**T.C.**
**CUKUROVA UNIVERSITY**
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# EXAMINATION OF VARIOUS MIRNA MARKERS IN THE DIFFERENTIATION OF MENSTRUAL BLOOD FROM PERIPHERAL BLOOD

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**MASTER'S THESIS**

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I dedicate this work to my grandmother Miss Nagawa Teddy, my Children, my mother Miss Namatovu Praxeda, my husband and friends. Thank you for your unconditional support, love, guidance and encouragement throughout my education and life.

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| | |
|---|---|
| EDTA | Ethylenediaminetetraacetic acid |
| RT | Room temperature |
| ALS | Alternate light sources |
| AP | Acid phosphatase |
| PCR | Polymerase Chain Reaction |
| RT-PCR | Realtime polymerase chain reaction |
| mRNA | messenger RNA |
| miRNA | microRNA |
| PB | Peripheral blood |
| MB | Menstrual blood |
| SA | Saliva |
| SE | Semen |
| VA | Vaginal secretion |
| cDNA | Complementary Deoxyribonucleic acid |

# ÖZET

## Menstruel Kan-Periferal Kan Ayırımında Çeşitli miRNA Belirteçlerinin İncelenmesi

Olay yerinde menstruel kan, periferal kan, semen, tükürük ve vajinal sekresyon gibi vücut sıvıları yaygın olarak bulunan en önemli biyolojik örneklerdir. Bunlar bir ceza davasının niteliksel olarak analiz edilmesine, olay yerinin yeniden canlandırılmasına ve mahkeme salonu için güçlü delillerin sağlanmasına yardımcı olacak belirleyici ipuçları sağlar. Benzer şekilde, menstruel kan ile periferal kan ayrımı, bazı ceza davalarının mahiyetini anlamak için güçlü veriler sunacağından, adli vaka çalışması için hayati öneme sahiptir.

Bu çalışmada, 5 farklı vücut sıvısından oluşan toplam 96 biyolojik materyalde (21'i periferik kan, 21'i adet kanı, 20'si tükürük, 13'ü meni ve 21'i vajinal sekresyon) beş farklı miRNA'nın (miR-451, miR-144, miR-214, miR-203, miR-205) ekspresyon seviyeleri SYBR green tabanlı gerçek zamanlı PCR ile analiz edildi. Endojen kontrol olarak SNORD48 kullanıldı. Daha sonra seçilen aday belirteçlerin ekspresyon seviyeleri SPSS 22.0 ile yapılan diskriminant fonksiyon analizi ile karşılaştırılarak menstrüel kan ile periferik kan ayrımının mümkün olup olamayacağı araştırıldı.

İlk adımda kan örneklerini kan dışı örneklerden ayırmak için miR451'in delta CT değerleri bağımlı değişken olarak, kan ve kan dışı örnekler de bağımsız değişkenler olarak seçildi. İkinci adım olarak iki farklı yol izlendi. İkinci adım için seçilen yollardan birinde, periferik kanı menstruel kandan ayırt etmek için bağımlı değişkenler olarak MB ve PB, bağımsız değişkenler olarak miR-214, miR203 ve miR205 seçildi. İkinci adım için seçilen diğer yolda ise bağımsız değişken olarak miRNA'lardan yalnızca miR203 seçildi. Kan örneklerini kan dışı örneklerden ayırmak için kullanılan fonksiyon testi Z1 ve Z2; menstrüel kanı periferal kandan ayırmak için kullanılan fonksiyon testi Y1 ve Y2 olarak adlandırıldı. Takip edilen iki aşamalı yöntem ile 21 test numunesi kullanıldığında %100 doğruluk oranına ulaşıldı. Bu başarı ikinci adımda hem miR-214, miR-203 ve miR-205'in birlikte bağımsız değişken olarak kullanılması durumunda hem de değişken olarak sadece miR-203'ün kullanılması durumunda geçerliydi.

Bu sonuçlar, miR451 ve miR203'ün tek kaynak kanı menstrüel kandan ayırmada yeterli olacağını ve adli vakaların analizinde başarıyla kullanılabileceğini gösterdi. Çalışmada 5 ng RNA ile sonuç alınabildiği için az miktardaki adli örneklerde de kullanılması mümkün olmaktadır.



**Anahtar Kelimeler:** Adli bilim, mikroRNA, menstruel kan, ters transkripsiyon, kantitatif Gerçek Zamanlı polimeraz zincir reaksiyonu.

## ABSTRACT

### Examination of Various miRNA Markers in the Differentiation of Menstrual Blood from Peripheral Blood

Body fluids such as peripheral blood, menstrual blood, semen, saliva, and vaginal secretions are the most significant biological samples commonly found at the scene of a crime. These provide decisive clues to help qualitatively analyse a criminal case, restructure the crime scene, and provide strong evidence for the courtroom. Similarly, the distinction between menstrual blood and peripheral blood is vital for forensic casework, as it could provide strong evidence to figure out the nature of some criminal cases.

In this study, the relative expression levels of five different miRNAs (miR-451, miR-144, miR-205, miR-214, and miR-203) were analysed by SYBR green-based real-time PCR with 96 samples from 5 different body fluids, including 21 peripheral blood, 21 menstrual blood, 20 saliva, 13 semen, and 21 vaginal secretions. Then, it was investigated whether menstrual blood could be distinguished from peripheral blood with candidate markers selected by discriminant function analysis performed with SPSS 22.0.

In the first step, delta CT values of miR451 were selected as the dependent variable, and blood and non-blood samples were selected as the independent variables to distinguish blood samples from non-blood samples. As the second step, two different paths were followed. The first was to select miR-214, miR203, and miR205 as independent variables and MB and PB as dependent variables to distinguish peripheral blood from menstrual blood. The other way was to select only miR203 from the miRNAs as an independent variable. The first of the two function test sets used was named Z1 and Z2, to separate blood samples from non-blood samples, and the second set, named Y1 and Y2, was used to separate menstrual blood from peripheral blood. The two-step method was followed, and a 100% accuracy rate was achieved when 21 test samples were used. This success was valid in the second step both when miR-214, miR-203, and miR-205 were used as variables and when only 203 was used as a variable.

These results indicated that miR451 and miR203 would be sufficient to distinguish single source blood from menstrual blood and could be used successfully in the analysis of forensic cases. Since results can be obtained with 5 ng of RNA in the study, it is possible to use it in small amounts in forensic samples.



**Keywords:** Forensic science, microRNA, menstrual blood, reverse transcription, quantitative real-time polymerase chain reaction

# 1. INTRODUCTION

## 1.1. Menstrual Blood in Forensics

Body fluids are the most and significant biological samples commonly found at the scene of crime, (1). The commonly body fluids found at crime scenes comprise of the peripheral blood (PB), menstrual blood (MB), saliva (SA), semen (SE) and vaginal secretion (VS). These samples provide decisive clues to help qualitatively analyse a criminal case, restructure the crime scene and provide strong evidence for the courtroom, (2).

At the scene of crime, blood is considered to be the mostly common and significant sample and visually distinguished easily from the other fluids. Nevertheless, whether blood samples belong to PB or MB, it is very important issue in forensic science, (1) since it can provide evidence especially in cases such as sexual assault, female disappearance, and some injuries where female victims may have haematuria due to a violent assault, (1). Therefore, it is crucial, for an investigator to determine whether the blood cell source in the urine is contaminated by MB, (3).

## 1.2. Methods to differentiate menstrual blood from peripheral blood

Menstrual blood is a complex body fluid that comprehends peripheral blood, old uterine tissue, cells from the mucus lining of the vagina, matrix degradation factors involved in the onset of menses, and bacteria from the vaginal flora, (4, 5). Menstrual blood does not contain an active fibrinolytic system thus it does not coagulate like robust peripheral blood, (3).

So, the ability to identify menstrual blood in forensics is vital for crime reconstruction, determine the nature and severity of the case especially in sexual assaults and the following are some techniques that have been used to differentiate menstrual from peripheral blood.

Initially, microscopic methods were performed to differentiate peripheral blood from menstrual blood basing on the identification of either endometrial or vaginal epithelial cells in the menstrual blood, (6). Nevertheless, this technique had low discriminatory power hence proven precluded in cases of sexual assault since epithelial

cells from the buccal cavity and male urethra cannot be distinguished from vaginal epithelial cells, (6).

Microscopy analysis and the Teichmann test was used to distinguish between peripheral blood and menstrual blood using a combination of haemoglobin content, red blood cells and white blood cell count, (7). It was found that the Teichmann test was a confirmatory blood test based on the formation of haematin crystals observed under a microscope, (6).

Menstrual blood tested negative using the Teichmann test because menstrual secretions interfered with crystal formation. Menstrual blood also had less red blood cells and white blood cells and more debris compared to peripheral blood when viewed under a microscope, (7).

The immunochromatographic method is another technique used to differentiate menstrual from peripheral blood. This technique uses the D-dimer assay to identify menstrual blood.

The D-dimers are produced during fibrinolysis, the process of breaking down fibrin in blood clots. Fibrinolysis prevents blood coagulation in order for menstrual blood to easily exodus the body, (6). The mean plasma concentration of D-dimers in peripheral blood is 0.047 μg/mL compared to 102 μg/mL in menstrual blood demonstrating that D-dimers are present in menstrual blood at levels that are 200 times than that of peripheral blood, (8, 9).

### 1.3. Different techniques to identify body fluids

Previously, the normal analysis of forensic cases involved the preliminary screening of evident materials obtained during the investigation of criminal offenses was purposely to identify the presence, and possible tissue origin of biological material. These biological materials exhibited the location of potential sources of DNA that, once recovered, could be used to find the donor of the biological material, (10).

Originally, different conventional methods techniques such as immunological, chemical and enzymatic have been applied recently in identifying menstrual blood and other body fluids in forensics. These methods have been performed in a serial manner, with a portion of the stain being tested for only one body fluid at a time.

Normally, it was mandatory to perform multiple tests first to presumptively identify the presence of biological fluids, then, followed by additional testing to confirm the presence of the fluid or identify the species of origin, (10).

These methods differed greatly in terms of sensitivity and specificity, costly in terms of time and labour, and also a large amounts of sample material required to perform the assays hence becoming disadvantageously, (10, 11). It is also documented that, none of the routinely used serological and immunological tests can definitely identify the presence of human saliva or vaginal secretions, (10). Therefore, this created a very big gap in the field of forensics, and other approaches such as use of microRNA (miRNA) that could use less samples and stable had to be explored and hence found confirmed as ideal biomarker for body fluid identification, (3, 11). This approach is specific, labour-efficient and it is not time costly however, it is extremely expensive.

The Raman spectroscopy and messenger RNA (mRNA1) profiling is another approach currently used in the forensic laboratories to identify trace evidence materials such as drugs, fibres, paint, and ink, (12, 13, 14). However, this method has not been extensively explored for use with biological samples, (15).

### 1.4. Problem statement and justification

In criminal cases such as sexual assault and murder, blood has been confirmed as the most frequently encountered in crime scenes, and it faces the influence of physical, chemical and biological factors once it has left the human body, (16).

Several body fluid stains are either invisible to the naked eyes or similar in appearance with other body fluids at the crime scene which creates very tough situation for a forensic investigator. Therefore, there is a need for absolute confirmation in order for the evidence to be applied to either prove or disprove a fact of a case, (17). Our study examined various miRNA markers in the differentiation of peripheral blood from menstrual blood. The different miRNAs used included miR-451, miR-144, miR-205, miR-214, and miR- 203, plus SNORD-48 as our reference gene using a RT-PCR to achieve the study objectives. Thus, the findings from our study will assist the forensic investigator to provide the evidence to courts of law on which they will base to draw the judgement.

# 2. GENERAL INFORMATION

## 2.1. The difference between peripheral and menstrual blood

Peripheral blood is a constantly circulating fluid that provides the body with nutrition, oxygen and waste removal. While menstrual blood is a complex biological fluid consisting of blood, vaginal secretions, and the endometrial cells of the uterine wall as they leave immediately prior to menses. Menstruation is the process of excluding of the endometrial lining of the uterus following a nearly month-long preparation for embryo implantation and pregnancy, (18).

Identification of menstrual blood from peripheral blood is critical especially in crime reconstruction, determination of the nature and severity of the case in criminal cases such as sexual assault, (19). Blood is a body fluid that often means traumatic injury but menstrual blood is the blood that does not coagulate like robust peripheral blood since it lacks active fibrinolytic system, (3). Menstrual blood appears more often in sexual assaults, (20).

## 2.2. Composition of body fluids

The Common body fluids identified at the scene of crime include peripheral blood (PB), menstrual blood (MB), saliva (SA), semen (SE) and vaginal secretion (VS). Their discovery and identification in forensics give vital evidence to help qualitatively investigate criminal cases, rebuild crime scene and provide stout evidence for courtroom, (2).

Blood samples have been considered the most frequently and extensively explored among the body fluids found at the crime scene. However, whether blood samples belong to peripheral or menstrual blood remains an important issue in forensic science, (1) .

The determination of the presence of the body fluid allows the sample to undergo further laboratory testing such as DNA analysis which is a very critical step in a wide range of investigations, (17). Each body fluid has an exclusive composition, and the presence of specific components in one fluid versus another is the basis of its identification, (21).

There are numerous components that are shared among more than one fluid, but it is the difference in relative contribution which brands tests for these components effective. For instance, saliva contains a large amount of amylase compared to what is found in semen and vaginal fluid. Also, the ratio of citrate to lactate when comparing semen and vaginal fluid showing the major difference, (17, 22).

**Table 2.1.** Showing components of body fluids

| **Semen** | **Blood** | **Vaginal fluid** | **Saliva** |
|---|---|---|---|
| ● **Acid phosphate**<br>● **Prostate specific antigen**<br>● **Spermatozoa**<br>● **Choline**<br>● **Spermine**<br>● **Zinc**<br>● **Semenogelin**<br>● **Urea**<br>● **Fructose**<br>● **Ascorbic acid**<br>● **Lactic acid**<br>● **Immunoglobulins** | ● Haemoglobin<br>● Fibrinogen<br>● Erythrocytes<br>● Albumin<br>● Immunoglobulins | ● Acid phosphate<br>● Lactic acid<br>● Citric acid<br>● Urea<br>● Vaginal peptidase<br>● Glycogenated epithelial cells<br>● Acetic acid<br>● Pyridine<br>● Squalene<br>● Immunoglobulins | ● Amylase<br>● Lysozyme<br>● Mucin<br>● Buccal epithelial cells<br>● Thiocyanate<br>● Potassium<br>● Bicarbonate<br>● Phosphorus<br>● Immunoglobins |

### 2.3. What is miRNAs.

The miRNAs are small, single-stranded, non-coding RNAs of 18 to 24 nucleotides in length, well conserved in eukaryotic organisms, (23). The miRNAs are have considered as a viable tool for body fluid identification in forensic casework in recent years because

of their short size, and are particularly valuable for degraded samples or complex mixtures, (3, 16, 23).

The miRNA**s** are employed or used to solve problems such as identification of body fluid sources, (24), inference of death or injury time, (25), analysis of the cause of death, (26), identification of mental illness and age inference, (27). They can act at the post-transcriptional level and regulate the expression of many genes in various biological processes, binding complementary sequences of mRNA target and silencing them via degradation through mRNA cleavage or by preventing protein synthesis, (23).

### 2.4. The synthesis of miRNA

The miRNA are single-stranded RNA molecules with an average of 22 nucleotides in length. miRNAs are produced from double-stranded RNA hairpin precursors (the primary miRNA or pri-miRNA) through a multi-step maturation process, (28). These miRNA precursors are characteristically found in clusters, most commonly within intronic regions of protein-coding genes and intergenic regions of the genome, (29).

