expression level of micro RNA 451A biomarker was tried to be determined to identify the blood fluid. At every ratio, earlier expression was achieved in the mixture with higher blood density than in the mixture with higher saliva density. Except for the 1x50 ratio, the expression difference between blood and saliva increased as the dilution ratio increased.

Female saliva-male blood samples in GROUP 3 were mixed in different ratios and the expression level of micro RNA 451A biomarker was tried to be determined to identify the blood fluid. Except for the 1x10 ratio where samples 9 and 10 were studied, earlier expression was achieved in the mixture with higher blood

DOI: 10.5455/medscience.2024.03.029

density than in the mixture with higher saliva density. Except for 1x100 samples, the expression difference between blood and saliva increased as the dilution rate increased.

Male saliva-female blood samples in GROUP 4 were mixed in different proportions and the expression level of micro RNA 451A biomarker was tried to be determined to identify the blood fluid. At every ratio, expression was reached earlier in the mixture with higher blood density than in the mixture with higher saliva density. As the dilution rate increased, the difference in expression between blood and saliva also increased.

When the mixtures at different ratios in all groups were examined, it was seen that the hsa-miRNA-451A marker, which is considered a peripheral blood marker, reached earlier expression in all other studies, except for the 1x10 ratio in which samples 9 and 10 in GROUP 3 were studied. The mixtures where this result is obtained are the mixtures in which the blood density is higher than the saliva density.

Our studies to compare hsa-miR-451A expression levels according to ratios have shown that, regardless of whether they were prepared at 1x1, 1x10, 1x50, 1x100 dilution, the samples with the earliest expression and the lowest expression level belong to GROUP 4. These samples, where female blood was abundant and male saliva was low, gave the best results for miR-451A, except for the 1x20 ratio. Samples in which expression was measured at the latest and highest values belong to GROUP 1, regardless of whether they were prepared at 1x1, 1x10, 1x20, 1x100 dilution. These samples, where female saliva was dense and female blood was low, were the most difficult to identify for miR-451A, except for the 1x50 ratio.

As a result, samples with high blood density expressed hsa-miR-451A in the peripheral blood earliest, while samples with high saliva density were measured at the highest expression levels. This supports that miRNA-451A is a suitable biomarker for blood. Among the samples from all groups with high blood density, it was observed that the samples from GROUP 4 were expressed at the best rate. Female-male mixtures gave better results than female-female or male-male mixtures. When GROUP 3 and GROUP 4 were compared, it was seen that in male-female mixtures, mixtures with a high concentration of female blood gave better results than mixtures with a high concentration of male blood. Among the samples belonging to all groups with high saliva density, the highest value in the mixtures was measured in GROUP 1. Mixtures of female-female individuals have been more difficult to define than any other mixture.

In the literature, no attempt has been made to distinguish mixture samples by preparing different dilutions. We think that more studies should be conducted to confirm our results.

It has been observed in different samples with high blood and saliva density that the blood-saliva sample was expressed earlier in the mixtures that were equal (1x1) or at the highest value (1x10). The samples with the highest levels of expression were measured in mixtures with low values (1x50). When we examined the other dilutions except the 1x1 dilution samples, the samples in which female blood was predominant showed the earliest expression at a ratio of 1x100. In other words, the expression was measured at the highest level in the mixture with the highest amount of blood and the least amount of saliva. In samples with high saliva density, the latest expression was reached at a rate of 1x10. In other words, 451-A expression was measured at the best value from the highest amount of blood in the saliva density. Since the hsa-miR-451A marker is known to be a potential marker for blood fluid, our results are consistent with the literature and our previous results.

Mixtures of hsa-miR-451A, which is considered a biomarker for peripheral blood, from male and female individuals were prepared and analyzed separately (Group 1 and Group 2). The prepared 1x1 dilution mixtures showed that the mixture sample formed by male individuals was identified earlier than the mixture sample formed by female individuals. When the samples with high blood density and high saliva density were examined separately, in other dilutions except the 1x50 dilution, mixture samples consisting of male individuals were identified earlier. At 1x50 dilution, samples from female individuals were expressed earlier at both densities.

As a result, the hsa-miR-451A biomarker was expressed at different rates in female and male donors in samples containing the same ratios, same body fluids and with the same mixing density. In our study, looking at the Ct values obtained in the hsa-miR-203A-3p marker, which we preferred for saliva, shows that it is found after very high repetitions and is not a good biomarker for saliva. In our research when choosing a marker, we came across studies showing the suitability of miR 203A-3p [10,13,17,18] as well as studies stating the opposite [12,15].

In our study, our saliva samples were collected by spitting into appropriate containers. The samples were kept in the refrigerator at +4 degrees. Stabilization of saliva samples on miRNA markers has not been studied in the literature. It is possible that the enzymes found in saliva may become destructive to miRNA over time. Conducting such studies will help make clearer interpretations.

Conclusion

In this study, evidence that could be obtained from a possible crime scene was studied. These examples were prepared considering multi-participant injury and murder cases, especially sexual abuse cases. The body fluids of the victim and the perpetrator or perpetrators may be mixed together at the scene. The fact that the excess body fluid masks the small amount of body fluid can create misleading results in clarifying the cases and can also lead to a faulty plot.

According to our study, the 451A marker showed a good expression level even in samples with high saliva density. We

DOI: 10.5455/medscience.2024.03.029

think that it is a biomarker that is resistant to enzymes found in saliva. There is no study showing miRNA expression according to gender. The expression of the same miRNA in men, women and mixtures of women and men is included in our study for the first time. As a result of our study, it was found that mixtures consisting of male participants gave better results than mixtures consisting of female participants. We say that the miR-203A-3p marker is not suitable for saliva.

The saliva collected by spitting into containers suitable for our study was kept at +4 degrees for a week, regardless of our experimental planning. The fact that we could not obtain miR-203A-3p from saliva is supported by many studies, but considering the studies in the past years that say it exists, we think that we may not have been able to obtain it due to the enzymes in saliva. The lack of a study showing the stability of salivary fluid prevents us from making a definitive comment.

When all prepared dilutions were examined, the earliest expression was reached in each group mixture sample prepared as 250 µl blood-250 µl saliva (1x1). One 1x1 mixture was prepared for each of the four groups in our study. Even if the participant's gender changed, the same amount of blood and saliva was mixed and different expression results were detected for each group. Again, the mixture with only male participants showed the highest expression. This led us to think that miRNA markers may be expressed at different levels depending on gender. From a different perspective, it is thought that mixtures of same/different gender individuals may affect the stabilization of that body fluid.

In the literature, the separation of a mixture of body fluids has not been studied. In our study, we aimed to distinguish a mixture of body fluids using miRNA markers according to gender in different dilutions. We saw that two body fluids can be differentiated using the appropriate miRNA marker. Expression rates may vary depending on participant gender. The 451-A marker in the blood can protect itself in the presence of saliva.

Since the results we obtained are a first, we think that our study should be among the new study subjects in terms of being exemplary and the reliability of its results.

Conflict of Interests

The authors declare that there is no conflict of interest in the study.

Financial Disclosure

This study was supported by Istanbul University-Cerrahpasa Scientific Research Projects (BAP) (project number 36198).

Ethical Approval

It was stated that our study was found ethically appropriate by the Istanbul University-Cerrahpasa Clinical Research Ethics Committee, with the letter dated 12.02.2021 and numbered E-86669574-302.14.06-30503, provided that Scientific Research Projects (BAP) support was received.

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