The pri-miRNA is frequently more than 1000 nucleotides in length and comprises of a 60–120 nucleotide RNA hairpin which is cleaved by the enzyme Drosha in the nucleus of the cell, to generate the precursor-miRNA (pre-miRNA).

The Pre-miRNAs are then exported to the cytoplasm via the exportin-5 protein, where they are cleaved by Dicer enzyme to form mature miRNA, (29). Mature miRNA binds to Argonaute proteins to form the RNA-induced silencing complex (RISC) which functions to prevent the translation of target messenger RNA (mRNA), (30).

The miRNA in the RISC binds to a 13–16 nucleotide sequence in the 3’ untranslated region (UTR) region of one or more target mRNAs.

Usually, the 2nd–8th nucleotides (the seed sequence) of the mRNA have perfect complementarity to the miRNA in animal cells, (31). The binding of the miRNA-RISC to the mRNA causes the inhibition of translation and also accelerates the deadenylation of the mRNA polyA tail, resulting in earlier degradation of the mRNA, (29).

Thus, miRNAs are able to fine-tune gene expression at the post-transcriptional level.

One miRNA can potentially interact with several mRNAs and the same mRNA may be targeted by many different miRNAs. Thus, miRNAs and mRNAs form complex networks of gene regulation, (32).

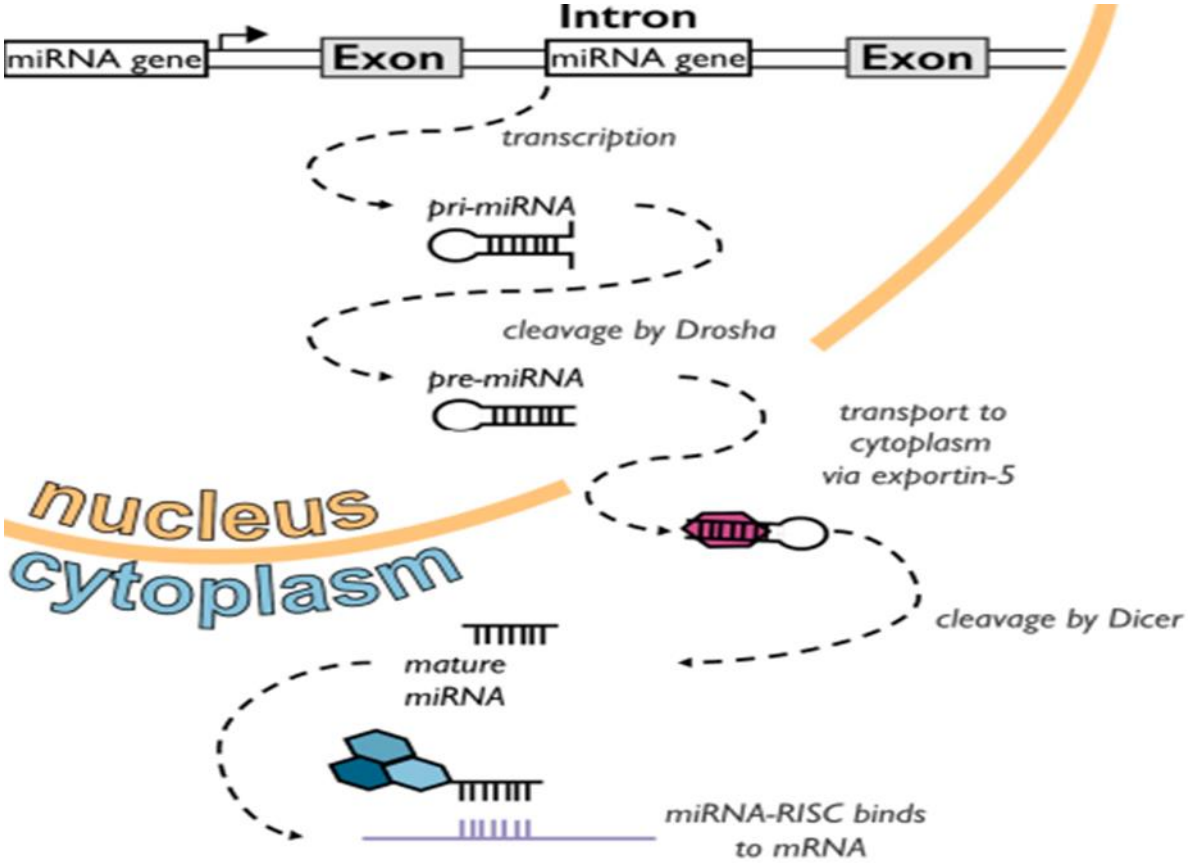



**Figure 2.1.** Shows the synthesis of miRNA

### 2.5 The miRNAs Analysis

The miRNAs analysis is done with profiling methods such as microarray or next generation sequencing (NGS) techniques and this allows simultaneous detection of even hundreds of low copy number miRNAs in a single experiment, (33).

When few designated miRNAs are analysed, the assay is performed by quantitative Real-Time PCR (qRT-PCR) and shows an increased sensitivity and reproducibility. To reduce technical variations introduced during the experimental

procedure, the target miRNA expressed is always normalized to a stable internal control, measured in the same sample at the same time. Also, an ideal internal control is expressed at a constant level, not dependent on biological variations, with length and expression range similar to the target miRNA, (23).

### 2.6. Factors that affects forensic RNA samples

RNA profiling has undergone vast progress in numerous fields of forensic science, such as identification of body fluid, wound age determination, and post-mortem interval (PMI) assessment, (23). Forensic samples such as blood are faced with many factors once it has oozed out of the body. There are many factors that can affect RNA.

These factors include; high humidity, temperature and rain. They can cause robust destructive effects on these samples hence causing a highly reduced miRNA content, (34). Rain has adverse effect on RNAs stability and accelerates its degradation rate, (35).

Therefore, the increased destruction of proteins and DNA leads to difficulty identification of individual samples. Other environmental factors that have always encountered RNA may include; heat and UV light, (2).

### 2.7. Other tools used in forensic science

At the scene of crime, there are many biological traces of human origin that are found and these can provide physical evidence or connect the suspect to the criminal offense or to vindicate an innocent person. Thus, various conventional presumptive tests such as chemical, enzymatic or immunological reactions have been performed routinely to isolate and analyse trace DNA to generate an individual-specific STR profile and, also to explicitly confirm the biological origin of this DNA for instance whether the recovered DNA stain is from peripheral or menstrual blood. The latter can help to clarify the circumstances of a criminal act, (36).

To a sad note, these tests have a disadvantage regarding the sensitivity and specificity resulting to destruction or complete consumption of valuable stain material, and an infirmity to recognize certain kinds of body fluids such as vaginal secretions, (36).

### 2.8. Forensic body fluid markers

The miRNA plays a role in the body fluid identification in forensics. In forensic settings, miRNA profiling renders mature miRNAs decidedly less susceptible against fractionation by chemical or physical strain, (37). Different body fluids such as semen, saliva, urine, peripheral blood, menstrual blood, and vaginal secretions encountered at the crime scene have different miRNA markers with the application of reference tools as stated below:

**Table 2.2.** Showing miRNA markers for different body fluids, (6, 10, 35, 37, 38).

| **Body fluid** | **miRNA** |
|---|---|
| Blood | miR-451, miR-16, miR-200b, miR-20a, miR-185, miR-205, miR-214, and miR-203 |
| Saliva | miR-658, miR-205, miR- 583, miR-518, miR-208b and miR-26b |
| Menstrual blood | miR-451, miR-412, miR-185, miR-144, miR-1246, miR-205, miR-214, and miR-203 |
| Semen | MiR-135b, miR-10b, miR-943, miR-135a miR-10a, miR-891a |
| Vaginal secretions | MiR-451, miR-412, miR-185, miR-144 |
| **Reference genes:** | RNU6b, SNORD 48, SNORD 47, SNORD 44, beta-2microglobulin and let-7g |

# 3. MATERIALS AND METHODS

## 3.1 Study Design

In our study, 96 samples were randomly selected from the participants following an approval from Çukurova University Ethics committee (Decision date and number: 2022.09.16/125-43). This study was carried out with the financial support of the research project of Cukurova University.

## 3.2 Supplies

### 3.2.1. Sample collection

96 samples were collected from volunteers of varying age and gender (between 20–40 years old). The samples included 21 peripheral blood (PB), 21 menstrual blood (MB), 20 saliva (SA), 20 vaginal secretions (VS) and 14 semen (SE). 51 of a total of 96 (10 PB, 11 MB, 11 SA, 11 VS and 8 SE) were used as training samples and the remaining 45 samples were used as test samples.

About 1 mL of peripheral blood samples were collected using vein puncture (the area was sterilized with an alcohol wipe and blood was collected using a needle and a syringe), then blood was drawn into a labelled EDTA vacutainers. The menstrual blood samples were collected by the female donors themselves using sterile cotton swabs. But for menstrual samples, day 2 or 3 was most significant sample. The saliva and semen samples were also collected in sterile clean containers by the volunteers themselves.

### 3.2.2. Marker selection

Five miRNA markers (miR-451, miR144, miR-214, miR- 203, and miR-205,) were selected based on previous studies, (1). SNORD48 was used as an internal reference gene, (38). The information for each miRNA and the internal reference gene is shown below in **Table 3**

**Table 3.1.** Information for the miRNA markers and the internal reference gene

| **miRNA** | **Mature miRNA sequence** | **miRbase** | **Gene Globe ID** |
|---|---|---|---|
| hsa-miR-451 | AAACCGUUACCAUUACUGAGUU | MIMAT0001631 | YP02119305 |
| hsa-miR-144 | 5'UACAGUAUAGAUGAUGUACU | MIMAT0000436 | YP00204754 |
| has-miR-214-3p | 5'ACAGCAGGCACAGACAGGCAGU | MIMAT0000271 | YP00204510 |
| has-miR-203a-3p | 5'GUGAAAUGUUUAGGACCACUAG | MIMAT0000264 | YP00205914 |
| has-miR-205-5p | 5'UCCUUCAUUCCACCGGAGUCUG | MIMAT0000266 | YP00204487 |
| SNORD48 | 3'GATGACCCCAGGTAACTCTGAGTGTGTCGCTGATGCCATCACCGCAGCGCTCTGACC | NR_002745.1 **(NCBI reference)** | YP00203903 |

### 3.2.3. Chemical substances used in the experiments

- ❖ miRNeasy® Mini Kit (50) QIAGEN
- ❖ miRCURY LNA ™ SYBR, Green PCR Kit (600) QIAGEN
- ❖ miRCURY LNA ™ RT Kit QIAGEN
- ❖ Quant-iT ™ RNA Assay Kit (5-100ng) Invitrogen
- ❖ Buffer EL-Erythrocyte Lysis Buffer (QIAGEN)
- ❖ Absolute ethanol (Merck)
- ❖ Chloroform (Merck)

### 3.2.4. Plastic and other materials used in the experiments

- ❖ Sterile Eppendorf tubes: (0.2, 0.5 and 1.5 mls)
- ❖ Pipette tips (10. 100, 200 and 1000µl)

- ❖ Eppendorf pipette (10, 100, 200 and 1000µl)
- ❖ Racks
- ❖ Ice mold containers
- ❖ Gauze
- ❖ Gloves
- ❖ Sterile plain swabs
- ❖ Optical adhesive film

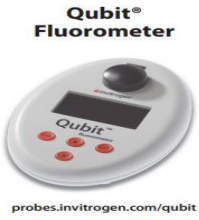
### 3.2.6. Equipment used during the experimental study

**VortexV-1 plus:** a perfect instrument for gentle mixing to vigorous resuspension of cells, biological and chemical liquid components in tubes using eccentric mechanism. It has fluoroplastic head for single tube (0.2 – 50 ml) vertexing. It works by connecting the external power supply to the main switch and then holding the sample tube gently by its upper part, and press the lower part to the vortex head.

When it is running or rotating, try as much as possible to control the intensity of shaking by varying applied pressure.

**Qubit Fluorimeter, Invitrogen:**

It was for quantitation of RNA with highly sensitive and accurate fluorescence-based Qubit™ quantitation assays.




**7500 Real Time PCR (Applied Biosystems):**

The Real-Time PCR System enables high-speed thermal cycling which runs many times for quantitative real-time PCR applications of fewer than 40 minutes. The

system is an open platform and can be used to analyze samples using custom made or COTs-based PCR assays.

The 7500 RT-PCR can analyse a batch of 96 samples per run. It is used for gene expression analysis, pathogen quantitation, SNP genotyping, isothermal and +/– assays utilizing internal positive controls.

**Thermal Cycler device (Applied Biosystems)**

Thermal cyclers sustain and regulate exact temperatures following pre-programmed settings to facilitate temperature-sensitive reactions such as the PCR.

**Centrifuge (Beckman Coulter Life Sciences):**

The centrifuge uses centrifugal forces to separate various components of a fluid. This is always achieved by spinning the fluids at a very high speed within the container hence separating the fluids of different densities.

**Deep freezer (Bosch):**

The deep freezer plays a vital role of storing reagents, frozen vaccines and other temperature sensitive specimen for research purposes that need to be stored at -20$^{o}$C.

### 3.3. Methods

#### 3.3.1. RNA Isolation using miRNeasy® mini-Kit (50)

The following steps were followed during the isolation of RNA on 96 samples of peripheral, menstrual blood, vaginal secretions, saliva and semen to achieve the study objectives.

**Peripheral Blood (RNA extraction procedure)**

- 100µl of whole blood was pipetted from the EDTA tubes into a 1.5 ml Eppendorf tubes

- To the sample, 500µl of buffer EL (erythrocyte lysis buffer) was added, followed by incubation on ice on the bench and vortexing the sample for around 20 minutes.
- The samples were centrifuged for 10 minutes at 400g at $4^{O}$C for 10 minutes.
- The supernatant was poured off, and then 350µl of buffer EL was added to the samples. This was followed by vortexing the samples for 10 seconds, and then centrifuging the samples at 400g for 10 minutes at $4^{O}$C.
- Supernatant was poured off, and 700µl of Qiazol ® Lysis buffer was added to the samples, followed by vortexing for 5 minutes. The samples were incubated on the bench at room temperature for 5 minutes.
- Then, 140µl of chloroform was added to the samples, then shaken for 3 minutes for proper mixture.
- The samples were centrifuged at 12000g for 15 minutes at $4^{O}$C so that 3 phases were obtained. Upper phase is RNA.
- Then, 350µl of the sample (RNA) was pipetted off into another labelled microcentrifuge tubes, and then, 525µl of absolute ethanol was added to the samples, mixing the samples up and down by pipetting.
- Then, 700µl of the sample were pipetted into the labelled supplied filtered column tubes.
- The samples were centrifuged at 8000g room temperature for 10-15 seconds, and then the supernatant will be poured off.
- After, 700µl of RWT (optional) wash buffer was added to the samples, centrifuged for 15 seconds at 8000g and supernatant poured off.
- Again, 500µl of RPE wash buffer was added to the samples, centrifuged at 8000g for 2 minutes at room temperature and supernatant will be poured.
- Then, the samples were centrifuged at 12000g for 1 minute. Then, finally 30µl of RNease free water was added to the samples, centrifuged at 8000g for 1 minute. Then, the supernatant will be poured.

**Menstrual Blood (RNA extraction procedure)**

- The swab sample was put into a labelled microcentrifuge tube.

- (500 EL lysis reagent was added to the samples and then vortexed for 10 seconds.
- To the sample, 500µl of buffer EL (erythrocyte lysis buffer) was added, followed by incubation on ice on the bench for around 20 minutes
- The samples were centrifuged for 10 minutes at 400g at $4^{O}$C.
- The supernatant was poured off, and then 350µl of buffer EL was added to the samples. This was followed by vortexing the samples for 10 seconds, and then centrifuging the samples at 400g for 10 minutes at $4^{O}$C.
- The supernatant was poured off
- Then, 700µl Quiazol lysis buffer reagent was added to the samples.
- The samples were then vortexed for 1minute
- The samples were left to incubate on bench at RT for 5 minutes.
- 140µl of chloroform was added to the samples for separation into phases.
- The samples were shaken well for about 3 minutes to have a uniform mixture.
- The samples were then centrifuged for 15 minutes at 12000g at $4^{O}$C.
- 350µl of the upper phase (RNA) was then pipetted off to new labelled microcentrifuge tubes.
- Then, 525µl of absolute ethanol was added to the samples and mixed well by up and down pipetting.
- Then, 700µl of the sample was transferred into labelled column tubes.
- The samples were then centrifuged for 15 seconds at 8000g at $4^{O}$C.
- The supernatant was poured off
- 700µl RWT wash buffer was added to the samples and again centrifuged for 15 seconds at 8000g at $4^{O}$C and the supernatant was poured.
- Then 500µl of RPE wash buffer was added to the samples centrifuged for 15 seconds at 8000g and supernatant poured off
- Again, 500µl RPE was added to the samples and then centrifuged for 2 minutes at 8000g at $4^{O}$C and then the supernatant was poured off.
- The samples were again centrifuged at 20000g for 1 minute to remove excess alcohol.
- Then, finally 30µl of RNase free water was added to the samples and incubated for 1 minute at RT, centrifuged at 8000g for 1 minute.

**Saliva (RNA extraction procedure)**

- 100 µl saliva sample was collected in a RNase-free eppendorf tubes.
- To the sample, 700 µl of QIAzol lysis reagent (Qiagen) was added to extract RNA
- The samples were briefly vortex-mixed and incubated for 5 min at room temperature.
- 140µl chloroform was added to the Eppendorf tube samples, then shaken for three minutes for proper mixture.
- The samples were then centrifuged at 12000g for 15 minutes at 4 °C. The upper phase is now the RNA.
- Then, 350µl of sample (RNA) was pipetted off into another labelled microcentrifuge tubes, and then, 525µl of absolute ethanol was added to the samples, mixing the samples up and down by pipetting.
- Then, 700µl of the samples was pipetted into the labelled supplied filtered column tubes.
- The samples were centrifuged at 8000g room temperature for 10-15 seconds, and then the supernatant was poured off.
- After, 700µl of RWT (optional) wash buffer was added to the samples, centrifuged for 15 seconds at 8000g and supernatant poured off.
- Then 500µl of RPE wash buffer was added to the samples centrifuged for 15 seconds at 8000g and supernatant poured off
- Again, 500µl of RPE wash buffer was added to the samples, centrifuged at 8000g for 2 minutes at room temperature and supernatant will be poured.
- The samples were again centrifuged at 20000g for 1 minute to remove excess alcohol
- Then, finally 30µl of RNease free water was added to the samples, centrifuged at 8000g for 1 minute

**Vaginal Secretions (RNA extraction procedure)**

- Using scalpel blade, cut the sterile swab sample and place it into a labelled Eppendorf tube.

- Add 700µl QIAzol lysis buffer reagent to the sample, and then leave the samples at room temperature for 5 minutes to allow incubation.
- Then, to the samples, add 140µl chloroform, then mix samples well for about 3 minutes to have a uniform mixture.
- Then, centrifuge the samples for 15 minutes at 12000g at 4$^{o}$C to have 3 separate phases: an upper, colourless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. ***(NB: the centrifuge can be re warmed if it is to be used for the next steps).***
- Then, pipette off 350µl of the upper phase (containing RNA) into labelled microcentrifuge tubes.
- To the samples, then add 525µl of absolute ethanol and mix thoroughly by pipetting up and down several times.
- Then, pipette 700µl of the sample into an RNeasy Mini spin column tubes. Close the lid of the column tubes gently.
- Then, centrifuge the samples at 8000g for 15 seconds at room temperature.
- After, the spinning of the samples, the flow through is discarded.
- After, 700µl of RWT (optional) wash buffer was added to the samples, centrifuged for 15 seconds at 8000g and supernatant poured off.
    - Add 500µl Buffer RPE to the samples, close the lid gently and centrifuge for 15 seconds at 8000g at 4$^{o}$C. After, pour off the supernatant.
    - Again, 500µl of RPE wash buffer was added to the samples, centrifuged at 8000g for 2 minutes at room temperature and supernatant will be poured.
    - The samples were again centrifuged at 20000g for 1 minute to remove excess alcohol
- Transfer the RNeasy Mini spin column to new microcentrifuge labelled tube, then, pipette 30 µl RNase-free water to the samples and close the lid tightly.
- Incubate then samples for one 1 minute at room temperature on bench.
- Centrifuge the samples for 1 minute at 8000g.
- Then, finally discard the RNeasy mini spin column tubes since the samples are already in the microcentrifuge tubes.

**Semen (RNA extraction procedure)**

- 100 µl semen sample was put into a labelled microcentrifuge tube
- To the sample, 700µl of QIAzol lysis reagent (Qiagen) was added to extract RNA
- The samples were briefly vortex-mixed and incubated for 5 min at room temperature on.
- The samples were centrifuged for 10 minutes at 400g at $4^{O}C$ for 10 minutes and the supernatant was poured off.
- 140µl chloroform was added to the Eppendorf tube samples, then shaken for three minutes for proper mixture.
- The samples were then centrifuged at 12000g for 15 minutes at 4 °C. The upper phase is now the RNA.
- Then, 350µl of sample (RNA) was pipetted off into another labelled microcentrifuge tubes, and then, 525µl of an absolute ethanol was added to the samples, mixing the samples up and down by pipetting.
- Then, 700µl of the samples was pipetted into the labelled supplied filtered column tubes.
- The samples were centrifuged at 8000g room temperature for 10-15 seconds, and then the supernatant was poured off.
- After, 700µl of RWT (optional) wash buffer was added to the samples, centrifuged for 15 seconds at 8000g and supernatant poured off.
- Then 500µl of RPE wash buffer was added to the samples centrifuged for 15 seconds at 8000g and supernatant poured off
- Again, 500µl of RPE wash buffer was added to the samples, centrifuged at 8000g for 2 minutes at room temperature and supernatant will be poured.
- The samples were centrifuged at 20000g for 1 minute to remove excess alcohol
- Then, finally 30µl of RNease free water was added to the samples, centrifuged at 8000g for 1 minute and then, the supernatant will be poured.

### 3.3.2. Quantification of RNA

Our study samples for RNA were quantified using the Quant-IT RNA buffer and the RNA reagent, following the manufacture's protocol (Quant-iT ™ RNA Assay Kit).

**Preparation for the standard**

**N.B** For quantification of RNA using the Quant-iT™RNA Assay kit, two standards for calibration of the Qubit® Fluorometer were required

A dilution of 0ng/µl *E. coli* (Standard1) and 10ng/µl *E. coli* rRNA from component C (Standard2) was performed as shown in the table below:

**Table 3.2.** Preparation of standards for RNA Quantification

| | Standard assay tubes | User Sample assay tubes |
|---|---|---|
| Volume of working solution | 190 µl | 198 µl |
| Volume of Standard (from kit) | 10 µl | - |
| Volume of User Sample to add | - | 2 µl |
| Total volume of each assay tube | 200µl | 200 µl |

**Preparation of the working solution**

- To each sample, put 199µl of the Quant-IT RNA buffer.
- Add 1µl RNA reagent to each sample.
- Then, pipette 198µl of the working solution to a new labelled microcentrifuge tube, and then add 2µl of the sample to it.
- Vortex the samples for the at least 2 minutes at room temperature.
- Then, the sample was put into the Qubit fluorometer and the concentration was recorded.

### 3.3.3. Reverse transcription

The reverse transcription was done on the samples purposely to quantify the number of miRNAs present in the RNA extracts using the miRCURY LNA RT Kit while following the manufacturer's instructions.

**Table 3.3.** Components of reverse transcription reaction

| **Components of miRCURY LNA RT kit** | | |
|---|---|---|
| **Number** | **Component** | **Amount** |
| 1 | 5 x miRCURY RT reaction Buffer | 2µl |
| 2 | RNase free water | 4.5µl |
| 3 | 10x miRCURY RT Enzyme | 1µl |
| 4 | Synthetic RNA spike-ins, optional | 0.5µl |
| 5 | Template RNA (5ng/µl) | 2µl |
| **Total reaction volume** | | 10µl |

Then, Reverse transcription quantitative PCR was performed with 10µl to each sample following the manufacturer's protocol using the Veriti Thermal Cycler (Applied biosystems).

The incubation for 60 minutes was at 42$^{O}$C, and then 95°C for five minutes.

### 3.3.4 miRNA analysis

miRCURYLNA miRNA PCR assay was used in the miRNA analysis

**Procedure**

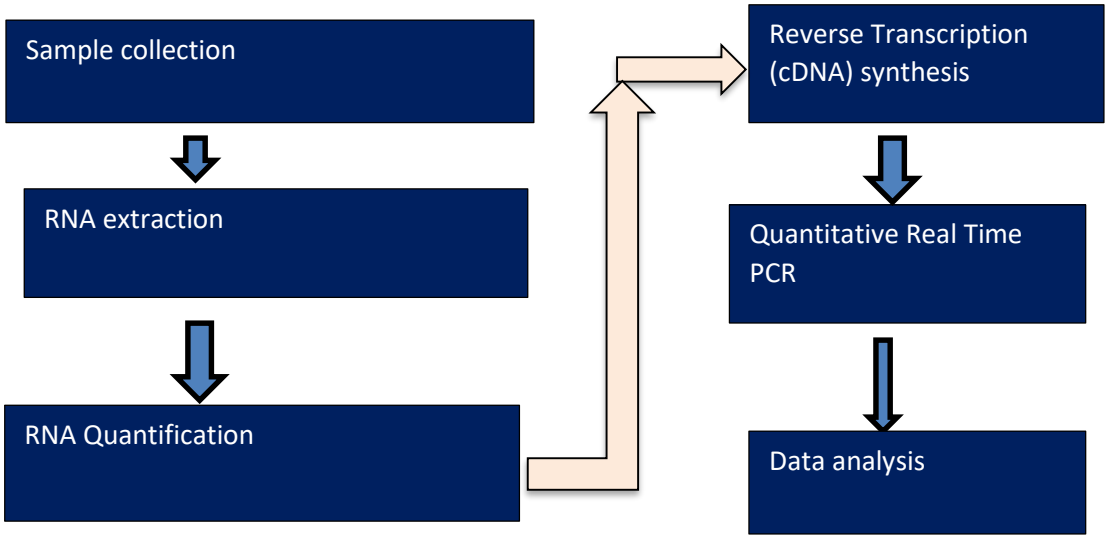
- Initially, cDNA was diluted 1:60 by adding 59µl RNase free water to the 1µl RT reaction.
- The cocktail or working solution was prepared as shown in **(table 6)** below.

- **7** µl of the working solution was put into the plate wells
- 3µl of the cDNA was added to the wells
- Plate was centrifuged well for 5 seconds for proper mixing
- Then, the plate well was sealed properly with Optical adhesive film.
- The well sample was then taken to the RT- PCR to run for 2 hours.

**Table 3.4.** Reaction mixture of miRCURY LNA miRNA PCR assays

| **Compound** | **Volume** |
|---|---|
| 2x miRCURY SYBR® Green Master Mix | 5 µl |
| ROX Reference Dye (ABI instruments only) | 0.05 µl |
| Resuspended PCR primer mix | 1 µl |
| cDNA template | 3 µl |
| RNase free water | 1 µl |
| Total reaction water | 10 µl |

### 3.4. Summary of Laboratory work flow



### 3.5. Storage of samples

All the above liquid samples were stored directly at - 80°C for preservation, (1). Forensic sample materials should be stored well. For instance, blood-stained samples can be stored at -20 C, low humidity if storage is for a long period of time. However, it has been realised that the long-term storage causes minor changes in the levels of individual miRNAs (16).

### 3.6. Data analysis

To calculate the relative miRNA expression, we used the delta Ct (ΔCt) method: ΔCt = Ct (miRNA)-Ct (SNORD 48). The SNORD48 reference gene was used as an endogenous control to normalise the data to ensure that the results are scientific and reasonable. Statistical analyses, including two-sample t tests, Mann–Whitney U tests was performed using SPSS 22.0, and $P < 0.05$ was considered significant at 95% confidential interval. Further statistical analyses also included discriminant function analysis.

### 3.7. Ethical considerations

Ethical approval to conduct this study was obtained from Legal Medicine department, Cukurova University. Written informed consent was obtained from all participants before the interviews. Participants were assured of their anonymity and confidentiality. The right to withdraw at any time and moral commitments was explained in all interviews.

### 3.8. Confidentiality

We used codes for the study participants instead of their names and findings / results were not disclosed to any unauthorized personnel.

### 3.9. Conflict of interest

There was no conflict of interest in this work.

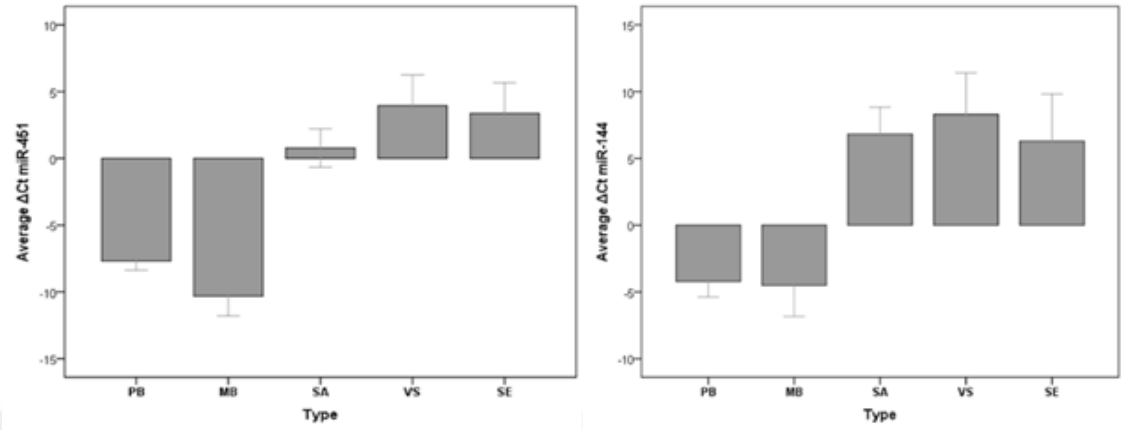
# 4. RESULTS

We examined the expression levels of five different miRNAs (miR-451a, miR-144, miR-214-3p, miRNA-203, and miR-205) and SNORD-**48** (reference gene) to differentiate peripheral blood from menstrual blood.

## 4.1. Analysis of the expression of the five candidate miRNAs in five body fluids

Analysis of the expression of the five candidate miRNAs in five body fluids included 10 PB samples, 11 MB samples, 11 SA samples, 11 VS samples, and 8 SE samples.

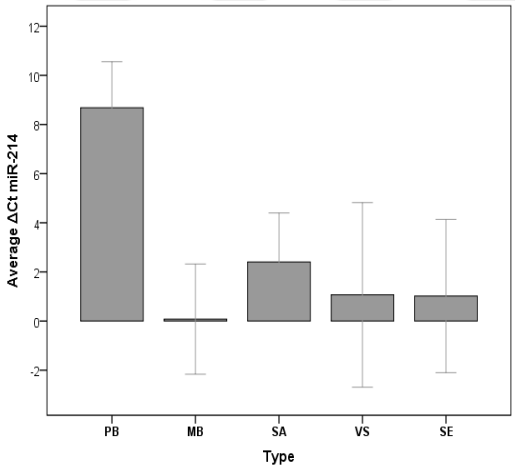
**Figure 4.1: (A-E)**: The CT values of each miRNA in all studied samples are given in appendix 1.



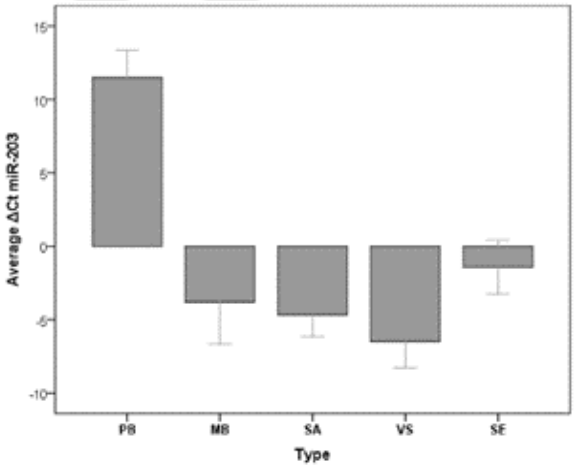

A



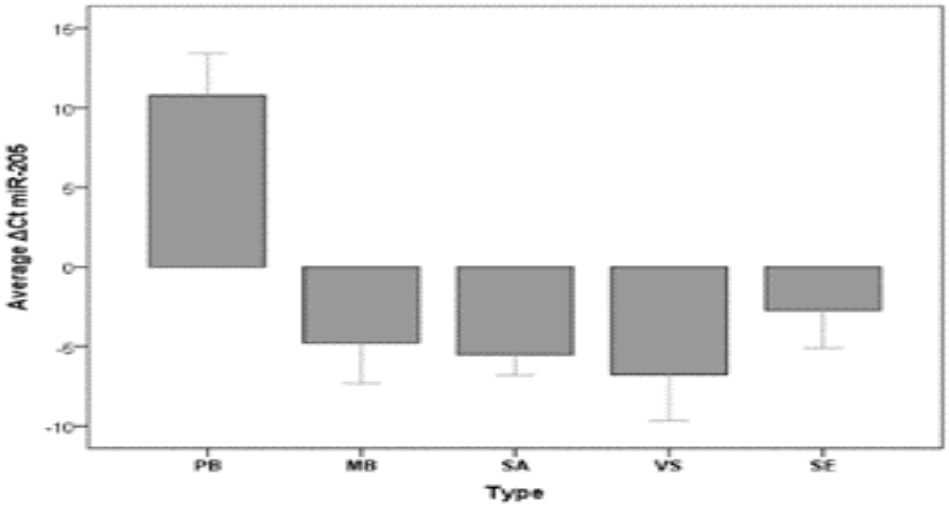

B




C




D




E

**Figure 4.1.** (A-E): *The expression of candidate genes in five kinds of body fluids*

***Ordinate:*** *Relative expression of microRNA Ct values.* ***Abscissa****: PB: Peripheral blood; MB: Menstrual blood; SA: Saliva; VS: Vaginal secretion SE: Semen;*

The miR-451 expression was significantly different ($p < 0.05$) between blood samples and non-blood samples. MiR-451 was highly expressed in both the PB and MB samples but showed almost no expression or very low expression in the three non-blood samples.

On the other hand, in addition to the high expression of miR144 in PB and MB, it is also expressed in SA, VS and SE samples, albeit at lower levels. (Fig 4.1, B

As seen in Fig 4.: from C to E, the expression levels of three miRNAs (miR-214, miR-203, miR-205) in PB and MB were significantly different and much higher in MB than in PB ($p < 0.05$). Detailed analysis procedures and results are shown in **Appendices 2**.

The ΔCt values were used to generate two-dimensional (2D) scatter plots to determine whether these miRNAs showed differential expression between blood and nonblood samples and between MB and PB. Those plots were presented in Fig 4.2, 4.3 and 4.4.

**Figure 4. 2**: **(A-D): Expression o**f the five miRNAs (miR-451, miR-144, miR-214, miR-203, miR-205) in all samples from the training set. The ΔCt values were used to generate two-dimensional (2D) scatter plots, and also to determine whether these miRNAs exhibited differential expression between MB and PB.

According to the expression data shown in **Figure 4.2: A-C,** the MB samples were evidently separated from the PB samples by the distinct clustering of the two different blood samples in each miRNA pair. This meant that, the levels of miRNAs were higher in MB than in PB.

A

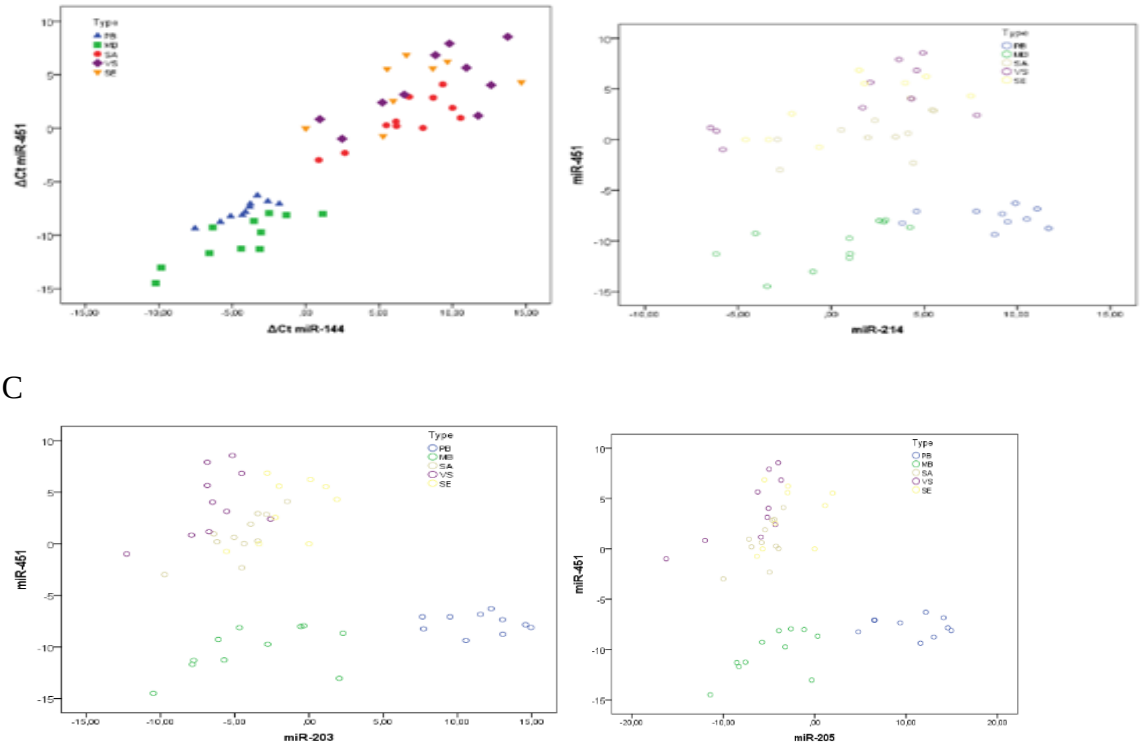



**Figure 4.2.** Scatter plots of ΔCt miR-451 to distinguish non-blood from blood (blue circles and triangles).

a A two-dimensional scatter plot consisting of ΔCt miR- 451 (ordinate) and ΔCt miR-144 (abscissa). b A two-dimensional scatter plot consisting of ΔCt miR-451 (ordinate) and ΔCt miR-214 (abscissa). c A two-dimensional scatter plot consisting ofΔCtmiR-451 (ordinate) and ΔCt miR-203 (abscissa).

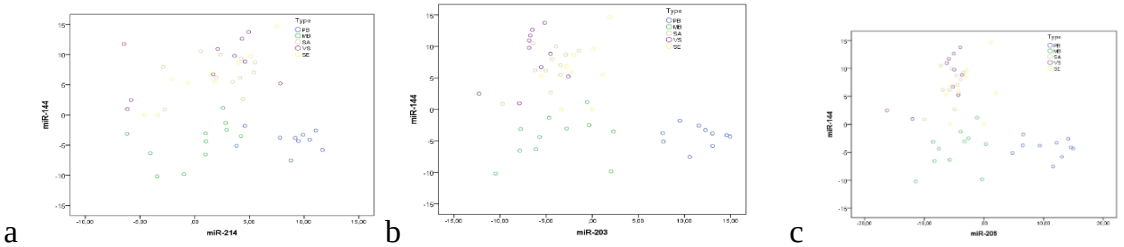
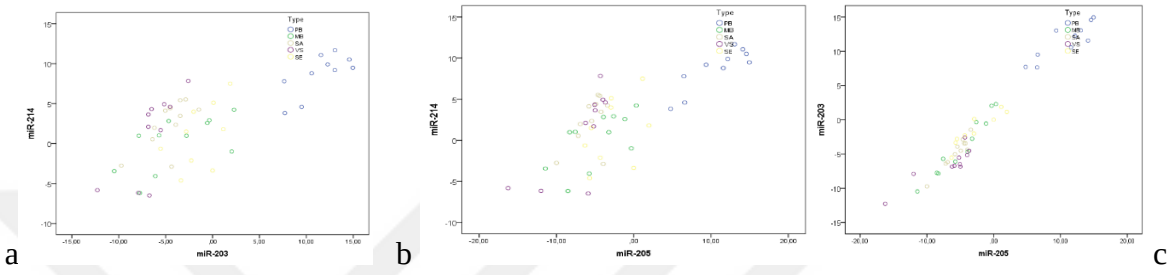



**Figure 4.3.** Scatter plots of ΔCt miR-144 to distinguish blood from non-blood

**(a).** A two-dimensional scatter plot consisting of ΔCt miR- 144 (ordinate) and ΔCt miR-214 (abscissa). **(b).** A two-dimensional scatter plot consisting of ΔCt miR-144 (ordinate) and ΔCt miR-203 (abscissa). **(c).** A two-dimensional scatter plot consisting of ΔCtmiR-144 (ordinate) and ΔCt miR-205 (abscissa)

The body fluid samples shown included 21 MB samples and 21 PB samples from the sample set, and the expression of three miRNAs (miR-205, miR-214, miR-203) in

MB and PB is also shown in Fig. **4.4:** The ΔCt values were used to generate two-dimensional (2D) scatter plots to determine whether these miRNAs showed differential expression between MB and PB. According to the expression data shown in Fig. 4:4 (a-c), the MB samples were clearly separated from the PB samples by the distinct clustering of the two different blood samples in each miRNA pair, suggesting that these miRNAs had higher levels in MB than in PB.




a b c

**Figure 4.4.** Scatter plots of ΔCt miR-214, ΔCt miR-203, ΔCt miR-205 to distinguish MB from PB.
(a) A two-dimensional scatter plot consisting of ΔCt miR- 214 (ordinate) and ΔCt miR-203 (abscissa). (b) A two-dimensional scatter plot consisting of ΔCt miR-214 (ordinate) and ΔCt miR-2205 (abscissa). (c) A two-dimensional scatter plot consisting of ΔCt miR-203 (ordinate) and ΔCt miR-205(abscissa).

### 4.2. A discriminant function to distinguish blood samples from non-blood samples

The discriminant function analysis to predict the group affiliations of unknown samples obtained by a training set of 51 samples was applied to the data subsets, with the ΔCt values of miR-451 as independent variables and the blood samples and non-blood samples as dependent variables.

The following results for the blood samples and non-blood samples were obtained:

$$Z1 = x - 1.225\Delta Ct*(miR\text{-}451) - 6.251;$$

$$Z2 = 0.353*\Delta Ct(miR\text{-}451) - 1.154.$$

When the ΔCt values of miR-451 are placed into the functions, if Z1>Z2, the unknown sample was classified as a blood sample, and if Z1<Z2, the sample was classified as a non-blood sample. The accuracy rate of the function in the data subset was

100%. The accuracy of the function was validated by using test samples (n=45), and 100% accuracy for the blood and 95.9% accuracy for the non-blood were obtained, as shown in **Table 4.1** below

Detailed analysis procedures and results are shown in **Appendices 3.**

**Table 4.1.** Results of the analysis of the blood and non-blood samples using Z1/Z2 discriminant functions

| Sample set | Sample type | Prediction classification | | |
|---|---|---|---|---|
| | | Blood sample | Non-blood sample | Accuracy rate |
| Training set (n=51) | Blood sample (n=21) | 21 | 0 | 100% |
| | Non-blood sample (n=30) | 0 | 30 | 100% |
| Test set (n=45) | Blood sample (n=21) | 21 | 0 | 100% |
| | Non-blood sample (n=24) | 1 | 23 | 95.9% |

### 4.3. A discriminant function to distinguish MB samples from PB samples

A training set of 21 samples was used to establish the discriminant function analysis, with the ΔCt values for miR-203, miR-205 and miR-214 as independent variables and MB samples and PB samples as dependent variables.

The following results for the MB samples and PB samples were obtained:

***Y1*** *= 0.601*ΔCt (miR-214) + 1.077*ΔCt (miR-203) + 0.522*ΔCt (miR-205) – 6.674;*

***Y2*** *= 0.661*ΔCt (miR-214) + 0.187*ΔCt (miR-203) + 0.878*ΔCt (miR-205) – 2.457.*

When the ΔCt values for miR-203, miR-205 and miR-214 were placed into the functions, if Y1 > Y2, the unknown sample was classified as a PB sample, and if Y1 < Y2, the sample was classified as an MB sample. The accuracy rate of the function for the data subset was 100%.

The accuracy of the function was validated by using test samples (n=21), and 100% accuracy was obtained, as shown in **Table 4.2** below.

**Table 4.2.** Results of analysis of menstrual blood and peripheral blood samples using the Y1/Y2 discriminant function

| Sample set | Sample type | Prediction classification | | |
|---|---|---|---|---|
| | | **Menstrual blood** | **Peripheral blood** | **Accuracy rate** |
| **Training set (n=21)** | Menstrual blood (n=11) | 11 | 0 | 100% |
| | Peripheral blood (n=10) | 0 | 10 | 100% |
| **Test set (n=21)** | Menstrual blood (n=10) | 10 | 0 | 100% |
| | Peripheral blood (n=11) | 0 | 11 | 100% |

A training set of 21 samples was used to establish the discriminant function analysis, with the ΔCt values for miR-203 as independent variables and MB samples and PB samples as dependent variables.

The following results for the MB samples and PB samples were obtained:

$$Y1 = 0.902 * \Delta Ct(miR203) - 5.871$$

$$Y2 = -0.299 * \Delta Ct(miR203) - 1.264$$

When the ΔCt values for miR-203 were placed into the functions, if Y1 > Y2, the unknown sample was classified as a PB sample, and if Y1 < Y2, the sample was classified as an MB sample. The accuracy rate of the function for the data subset was 100%.

# 5. DISCUSSION

Our study used the RT- PCR to examine the expression of different candidate miRNAs markers in 96 body fluid samples from (PB, MB, VS, SA, and SE). These candidate markers included miR-451, miR-144, miR-214, miR-203, and miR-205. Our study exhibited a great abundance of miR-451 expression in menstrual blood and peripheral blood samples compared with other non -blood body fluid samples, and this corresponded with work done in other studies, (10). Still, miR-451 is specifically expressed in mature red blood cells, (39, 40), and variable for erythrocyte differentiation and therefore, its expression levels in peripheral blood (reticulocytes and mature red blood cells) should be relatively constant, (10).

Although not as large as miR-451, MiR-144 is also expressed in both peripheral blood and menstrual blood (39). However, it is nonspecific since expression also occurs in biological samples that are not blood. These data are consistent with the study by Sauer et al(11). Ultimately, the Ct (miR-144) in our study didn't exhibit a significant expression value to distinguish blood and non-blood samples value.

MiR-203 and miR-205 are specific markers for epithelial cells (39). Our results showed that the expression levels of miR-203 and miR-205 in menstrual blood samples and peripheral blood samples were significantly different. However, the expression of these two markers was also found to be high in SA and VS. This means that although some markers, for example miR-205 and miR-203, are saliva-specific markers, specificity in saliva is controversial, as specificity sometimes does not reach a direct determination in the form of simply detecting presence or absence, so miR-205 may not be specific to saliva ( 24, 40, 41).

The expression levels of miR-214 in menstrual blood samples and peripheral blood samples were significantly different. The high expression of miR-214 in menstrual blood in our study, corresponds with other studies that exhibited significant expression levels of miR-214 in menstrual blood and hence considered it a potential biomarker for menstrual blood, (40).

The stepwise model was applied, as in the study published by The et al. in 2020. In this analysis, where miR-451 was used as the independent variable in the first step and blood and non-blood samples were used as the dependent variable, the functions were

named Z1 and Z2. In the analysis performed with 51 training samples, the distinction between blood and non-blood samples was achieved with 100% accuracy. With 45 test samples, discrimination of the samples was achieved with 95.9% accuracy.

Then, the functions were named Y1 and Y2 in the analysis of all blood samples in which miR-241, miR-203 and miR-205 were used as independent variables and MB and PB were used as dependent variables. Here, MB and PB separation was achieved with 100% accuracy.(Fig 5.1)

In addition, in the function analysis where only 203 was used as an independent variable for the second step, the functions were again named Y1 and Y2. Here, it was determined that the distinction between PB and MB of blood samples could be made with 100% success. (Fig 5.2)

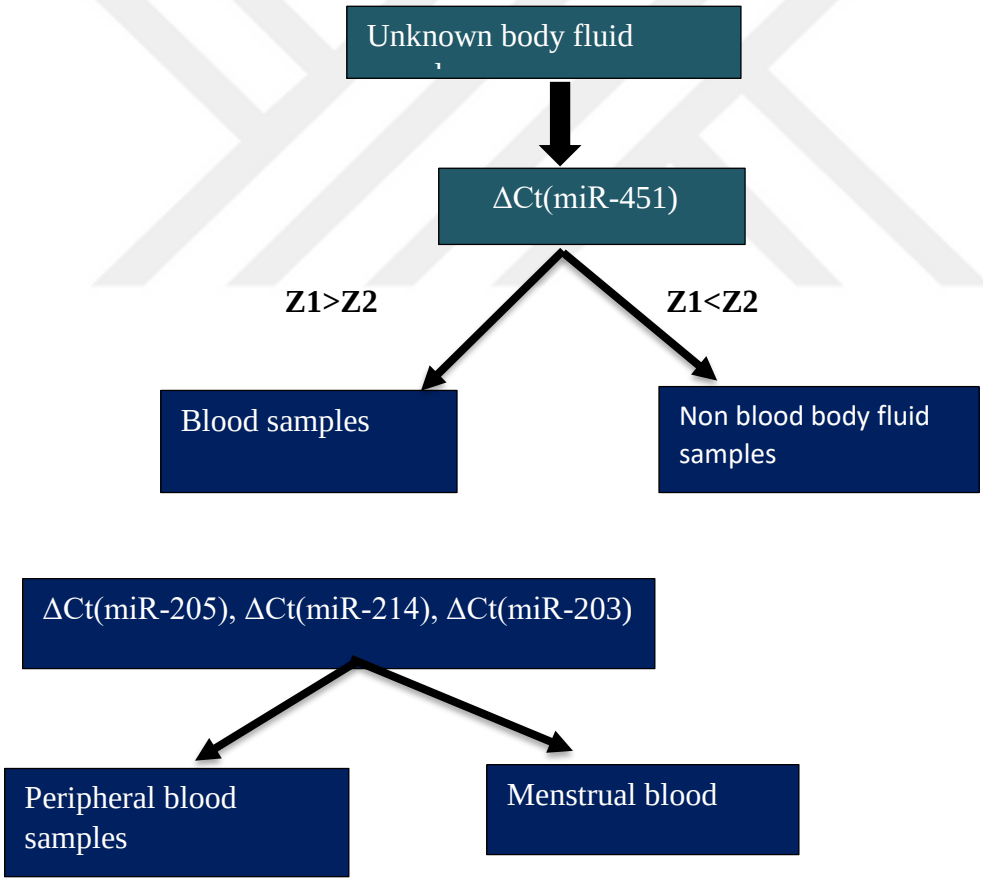



**Figure 5.1.** Diagram of the two-step analysis method

In the process of distinguishing MB from PB, to increase the accuracy of the results, we used the relative expression data of three kinds of miRNAs to establish a

discriminant function. The essence of the stepwise strategy is to combine multiple specific markers to identify various body fluids step by step and to improve the specificity and accuracy of the body fluid identification rate. The stepwise analysis strategy proposed here used 5 miRNAs and a reference gene, SNORD48. And this analysis can accurately identify samples as blood and non-blood samples, as well as MB and PB samples. The evaluation of the model provided encouraging results.

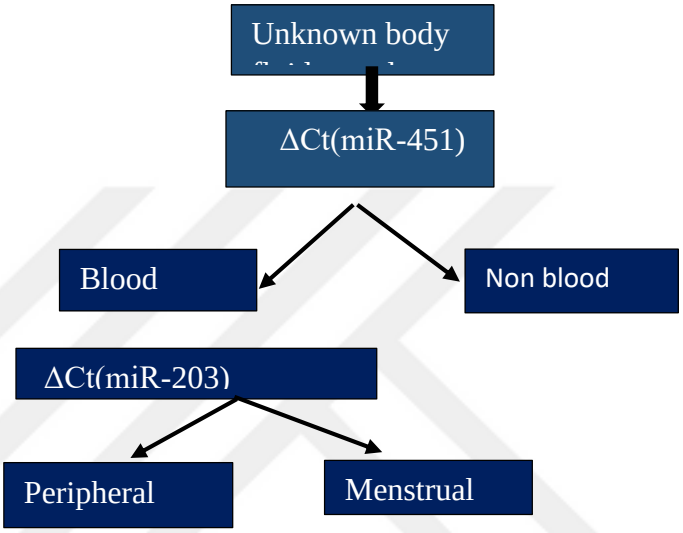



**Figure 5.2.** Diagram of the two-step analysis method

Also, strategy technique used by He *et al* 2020 remained valid in this study as well. In this study, it was observed that miR451 is sufficient for the discrimination of blood and non-blood samples, and miR203 is sufficient for the distinction between blood and MB in the second step, in single source samples.

Since the problems arising from possible expression differences can be partially overcome by studying more than one marker in the analysis of routine forensic samples, studying both miR-144 selected as a blood marker and other markers together will contribute to obtaining more reliable results.

As a result, the selected markers and the applied step wise model utilizes the identification potential of miRNAs, and the problem of the relative specificity of miRNAs can also be solved to a certain extent. Thus, this strategy shows strong application potential in forensic science. This set of markers is suitable for determining the type of body fluids in samples taken from a single body fluid source; However, it can be

concluded that it may not be sufficient for mixed samples. Therefore, future researchers should continue to identify more specific markers to distinguish complex samples.

# 6. CONCLUSION, RECOMMENDATION AND LIMITATIONS

## 6.1 Conclusion

Our study selected five potential miRNAs (miR-451, miR-203, miR-205-5, miR-144, and miR-214) to examine their expression in forensically relevant body fluids and to identify suitable miRNA biomarkers for distinguishing PB from MB. This was achieved when blood was determined as the unknown samples and then whether each blood sample is MB or PB. The accuracy rate of this strategy is 100%. Thus, this model is a reliable and accurate identification strategy for discriminating PB and MB) in forensic science.

## 6.2 Recommendation and Limitations

On the other hand, we proposed that the combination of two or three miRNAs to distinguish PB and MB as well as other different body fluids individually be developed. This strategy of combination of different body fluid miRNAs could be used as a new method for body fluid identification. Also, since the method used in our study accommodated for single samples, future researchers should focus on using more specific markers to distinguish complex samples. Still, sufficient tools should be used to carry out some tests such as sensitivity tests. The small sample size used in the study and the fact that its cost is much higher than the classical serological test can be considered as its limit.

# APPENDIXES

## Appendices 1

**Ct values of 5 genes in five different body fluids (51+45) samples**

| | | SNORD48 | 451 | 144 | 214 | 203 | 205 |
|---|---|---|---|---|---|---|---|
| Training set_51 | PB1 | 25.42 | 17.58 | 21.32 | 35.93 | Undetermined | Undetermined |
| | PB2 | 24.94 | 15.57 | 17.40 | 33.73 | 35.51 | 36.52 |
| | PB3 | 27.48 | 19.24 | 22.37 | 31.30 | 35.19 | 32.26 |
| PB_10 | PB4 | 26.97 | 19.89 | 25.17 | 31.56 | 36.47 | 33.56 |
| | PB5 | 26.95 | 18.19 | 21.14 | 38.63 | Undetermined | Undetermined |
| | PB6 | 25.03 | 17.95 | 21.26 | 32.83 | 32.67 | 31.53 |
| | PB7 | 24.28 | 16.93 | 20.46 | 33.47 | 37.31 | 33.64 |
| | PB8 | 25.05 | 16.93 | 20.74 | 34.53 | Undetermined | Undetermined |
| | PB9 | 24.12 | 17.83 | 20.83 | 34.02 | 36.39 | 36.29 |
| | PB10 | 25.88 | 19.04 | 23.30 | 36.95 | 37.43 | Undetermined |
| | MB 1 | 24.03 | 16.02 | 25.20 | 26.61 | 23.47 | 22.88 |
| MB_11 | MB 2 | 32.54 | 18.05 | 22.34 | 29.10 | 22.06 | 21.11 |
| | MB 3 | 26.18 | 18.23 | 23.69 | 29.10 | 25.83 | 23.58 |
| | MB 4 | 25.69 | 17.02 | 22.17 | 29.92 | 27.98 | 26.02 |
| | MB 5 | 25.34 | 15.60 | 22.29 | 26.32 | 22.56 | 22.11 |
| | MB 6 | 28.14 | 16.88 | 23.74 | 29.16 | 22.43 | 20.57 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MB 7 | 30.12 | 18.43 | 23.57 | 31.10 | 22.26 | 21.83 | |
| | MB 8 | 28.65 | 19.38 | 22.31 | 24.59 | 22.54 | 22.90 | |
| | MB 9 | 32.36 | 21.06 | 29.24 | 26.18 | 24.60 | 23.84 | |
| | MB 10 | 26.79 | 18.66 | 25.47 | 29.62 | 22.11 | 22.87 | |
| | MB 11 | 29.15 | 16.11 | 19.32 | 28.17 | 31.19 | 28.83 | |
| | | | | | | | | |
| | SA 1 | 26.15 | 26.77 | 32.30 | 30.28 | 21.11 | 20.35 | |
| SA_11 | SA2 | 29.46 | 30.42 | Undetermined | 30.01 | 23.05 | 22.27 | |
| | SA 3 | 28.87 | 31.80 | 35.91 | 34.29 | 25.42 | 24.45 | |
| | SA 4 | 27.89 | 28.16 | 33.38 | 31.35 | 24.44 | 23.66 | |
| | SA 5 | 28.06 | 25.74 | 30.73 | 32.47 | 23.54 | 23.13 | |
| | SA 6 | 24.81 | 28.91 | 34.14 | 29.06 | 23.35 | 21.39 | |
| | SA 7 | 23.93 | 26.78 | 32.61 | 29.45 | 21.06 | 19.29 | |
| | SA 8 | 27.66 | 27.86 | 33.84 | 29.63 | 21.47 | 20.75 | |
| | SA 9 | 30.01 | 31.91 | Undetermined | 32.36 | 26.08 | 24.57 | |
| | SA 10 | 33.80 | 30.82 | 34.69 | 31.04 | 24.07 | 23.81 | |
| | SA 11 | 29.02 | 29.04 | 37.00 | 26.14 | 24.65 | 25.07 | |
| | | | | | | | | |
| | VS 1 | 30.05 | 33.19 | 36.76 | 31.74 | 24.51 | 24.86 | |
| VS_11 | VS 2 | 28.26 | 29.43 | Undetermined | 21.78 | 21.53 | 22.37 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | VS 3 | 26.15 | 28.55 | 31.37 | 33.98 | 23.56 | 21.84 | |
| | VS 4 | 27.53 | 35.44 | 37.31 | 31.17 | 20.68 | 22.54 | |
| | VS 5 | 29.06 | 34.71 | Undetermined | 31.16 | 22.21 | 22.83 | |
| | VS 6 | 27.38 | 31.41 | Undetermined | 31.70 | 20.88 | 22.32 | |
| | VS 7 | 27.26 | 19.20 | 23.51 | 28.23 | 20.36 | 17.40 | |
| | VS 8 | 37.52 | 36.53 | Undetermined | 31.70 | 25.24 | 21.27 | |
| | VS 9 | 27.66 | 34.49 | 36.49 | 32.26 | 23.13 | 23.98 | |
| | VS 10 | 26.25 | 34.80 | Undetermined | 31.18 | 21.09 | 22.27 | |
| | VS 11 | 35.70 | 36.54 | 36.66 | 29.54 | 27.79 | 23.71 | |
| | | | | | | | | |
| | SE 1 | 25.32 | 29.62 | Undetermined | 32.82 | 27.20 | 26.46 | |
| SE-7 | SE 2 | 30.35 | 36.58 | Undetermined | 35.46 | 30.45 | 27.43 | |
| | SE 3 | 28.75 | 34.34 | 37.40 | 32.73 | 26.73 | 25.79 | |
| | SE 4<br>SE5 | 28.38<br>Undetermined | 30.93<br>Undetermined | 34.34<br>Undetermined | 26.27<br>35.40 | 26.11<br>36.63 | 24.05<br>34.30 | |
| | SE 6 | 34.46 | Undetermined | Undetermined | 36.26 | 35.60 | 36.43 | |
| | SE 7 | 34.73 | 33.99 | Undetermined | 34.09 | 29.18 | 28.41 | |
| | SE 8 | 33.15 | Undetermined | Undetermined | 34.65 | 30.34 | 27.65 | |
| | | | | | | | | |
| Test set_45 | | SNORD48 | miR-451 | miR-144 | miR-214 | miR-203 | miR-205 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | PB11 | 24.28 | 17.13 | 18.42 | 33.67 | Undetermined | 24 | |
| PB_11 | PB12 | 25.30 | 18.00 | 22.80 | 34.66 | Undetermined | 35.22 | |
| | PB13 | 24.75 | 17.34 | 21.73 | 33.12 | Undetermined | 33.37 | |
| | PB14 | 25.25 | 16.73 | 21.14 | 33.55 | 37.82 | 34.08 | |
| | PB15 | 25.88 | 18.22 | 23.81 | 33.92 | Undetermined | 33.77 | |
| | PB16 | 24.97 | 15.97 | 19.81 | 33.60 | Undetermined | 33.55 | |
| | PB17 | 24.69 | 16.30 | 20.31 | 32.89 | 37.61 | 34.55 | |
| | PB18 | 24.49 | 17.05 | 21.19 | 33.43 | Undetermined | 34.11 | |
| | PB19 | 25.47 | 17.04 | 21.70 | 35.10 | 35.50 | 34.90 | |
| | PB20 | 27.41 | 19.70 | 22.20 | 33.85 | Undetermined | 37.18 | |
| | PB21 | 24.12 | 18.87 | 20.14 | 34.65 | 37.05 | 32.52 | |
| | | | | | | | | |
| | MB11 | 28.95 | 19.82 | 26.42 | 27.91 | 27.87 | 28.33 | |
| MB_10 | MB12 | 33.43 | 17.54 | 21.40 | 28.25 | 26.23 | 24.28 | |
| | MB13 | 27.36 | 15.69 | 22.73 | 28.43 | 27.32 | 26.34 | |
| | MB14 | 32.33 | 18.98 | 24.63 | 30.12 | 26.83 | 25.61 | |
| | MB15 | 31.42 | 20.06 | 27.80 | 29.77 | 22.83 | 22.95 | |
| | MB16 | 26.72 | 18.12 | 23.01 | 28.83 | 22.28 | 22.06 | |
| | MB17 | 26.56 | 18.50 | 23.03 | 27.42 | 26.57 | 25.50 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MB18 | 28.91 | 18.22 | 23.50 | 26.23 | 21.98 | 22.32 | |
| | MB19 | 26.42 | 18.96 | 23.05 | 31.41 | 21.82 | 21.92 | |
| | MB20 | 32.47 | 18.41 | 24.07 | 30.27 | 22.39 | 22.42 | |
| | | | | | | | | |
| | SA12 | 23.33 | 29.11 | 35.32 | 32.05 | 22.51 | 22.09 | |
| SA_9 | SA13 | 30.36 | 23.54 | 29.15 | 29.90 | 22.54 | 21.22 | |
| | SA14 | 24.17 | 24.90 | 31.25 | 29.47 | 23.04 | 21.39 | |
| | SA15 | 26.03 | 22.92 | 31.27 | 30.56 | 23.45 | 21.92 | |
| | SA16 | 26.73 | 24.99 | 31.76 | 30.18 | 22.49 | 21.96 | |
| | SA17 | 31.65 | 28.82 | 34.28 | 31.54 | 23.98 | 23.16 | |
| | SA18 | 27.54 | 29.96 | 36.07 | 31.82 | 22.12 | 21.41 | |
| | SA19 | 26.77 | 27.16 | 33.07 | 34.10 | 26.71 | 24.90 | |
| | SA20 | 29.14 | 30.33 | 37.35 | 29.92 | 22.02 | 22.23 | |
| | | | | | | | | |
| | VS11 | 36.30 | 35.89 | 37.99 | 32.76 | 27.18 | 23.50 | |
| VS_10 | VS12 | 29.08 | 36.57 | 37.96 | 33.39 | 22.69 | 24.57 | |
| | VS13 | 26.40 | 26.29 | 30.95 | 28.79 | 21.65 | 20.31 | |
| | VS14 | 29.22 | Undetermined | Undetermined | 30.92 | 24.07 | 24.36 | |
| | VS15 | 29.12 | 33.48 | 36.82 | 30.04 | 19.50 | 19.87 | |
| | VS16 | 31.44 | 30.33 | 34.03 | 31.16 | 21.21 | 20.79 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | VS17 | 29.90 | 36.63 | Undetermined | 33.15 | 22.66 | 24.35 | |
| | VS18 | 25.68 | 36.63 | 36.74 | 32.56 | 21.60 | 23.35 | |
| | VS19 | 25.39 | 32.92 | 37.29 | 32.71 | 19.55 | 20.54 | |
| | VS20 | 28.31 | 34.79 | Undetermined | 31.88 | 22.82 | 25.46 | |
| | | | | | | | | |
| | SE 9 | 35.01 | 35.58 | Undetermined | 34.34 | 29.75 | 28.41 | |
| SE_5 | SE 10 | 30.07 | 34.57 | Undetermined | 31.44 | 30.43 | 25.29 | |
| | SE 11 | 27.77 | 32.99 | Undetermined | 28.09 | 27.68 | 25.64 | |
| | SE12 | 30.36 | 33.13 | 35.91 | 29.65 | 28.76 | 25.12 | |
| | SE 13 | 29.06 | 33.39 | Undetermined | 28.37 | 29.27 | 26.03 | |
| | | | | | | | | |
| | | | | | | | | |

**Appendices: 2**

**Explore**

**Tests of normality**

| | Type | Kolmogorov-Smirnov[a] | | | Shapiro-Wilk | | |
|---|---|---|---|---|---|---|---|
| | | Statistic | df | Sig. | Statistic | df | Sig. |
| miR-451 | PB | ,143 | 10 | ,200* | ,974 | 10 | ,925 |
| | MB | ,150 | 11 | ,200* | ,913 | 11 | ,268 |
| miR-144 | PB | ,176 | 10 | ,200* | ,964 | 10 | ,828 |
| | MB | ,158 | 11 | ,200* | ,950 | 11 | ,645 |
| miR-214 | PB | ,216 | 10 | ,200* | ,882 | 10 | ,138 |
| | MB | ,243 | 11 | ,070 | ,911 | 11 | ,248 |
| miR-203 | PB | ,127 | 10 | ,200* | ,936 | 10 | ,506 |
| | MB | ,141 | 11 | ,200* | ,948 | 11 | ,622 |
| miR-205 | PB | ,186 | 10 | ,200* | ,899 | 10 | ,213 |
| | MB | ,133 | 11 | ,200* | ,953 | 11 | ,676 |

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

**T-Test**

**Group Statistics**

| | Type | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| miR-451 | PB | 10 | -7,6970 | ,94393 | ,29850 |
| | MB | 11 | -10,3227 | 2,20042 | ,66345 |
| miR-144 | PB | 10 | -4,2130 | 1,63768 | ,51788 |
| | MB | 11 | -4,5136 | 3,46069 | 1,04344 |
| miR-214 | PB | 10 | 8,6830 | 2,61584 | ,82720 |
| | MB | 11 | ,0800 | 3,33834 | 1,00655 |
| miR-203 | PB | 10 | 11,4850 | 2,60030 | ,82229 |
| | MB | 11 | -3,8145 | 4,25650 | 1,28338 |
| miR-205 | PB | 10 | 10,7680 | 3,72080 | 1,17662 |
| | MB | 11 | -4,7682 | 3,82529 | 1,15337 |

**Independent samples tests**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | F | Sig. | T | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | 95% Confidence Interval of the Difference | |
| | | | | | | | | | Lower | Upper |
| miR-451 | Equal variances assumed | 8,961 | ,007 | 3,487 | 19 | ,002 | 2,62573 | ,75304 | 1,04959 | 4,20187 |
| | Equal variances not assumed | | | 3,609 | 13,829 | **,003** | 2,62573 | ,72751 | 1,06356 | 4,18790 |
| miR-144 | Equal variances assumed | 4,637 | ,044 | ,250 | 19 | ,805 | ,30064 | 1,20246 | -2,21613 | 2,81741 |
| | Equal variances not assumed | | | ,258 | 14,552 | **,800** | ,30064 | 1,16489 | -2,18893 | 2,79020 |
| miR-214 | Equal variances assumed | 1,079 | ,312 | 6,525 | 19 | **,000** | 8,60300 | 1,31855 | 5,84325 | 11,36275 |
| | Equal variances not assumed | | | 6,603 | 18,628 | ,000 | 8,60300 | 1,30284 | 5,87243 | 11,33357 |
| miR-203 | Equal variances assumed | 3,862 | ,064 | 9,811 | 19 | **,000** | 15,29955 | 1,55946 | 12,03557 | 18,56352 |
| | Equal variances not assumed | | | 10,038 | 16,758 | ,000 | 15,29955 | 1,52421 | 12,08019 | 18,51890 |
| miR-205 | Equal variances assumed | ,005 | ,944 | 9,416 | 19 | **,000** | 15,53618 | 1,64992 | 12,08286 | 18,98951 |
| | Equal variances not assumed | | | 9,429 | 18,900 | ,000 | 15,53618 | 1,64763 | 12,08641 | 18,98595 |

**Discriminant**

**Analysis case processing summary**

| Unweighted Cases | | N | Percent |
|---|---|---|---|
| Valid | | 51 | 100,0 |
| | Missing or out-of-range group codes | 0 | ,0 |
| | At least one missing discriminating variable | 0 | ,0 |
| Excluded | Both missing or out-of-range group codes and at least one missing discriminating variable | 0 | ,0 |
| | Total | 0 | ,0 |
| Total | | 51 | 100,0 |

**Group Statistics**

**Analysis case processing summary**

| Blood | | Mean | Std. Deviation | Valid N (listwise) | |
|---|---|---|---|---|---|
| | | | | Unweighted | Weighted |
| Non-blood | miR-451 | 2,6133 | 3,05296 | 30 | 30,000 |
| Blood | miR-451 | -9,0724 | 2,15118 | 21 | 21,000 |
| Total | miR-451 | -2,1984 | 6,40268 | 51 | 51,000 |

**Tests of Equality of Group Means**

| | Wilks' Lambda | F | df1 | df2 | Sig. |
|---|---|---|---|---|---|
| miR-451 | ,177 | 227,799 | 1 | 49 | ,000 |

**Analysis 1: Summary of Canonical Discriminant Functions**

**Eigenvalues**

| Function | Eigenvalue | % of Variance | Cumulative % | Canonical Correlation |
|---|---|---|---|---|
| 1 | 4,649[a] | 100,0 | 100,0 | ,907 |

**a. First 1 canonical discriminant functions were used in the analysis**.

**Wilks' Lambda**

| Test of Function(s) | Wilks' Lambda | Chi-square | df | Sig. |
|---|---|---|---|---|
| 1 | ,177 | 83,976 | 1 | ,000 |

**Standardized Canonical Discriminant Function Coefficients**

| | Function |
|---|---|
| | 1 |
| miR-451 | 1,000 |

**Structure Matrix**

| | Function |
|---|---|
| | 1 |
| miR-451 | 1,000 |

Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions. Variables ordered by absolute size of correlation within function.

**Functions at Group Centroids**

| Blood | Function |
|---|---|
| | 1 |
| Non-blood | 1,768 |
| Blood | -2,526 |

Unstandardized canonical discriminant functions evaluated at group means

**Classification Statistics**

**Classification Processing Summary**

| | | |
|---|---|---|
| Processed | | 51 |
| Excluded | Missing or out-of-range group codes | 0 |
| | At least one missing discriminating variable | 0 |
| Used in Output | | 51 |

**Prior Probabilities for Groups**

| Blood | Prior | Cases Used in Analysis | |
|---|---|---|---|
| | | Unweighted | Weighted |
| Non-blood | ,500 | 30 | 30,000 |
| Blood | ,500 | 21 | 21,000 |
| Total | 1,000 | 51 | 51,000 |

**Classification Function Coefficients**

| | blood | |
|---|---|---|
| | Non-blood | Blood |
| miR-451 | ,353 | -1,225 |
| (Constant) | -1,154 | -6,251 |

Fisher's linear discriminant functions

**Casewise statistics**

| | Case Number | Actual Group | Highest Group | | | | | Second Highest Group | | | Discriminant Scores |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Predicted Group | P(D>d \| G=g) | | P(G=g \| D=d) | Squared Mahalanobis Distance to Centroid | Group | P(G=g \| D=d) | Squared Mahalanobis Distance to Centroid | Function 1 |
| | | | | p | df | | | | | | |
| Original | 1 | 1 | 1 | ,651 | 1 | ,999 | ,205 | 0 | ,001 | 14,756 | -2,073 |
| | 2 | 1 | 1 | ,760 | 1 | 1,000 | ,094 | 0 | ,000 | 15,907 | -2,220 |
| | 3 | 1 | 1 | ,464 | 1 | ,998 | ,536 | 0 | ,002 | 12,689 | -1,794 |
| | 4 | 1 | 1 | ,909 | 1 | 1,000 | ,013 | 0 | ,000 | 17,468 | -2,411 |
| | 5 | 1 | 1 | ,464 | 1 | ,998 | ,536 | 0 | ,002 | 12,689 | -1,794 |
| | 6 | 1 | 1 | ,527 | 1 | ,999 | ,401 | 0 | ,001 | 13,405 | -1,893 |
| | 7 | 1 | 1 | ,726 | 1 | 1,000 | ,122 | 0 | ,000 | 15,558 | -2,176 |
| | 8 | 1 | 1 | ,307 | 1 | ,992 | 1,045 | 0 | ,008 | 10,705 | -1,504 |
| | 9 | 1 | 1 | ,412 | 1 | ,997 | ,673 | 0 | ,003 | 12,068 | -1,706 |
| | 10 | 1 | 1 | ,913 | 1 | 1,000 | ,012 | 0 | ,000 | 19,392 | -2,635 |
| | 11 | 1 | 1 | ,696 | 1 | ,999 | ,152 | 0 | ,001 | 15,240 | -2,136 |
| | 12 | 1 | 1 | ,046 | 1 | 1,000 | 3,964 | 0 | ,000 | 39,503 | -4,517 |
| | 13 | 1 | 1 | ,680 | 1 | ,999 | ,170 | 0 | ,001 | 15,069 | -2,114 |
| | 14 | 1 | 1 | ,882 | 1 | 1,000 | ,022 | 0 | ,000 | 17,193 | -2,378 |
| | 15 | 1 | 1 | ,806 | 1 | 1,000 | ,060 | 0 | ,000 | 20,608 | -2,771 |
| | 16 | 1 | 1 | ,421 | 1 | 1,000 | ,646 | 0 | ,000 | 25,992 | -3,330 |
| | 17 | 1 | 1 | ,336 | 1 | 1,000 | ,925 | 0 | ,000 | 27,628 | -3,488 |
| | 18 | 1 | 1 | ,942 | 1 | 1,000 | ,005 | 0 | ,000 | 19,070 | -2,599 |
| | 19 | 1 | 1 | ,413 | 1 | 1,000 | ,670 | 0 | ,000 | 26,142 | -3,345 |
| | 20 | 1 | 1 | ,729 | 1 | 1,000 | ,120 | 0 | ,000 | 15,587 | -2,180 |
| | 21 | 1 | 1 | ,145 | 1 | 1,000 | 2,126 | 0 | ,000 | 33,089 | -3,984 |
| | 22 | 0 | 0 | ,464 | 1 | ,998 | ,537 | 1 | ,002 | 12,686 | 1,036 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 23 | 0 | 0 | ,543 | 1 | ,999 | ,369 | 1 | ,001 | 13,592 | 1,161 |
| 24 | 0 | 0 | ,907 | 1 | 1,000 | ,014 | 1 | ,000 | 19,454 | 1,885 |
| 25 | 0 | 0 | ,389 | 1 | ,996 | ,742 | 1 | ,004 | 11,787 | ,907 |
| 26 | 0 | 0 | ,070 | 1 | ,808 | 3,287 | 1 | ,192 | 6,157 | -,045 |
| 27 | 0 | 0 | ,585 | 1 | 1,000 | ,298 | 1 | ,000 | 23,432 | 2,315 |
| 28 | 0 | 0 | ,931 | 1 | 1,000 | ,008 | 1 | ,000 | 19,195 | 1,855 |
| 29 | 0 | 0 | ,375 | 1 | ,996 | ,787 | 1 | ,004 | 11,611 | ,881 |
| 30 | 0 | 0 | ,793 | 1 | 1,000 | ,069 | 1 | ,000 | 16,258 | 1,506 |
| 31 | 0 | 0 | ,040 | 1 | ,597 | 4,225 | 1 | ,403 | 5,012 | -,287 |
| 32 | 0 | 0 | ,341 | 1 | ,994 | ,908 | 1 | ,006 | 11,164 | ,815 |
| 33 | 0 | 0 | ,847 | 1 | 1,000 | ,037 | 1 | ,000 | 20,141 | 1,962 |
| 34 | 0 | 0 | ,596 | 1 | ,999 | ,281 | 1 | ,001 | 14,167 | 1,238 |
| 35 | 0 | 0 | ,938 | 1 | 1,000 | ,006 | 1 | ,000 | 17,774 | 1,690 |
| 36 | 0 | 0 | ,052 | 1 | 1,000 | 3,789 | 1 | ,000 | 38,947 | 3,715 |
| 37 | 0 | 0 | ,264 | 1 | 1,000 | 1,245 | 1 | ,000 | 29,270 | 2,884 |
| 38 | 0 | 0 | ,603 | 1 | 1,000 | ,271 | 1 | ,000 | 23,183 | 2,289 |
| 39 | 0 | 0 | ,185 | 1 | ,972 | 1,753 | 1 | ,028 | 8,822 | ,444 |
| 40 | 0 | 0 | ,121 | 1 | 1,000 | 2,401 | 1 | ,000 | 34,150 | 3,318 |
| 41 | 0 | 0 | ,029 | 1 | 1,000 | 4,759 | 1 | ,000 | 41,937 | 3,950 |
| 42 | 0 | 0 | ,515 | 1 | ,998 | ,425 | 1 | ,002 | 13,269 | 1,117 |
| 43 | 0 | 0 | ,535 | 1 | 1,000 | ,384 | 1 | ,000 | 24,148 | 2,388 |
| 44 | 0 | 0 | ,184 | 1 | 1,000 | 1,766 | 1 | ,000 | 31,622 | 3,097 |
| 45 | 0 | 0 | ,274 | 1 | 1,000 | 1,197 | 1 | ,000 | 29,032 | 2,862 |
| 46 | 0 | 0 | ,981 | 1 | 1,000 | ,001 | 1 | ,000 | 18,242 | 1,745 |
| 47 | 0 | 0 | ,337 | 1 | ,994 | ,922 | 1 | ,006 | 11,115 | ,808 |
| 48 | 0 | 0 | ,282 | 1 | 1,000 | 1,157 | 1 | ,000 | 28,835 | 2,844 |
| 49 | 0 | 0 | ,218 | 1 | ,981 | 1,519 | 1 | ,019 | 9,376 | ,536 |
| 50 | 0 | 0 | ,119 | 1 | 1,000 | 2,424 | 1 | ,000 | 34,236 | 3,325 |
| 51 | 0 | 0 | ,337 | 1 | ,994 | ,922 | 1 | ,006 | 11,115 | ,808 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 1 | 1 | ,637 | 1 | ,999 | ,222 | 0 | ,001 | 14,519 | |
| | 2 | 1 | 1 | ,750 | 1 | 1,000 | ,101 | 0 | ,000 | 15,614 | |
| | 3 | 1 | 1 | ,444 | 1 | ,998 | ,586 | 0 | ,002 | 12,574 | |
| | 4 | 1 | 1 | ,905 | 1 | 1,000 | ,014 | 0 | ,000 | 17,116 | |
| | 5 | 1 | 1 | ,444 | 1 | ,998 | ,586 | 0 | ,002 | 12,574 | |
| | 6 | 1 | 1 | ,509 | 1 | ,998 | ,436 | 0 | ,002 | 13,246 | |
| | 7 | 1 | 1 | ,716 | 1 | ,999 | ,133 | 0 | ,001 | 15,280 | |
| | 8 | 1 | 1 | ,283 | 1 | ,992 | 1,155 | 0 | ,008 | 10,727 | |
| | 9 | 1 | 1 | ,390 | 1 | ,996 | ,737 | 0 | ,004 | 11,995 | |
| | 10 | 1 | 1 | ,909 | 1 | 1,000 | ,013 | 0 | ,000 | 19,001 | |
| | 11 | 1 | 1 | ,684 | 1 | ,999 | ,165 | 0 | ,001 | 14,978 | |
| | 12 | 1 | 1 | ,031 | 1 | 1,000 | 4,678 | 0 | ,000 | 42,289 | |
| | 13 | 1 | 1 | ,668 | 1 | ,999 | ,184 | 0 | ,001 | 14,815 | |
| Cross- | 14 | 1 | 1 | ,878 | 1 | 1,000 | ,024 | 0 | ,000 | 16,850 | |
| validat | 15 | 1 | 1 | ,799 | 1 | 1,000 | ,065 | 0 | ,000 | 20,214 | |
| ed[a] | 16 | 1 | 1 | ,400 | 1 | 1,000 | ,708 | 0 | ,000 | 25,819 | |
| | 17 | 1 | 1 | ,313 | 1 | 1,000 | 1,020 | 0 | ,000 | 27,611 | |
| | 18 | 1 | 1 | ,940 | 1 | 1,000 | ,006 | 0 | ,000 | 18,683 | |
| | 19 | 1 | 1 | ,392 | 1 | 1,000 | ,734 | 0 | ,000 | 25,981 | |
| | 20 | 1 | 1 | ,719 | 1 | ,999 | ,130 | 0 | ,001 | 15,308 | |
| | 21 | 1 | 1 | ,121 | 1 | 1,000 | 2,405 | 0 | ,000 | 33,961 | |
| | 22 | 0 | 0 | ,451 | 1 | ,998 | ,569 | 1 | ,002 | 12,570 | |
| | 23 | 0 | 0 | ,532 | 1 | ,999 | ,390 | 1 | ,001 | 13,419 | |
| | 24 | 0 | 0 | ,905 | 1 | 1,000 | ,014 | 1 | ,000 | 19,062 | |
| | 25 | 0 | 0 | ,374 | 1 | ,996 | ,790 | 1 | ,004 | 11,730 | |
| | 26 | 0 | 0 | ,054 | 1 | ,801 | 3,702 | 1 | ,199 | 6,481 | |
| | 27 | 0 | 0 | ,575 | 1 | 1,000 | ,315 | 1 | ,000 | 23,099 | |
| | 28 | 0 | 0 | ,929 | 1 | 1,000 | ,008 | 1 | ,000 | 18,807 | |
| | 29 | 0 | 0 | ,360 | 1 | ,995 | ,838 | 1 | ,005 | 11,566 | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 30 | 0 | 0 | ,788 | 1 | 1,000 | ,072 | 1 | ,000 | 15,950 | |
| 31 | 0 | 0 | ,027 | 1 | ,566 | 4,863 | 1 | ,434 | 5,391 | |
| 32 | 0 | 0 | ,325 | 1 | ,994 | ,971 | 1 | ,006 | 11,150 | |
| 33 | 0 | 0 | ,843 | 1 | 1,000 | ,039 | 1 | ,000 | 19,745 | |
| 34 | 0 | 0 | ,586 | 1 | ,999 | ,297 | 1 | ,001 | 13,961 | |
| 35 | 0 | 0 | ,936 | 1 | 1,000 | ,006 | 1 | ,000 | 17,413 | |
| 36 | 0 | 0 | ,038 | 1 | 1,000 | 4,317 | 1 | ,000 | 41,469 | |
| 37 | 0 | 0 | ,247 | 1 | 1,000 | 1,341 | 1 | ,000 | 29,447 | |
| 38 | 0 | 0 | ,593 | 1 | 1,000 | ,286 | 1 | ,000 | 22,841 | |
| 39 | 0 | 0 | ,167 | 1 | ,972 | 1,909 | 1 | ,028 | 8,974 | |
| 40 | 0 | 0 | ,103 | 1 | 1,000 | 2,652 | 1 | ,000 | 35,240 | |
| 41 | 0 | 0 | ,019 | 1 | 1,000 | 5,547 | 1 | ,000 | 45,670 | |
| 42 | 0 | 0 | ,503 | 1 | ,998 | ,449 | 1 | ,002 | 13,115 | |
| 43 | 0 | 0 | ,524 | 1 | 1,000 | ,406 | 1 | ,000 | 23,849 | |
| 44 | 0 | 0 | ,165 | 1 | 1,000 | 1,923 | 1 | ,000 | 32,177 | |
| 45 | 0 | 0 | ,257 | 1 | 1,000 | 1,287 | 1 | ,000 | 29,177 | |
| 46 | 0 | 0 | ,981 | 1 | 1,000 | ,001 | 1 | ,000 | 17,869 | |
| 47 | 0 | 0 | ,321 | 1 | ,994 | ,986 | 1 | ,006 | 11,104 | |
| 48 | 0 | 0 | ,265 | 1 | 1,000 | 1,243 | 1 | ,000 | 28,953 | |
| 49 | 0 | 0 | ,200 | 1 | ,981 | 1,645 | 1 | ,019 | 9,489 | |
| 50 | 0 | 0 | ,102 | 1 | 1,000 | 2,678 | 1 | ,000 | 35,346 | |
| 51 | 0 | 0 | ,321 | 1 | ,994 | ,986 | 1 | ,006 | 11,104 | |

For the original data, squared Mahalanobis distance is based on canonical functions.
For the cross-validated data, squared Mahalanobis distance is based on observations.

a. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

**Classification Results**

| | | Blood | Predicted Group Membership | | Total |
|---|---|---|---|---|---|
| | | | Non-blood | Blood | |
| Original | Count | Non-blood | 30 | 0 | 30 |
| | | Blood | 0 | 21 | 21 |
| | % | Non-blood | 100,0 | ,0 | 100,0 |
| | | Blood | ,0 | 100,0 | 100,0 |
| Cross-validated[b] | Count | Non-blood | 30 | 0 | 30 |
| | | Blood | 0 | 21 | 21 |
| | % | Non-blood | 100,0 | ,0 | 100,0 |
| | | Blood | ,0 | 100,0 | 100,0 |

a. 100,0% of original grouped cases correctly classified.
b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.
c. 100,0% of cross-validated grouped cases correctly classified.

**Discriminant**

**Analysis Case Processing Summary**

| Unweighted Cases | | N | Percent |
|---|---|---|---|
| Valid | | 51 | 100,0 |
| Excluded | Missing or out-of-range group codes | 0 | ,0 |
| | At least one missing discriminating variable | 0 | ,0 |
| | Both missing or out-of-range group codes and at least one missing discriminating variable | 0 | ,0 |
| | Total | 0 | ,0 |
| Total | | 51 | 100,0 |

**Group Statistics**

| Blood | | Mean | Std. Deviation | Valid N (listwise) | |
|---|---|---|---|---|---|
| | | | | Unweighted | Weighted |
| Non-blood | miR-144 | 7,1523 | 3,93517 | 30 | 30,000 |
| Blood | miR-144 | -4,3705 | 2,68677 | 21 | 21,000 |
| Total | miR-144 | 2,4076 | 6,68374 | 51 | 51,000 |

**Tests of Equality of Group Means**

| | Wilks' Lambda | F | df1 | df2 | Sig. |
|---|---|---|---|---|---|
| miR-144 | ,266 | 135,424 | 1 | 49 | ,000 |

**Analysis 1: Summary of Canonical Discriminant Functions**

**Eigenvalues**

| Function | Eigenvalue | % of Variance | Cumulative % | Canonical Correlation |
|---|---|---|---|---|
| 1 | 2,764[a] | 100,0 | 100,0 | ,857 |

**a. First 1 canonical discriminant functions were used in the analysis.**

**Wilks' Lambda**

| Test of Function(s) | Wilks' Lambda | Chi-square | df | Sig. |
|---|---|---|---|---|
| 1 | ,266 | 64,283 | 1 | ,000 |

**Standardized Canonical Discriminant Function Coefficients**

| | Function |
|---|---|
| | 1 |
| miR-144 | 1,000 |

| **Structure Matrix** | |
|---|---|
| | Function |
| | 1 |
| miR-144 | 1,000 |

Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions

Variables ordered by absolute size of correlation within function.

| Functions at Group Centroids | |
|---|---|
| Blood | Function |
| | 1 |
| Non-blood | 1,363 |
| Blood | -1,948 |
| Unstandardized canonical discriminant functions evaluated at group means | |

**Classification Statistics**

**Classification Processing Summary**

| | | |
|---|---|---|
| Processed | | 51 |
| Excluded | Missing or out-of-range group codes | 0 |
| | At least one missing discriminating variable | 0 |
| Used in Output | | 51 |

**Prior Probabilities for Groups**

| Blood | Prior | Cases Used in Analysis | |
|---|---|---|---|
| | | Unweighted | Weighted |
| Non-blood | ,500 | 30 | 30,000 |
| Blood | ,500 | 21 | 21,000 |
| Total | 1,000 | 51 | 51,000 |

**Classification Function Coefficients**

| | blood | |
|---|---|---|
| | Non-blood | Blood |
| miR-144 | ,591 | -,361 |
| (Constant) | -2,805 | -1,482 |

Fisher's linear discriminant functions

**Casewise Statistics**

| | Case Number | Actual Group | Highest Group | | | | | Second Highest Group | | | Discriminant Scores |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Predicted Group | P(D>d \| G=g) | | P(G=g \| D=d) | Squared Mahalanobis Distance to Centroid | Group | P(G=g \| D=d) | Squared Mahalanobis Distance to Centroid | Function 1 |
| | | | | p | df | | | | | | |
| Original | 1 | 1 | 1 | ,938 | 1 | ,995 | ,006 | 0 | ,005 | 10,454 | -1,870 |
| | 2 | 1 | 1 | ,832 | 1 | ,998 | ,045 | 0 | ,002 | 12,415 | -2,160 |
| | 3 | 1 | 1 | ,460 | 1 | ,954 | ,546 | 0 | ,046 | 6,617 | -1,209 |
| | 4 | 1 | 1 | ,679 | 1 | ,999 | ,171 | 0 | ,001 | 13,873 | -2,361 |
| | 5 | 1 | 1 | ,863 | 1 | ,993 | ,030 | 0 | ,007 | 9,850 | -1,775 |
| | 6 | 1 | 1 | ,874 | 1 | ,993 | ,025 | 0 | ,007 | 9,940 | -1,789 |
| | 7 | 1 | 1 | ,986 | 1 | ,996 | ,000 | 0 | ,004 | 10,848 | -1,930 |
| | 8 | 1 | 1 | ,756 | 1 | ,988 | ,096 | 0 | ,012 | 9,003 | -1,637 |
| | 9 | 1 | 1 | ,607 | 1 | ,978 | ,265 | 0 | ,022 | 7,821 | -1,433 |
| | 10 | 1 | 1 | ,362 | 1 | 1,000 | ,829 | 0 | ,000 | 17,823 | -2,858 |
| | 11 | 1 | 1 | ,111 | 1 | ,552 | 2,535 | 0 | ,448 | 2,955 | -,356 |
| | 12 | 1 | 1 | ,094 | 1 | 1,000 | 2,806 | 0 | ,000 | 24,861 | -3,623 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 13 | 1 | 1 | ,589 | 1 | ,976 | ,292 | 0 | ,024 | 7,677 | -1,407 |
| 14 | 1 | 1 | ,807 | 1 | ,991 | ,060 | 0 | ,009 | 9,404 | -1,703 |
| 15 | 1 | 1 | ,704 | 1 | ,986 | ,144 | 0 | ,014 | 8,594 | -1,568 |
| 16 | 1 | 1 | ,993 | 1 | ,996 | ,000 | 0 | ,004 | 11,019 | -1,956 |
| 17 | 1 | 1 | ,531 | 1 | ,999 | ,392 | 0 | ,001 | 15,502 | -2,574 |
| 18 | 1 | 1 | ,571 | 1 | ,999 | ,320 | 0 | ,001 | 15,031 | -2,514 |
| 19 | 1 | 1 | ,719 | 1 | ,987 | ,129 | 0 | ,013 | 8,713 | -1,588 |
| 20 | 1 | 1 | ,381 | 1 | ,930 | ,768 | 0 | ,070 | 5,927 | -1,071 |
| 21 | 1 | 1 | ,117 | 1 | 1,000 | 2,461 | 0 | ,000 | 23,812 | -3,516 |
| 22 | 0 | 0 | ,773 | 1 | ,989 | ,083 | 1 | ,011 | 9,139 | 1,075 |
| 23 | 0 | 0 | ,330 | 1 | 1,000 | ,948 | 1 | ,000 | 18,357 | 2,337 |
| 24 | 0 | 0 | ,974 | 1 | ,995 | ,001 | 1 | ,005 | 10,750 | 1,331 |
| 25 | 0 | 0 | ,633 | 1 | ,980 | ,228 | 1 | ,020 | 8,028 | ,886 |
| 26 | 0 | 0 | ,198 | 1 | ,772 | 1,659 | 1 | ,228 | 4,093 | ,075 |
| 27 | 0 | 0 | ,531 | 1 | ,999 | ,392 | 1 | ,001 | 15,498 | 1,989 |
| 28 | 0 | 0 | ,661 | 1 | ,999 | ,193 | 1 | ,001 | 14,062 | 1,802 |
| 29 | 0 | 0 | ,780 | 1 | ,990 | ,078 | 1 | ,010 | 9,191 | 1,084 |
| 30 | 0 | 0 | ,415 | 1 | 1,000 | ,665 | 1 | ,000 | 17,027 | 2,179 |
| 31 | 0 | 1** | ,131 | 1 | ,617 | 2,285 | 0 | ,383 | 3,238 | -,436 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 32 | 0 | 0 | ,812 | 1 | ,998 | ,057 | 1 | ,002 | 12,594 | 1,601 |
| 33 | 0 | 0 | ,899 | 1 | ,994 | ,016 | 1 | ,006 | 10,137 | 1,236 |
| 34 | 0 | 0 | ,187 | 1 | 1,000 | 1,738 | 1 | ,000 | 21,430 | 2,682 |
| 35 | 0 | 0 | ,579 | 1 | ,974 | ,308 | 1 | ,026 | 7,594 | ,808 |
| 36 | 0 | 0 | ,450 | 1 | 1,000 | ,570 | 1 | ,000 | 16,533 | 2,118 |
| 37 | 0 | 0 | ,276 | 1 | 1,000 | 1,185 | 1 | ,000 | 19,355 | 2,452 |
| 38 | 0 | 0 | ,116 | 1 | 1,000 | 2,468 | 1 | ,000 | 23,835 | 2,934 |
| 39 | 0 | 0 | ,179 | 1 | ,738 | 1,803 | 1 | ,262 | 3,875 | ,021 |
| 40 | 0 | 0 | ,630 | 1 | ,999 | ,232 | 1 | ,001 | 14,388 | 1,845 |
| 41 | 0 | 0 | ,058 | 1 | 1,000 | 3,594 | 1 | ,000 | 27,111 | 3,259 |
| 42 | 0 | 1** | ,126 | 1 | ,601 | 2,346 | 0 | ,399 | 3,166 | -,416 |
| 43 | 0 | 0 | ,031 | 1 | 1,000 | 4,679 | 1 | ,000 | 29,965 | 3,526 |
| 44 | 0 | 0 | ,473 | 1 | 1,000 | ,515 | 1 | ,000 | 16,231 | 2,081 |
| 45 | 0 | 0 | ,667 | 1 | ,999 | ,185 | 1 | ,001 | 13,998 | 1,794 |
| 46 | 0 | 0 | ,732 | 1 | ,987 | ,117 | 1 | ,013 | 8,811 | 1,021 |
| 47 | 0 | 1** | ,209 | 1 | ,790 | 1,577 | 0 | ,210 | 4,224 | -,692 |
| 48 | 0 | 0 | ,643 | 1 | ,981 | ,215 | 1 | ,019 | 8,110 | ,900 |
| 49 | 0 | 0 | ,589 | 1 | ,976 | ,293 | 1 | ,024 | 7,674 | ,822 |
| 50 | 0 | 0 | ,931 | 1 | ,994 | ,008 | 1 | ,006 | 10,395 | 1,276 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 51 | 0 | 1** | ,209 | 1 | ,790 | 1,577 | 0 | ,210 | 4,224 | -,692 |
| | 1 | 1 | 1 | ,936 | 1 | ,994 | ,007 | 0 | ,006 | 10,242 | |
| | 2 | 1 | 1 | ,825 | 1 | ,998 | ,049 | 0 | ,002 | 12,174 | |
| | 3 | 1 | 1 | ,440 | 1 | ,952 | ,596 | 0 | ,048 | 6,559 | |
| | 4 | 1 | 1 | ,667 | 1 | ,999 | ,185 | 0 | ,001 | 13,640 | |
| | 5 | 1 | 1 | ,858 | 1 | ,992 | ,032 | 0 | ,008 | 9,655 | |
| | 6 | 1 | 1 | ,869 | 1 | ,992 | ,027 | 0 | ,008 | 9,743 | |
| | 7 | 1 | 1 | ,986 | 1 | ,995 | ,000 | 0 | ,005 | 10,627 | |
| | 8 | 1 | 1 | ,747 | 1 | ,987 | ,104 | 0 | ,013 | 8,838 | |
| | 9 | 1 | 1 | ,592 | 1 | ,976 | ,288 | 0 | ,024 | 7,705 | |
| Cross-validated[b] | 10 | 1 | 1 | ,340 | 1 | 1,000 | ,912 | 0 | ,000 | 17,776 | |
| | 11 | 1 | 1 | ,089 | 1 | ,521 | 2,895 | 0 | ,479 | 3,061 | |
| | 12 | 1 | 1 | ,073 | 1 | 1,000 | 3,224 | 0 | ,000 | 25,912 | |
| | 13 | 1 | 1 | ,573 | 1 | ,974 | ,317 | 0 | ,026 | 7,567 | |
| | 14 | 1 | 1 | ,799 | 1 | ,990 | ,065 | 0 | ,010 | 9,224 | |
| | 15 | 1 | 1 | ,693 | 1 | ,984 | ,156 | 0 | ,016 | 8,445 | |
| | 16 | 1 | 1 | ,993 | 1 | ,995 | ,000 | 0 | ,005 | 10,794 | |
| | 17 | 1 | 1 | ,513 | 1 | ,999 | ,427 | 0 | ,001 | 15,315 | |
| | 18 | 1 | 1 | ,555 | 1 | ,999 | ,348 | 0 | ,001 | 14,826 | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 19 | 1 | 1 | ,708 | 1 | ,985 | ,140 | 0 | ,015 | 8,558 | |
| 20 | 1 | 1 | ,358 | 1 | ,926 | ,844 | 0 | ,074 | 5,903 | |
| 21 | 1 | 1 | ,094 | 1 | 1,000 | 2,806 | 0 | ,000 | 24,625 | |
| 22 | 0 | 0 | ,768 | 1 | ,988 | ,087 | 1 | ,012 | 8,968 | |
| 23 | 0 | 0 | ,314 | 1 | 1,000 | 1,014 | 1 | ,000 | 18,349 | |
| 24 | 0 | 0 | ,974 | 1 | ,995 | ,001 | 1 | ,005 | 10,531 | |
| 25 | 0 | 0 | ,624 | 1 | ,979 | ,240 | 1 | ,021 | 7,902 | |
| 26 | 0 | 0 | ,179 | 1 | ,764 | 1,802 | 1 | ,236 | 4,155 | |
| 27 | 0 | 0 | ,520 | 1 | ,999 | ,414 | 1 | ,001 | 15,308 | |
| 28 | 0 | 0 | ,652 | 1 | ,999 | ,203 | 1 | ,001 | 13,832 | |
| 29 | 0 | 0 | ,775 | 1 | ,989 | ,082 | 1 | ,011 | 9,018 | |
| 30 | 0 | 0 | ,400 | 1 | 1,000 | ,707 | 1 | ,000 | 16,917 | |
| 31 | 0 | 1** | ,121 | 1 | ,650 | 2,402 | 0 | ,350 | 3,644 | |
| 32 | 0 | 0 | ,808 | 1 | ,998 | ,059 | 1 | ,002 | 12,352 | |
| 33 | 0 | 0 | ,896 | 1 | ,993 | ,017 | 1 | ,007 | 9,934 | |
| 34 | 0 | 0 | ,169 | 1 | 1,000 | 1,891 | 1 | ,000 | 21,792 | |
| 35 | 0 | 0 | ,568 | 1 | ,973 | ,325 | 1 | ,027 | 7,488 | |
| 36 | 0 | 0 | ,437 | 1 | 1,000 | ,605 | 1 | ,000 | 16,393 | |
| 37 | 0 | 0 | ,259 | 1 | 1,000 | 1,274 | 1 | ,000 | 19,446 | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 38 | 0 | 0 | ,098 | 1 | 1,000 | 2,730 | 1 | ,000 | 24,632 | |
| 39 | 0 | 0 | ,161 | 1 | ,729 | 1,964 | 1 | ,271 | 3,946 | |
| 40 | 0 | 0 | ,621 | 1 | ,999 | ,245 | 1 | ,001 | 14,163 | |
| 41 | 0 | 0 | ,043 | 1 | 1,000 | 4,077 | 1 | ,000 | 28,738 | |
| 42 | 0 | 1** | ,117 | 1 | ,633 | 2,463 | 0 | ,367 | 3,557 | |
| 43 | 0 | 0 | ,020 | 1 | 1,000 | 5,442 | 1 | ,000 | 32,571 | |
| 44 | 0 | 0 | ,460 | 1 | 1,000 | ,546 | 1 | ,000 | 16,074 | |
| 45 | 0 | 0 | ,659 | 1 | ,999 | ,195 | 1 | ,001 | 13,766 | |
| 46 | 0 | 0 | ,725 | 1 | ,986 | ,123 | 1 | ,014 | 8,653 | |
| 47 | 0 | 1** | ,193 | 1 | ,830 | 1,696 | 0 | ,170 | 4,861 | |
| 48 | 0 | 0 | ,634 | 1 | ,980 | ,226 | 1 | ,020 | 7,980 | |
| 49 | 0 | 0 | ,579 | 1 | ,974 | ,309 | 1 | ,026 | 7,564 | |
| 50 | 0 | 0 | ,929 | 1 | ,994 | ,008 | 1 | ,006 | 10,185 | |
| 51 | 0 | 1** | ,193 | 1 | ,830 | 1,696 | 0 | ,170 | 4,861 | |

For the original data, squared Mahalanobis distance is based on canonical functions.

For the cross-validated data, squared Mahalanobis distance is based on observations.

**. Misclassified case

b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

**Classification Results[a,c]**

| | | blood | Predicted Group Membership | | Total |
|---|---|---|---|---|---|
| | | | Non-blood | Blood | |
| Original | Count | Non-blood | 26 | 4 | 30 |
| | | Blood | 0 | 21 | 21 |
| | % | Non-blood | 86,7 | 13,3 | 100,0 |
| | | Blood | ,0 | 100,0 | 100,0 |
| Cross-validated[b] | Count | Non-blood | 26 | 4 | 30 |
| | | Blood | 0 | 21 | 21 |
| | % | Non-blood | 86,7 | 13,3 | 100,0 |
| | | Blood | ,0 | 100,0 | 100,0 |

a. 92,2% of original grouped cases correctly classified.
b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.
c. 92,2% of cross-validated grouped cases correctly classified.

**Discriminant**

**Analysis Case Processing Summary**

| Unweighted Cases | | N | Percent |
|---|---|---|---|
| Valid | | 51 | 100,0 |
| Excluded | Missing or out-of-range group codes | 0 | ,0 |
| | At least one missing discriminating variable | 0 | ,0 |
| | Both missing or out-of-range group codes and at least one missing discriminating variable | 0 | ,0 |
| | Total | 0 | ,0 |
| Total | | 51 | 100,0 |

**Group Statistics**

| Blood | | Mean | Std. Deviation | Valid N (listwise) | |
|---|---|---|---|---|---|
| | | | | Unweighted | Weighted |
| Non-blood | miR-214 | 1,5413 | 4,07500 | 30 | 30,000 |
| | miR-203 | -4,3087 | 3,08008 | 30 | 30,000 |
| | miR-205 | -5,1037 | 3,43534 | 30 | 30,000 |
| Blood | miR-214 | 4,1767 | 5,29485 | 21 | 21,000 |
| | miR-203 | 3,4710 | 8,56780 | 21 | 21,000 |
| | miR-205 | 2,6300 | 8,76146 | 21 | 21,000 |
| Total | miR-214 | 2,6265 | 4,74987 | 51 | 51,000 |
| | miR-203 | -1,1053 | 7,05818 | 51 | 51,000 |
| | miR-205 | -1,9192 | 7,23371 | 51 | 51,000 |

**Tests of Equality of Group Means**

| | Wilks' Lambda | F | df1 | df2 | Sig. |
|---|---|---|---|---|---|
| miR-214 | ,924 | 4,033 | 1 | 49 | ,050 |
| miR-203 | ,700 | 21,015 | 1 | 49 | ,000 |
| miR-205 | ,718 | 19,282 | 1 | 49 | ,000 |

**Analysis 1**

**Summary of Canonical Discriminant Functions**

**Eigenvalues**

| Function | Eigenvalue | % of Variance | Cumulative % | Canonical Correlation |
|---|---|---|---|---|
| 1 | ,599[a] | 100,0 | 100,0 | ,612 |

a. First 1 canonical discriminant functions were used in the analysis.

**Wilks' Lambda**

| Test of Function(s) | Wilks' Lambda | Chi-square | df | Sig. |
|---|---|---|---|---|
| 1 | ,625 | 22,062 | 4 | ,000 |

**Standardized Canonical Discriminant Function Coefficients**

| | Function |
|---|---|
| | 1 |
| miR-214 | -,725 |
| miR-203 | 1,501 |
| miR-205 | -,346 |

**Structure Matrix**

| | Function |
|---|---|
| | 1 |
| miR-203 | ,846 |
| miR-205 | ,811 |
| miR-214 | ,371 |

Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions
Variables ordered by absolute size of correlation within function.

**Classification Statistics**

**Classification Processing Summary**

| | | |
|---|---|---|
| Processed | | 51 |
| Excluded | Missing or out-of-range group codes | 0 |
| | At least one missing discriminating variable | 0 |
| Used in Output | | 51 |

**Prior Probabilities for Groups**

| Blood | Prior | Cases Used in Analysis | |
|---|---|---|---|
| | | Unweighted | Weighted |
| Non-blood | ,500 | 30 | 30,000 |
| Blood | ,500 | 21 | 21,000 |
| Total | 1,000 | 51 | 51,000 |

**Classification Function Coefficients**

| | blood | |
|---|---|---|
| | Non-blood | Blood |
| miR-214 | ,403 | ,161 |
| miR-203 | ,550 | ,938 |
| miR-205 | -1,013 | -1,099 |
| (Constant) | -2,983 | -3,106 |

Fisher's linear discriminant functions

## Casewise Statistics

| | Case Number | Actual Group | Highest Group | | | | | Second Highest Group | | | Discriminant Scores |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Predicted Group | P(D>d \| G=g) | | P(G=g \| D=d) | Squared Mahalanobis Distance to Centroid | Group | P(G=g \| D=d) | Squared Mahalanobis Distance to Centroid | Function 1 |
| | | | | P | df | | | | | | |
| Original | 1 | 1 | 1 | ,209 | 1 | ,958 | 1,578 | 0 | ,042 | 7,827 | 2,163 |
| | 2 | 1 | 1 | ,328 | 1 | ,937 | ,956 | 0 | ,063 | 6,346 | 1,884 |
| | 3 | 1 | 1 | ,190 | 1 | ,961 | 1,718 | 0 | ,039 | 8,135 | 2,217 |
| | 4 | 1 | 1 | ,567 | 1 | ,888 | ,328 | 0 | ,112 | 4,470 | 1,480 |
| | 5 | 1 | 1 | ,663 | 1 | ,865 | ,190 | 0 | ,135 | 3,908 | 1,342 |
| | 6 | 1 | 1 | ,190 | 1 | ,961 | 1,716 | 0 | ,039 | 8,131 | 2,217 |
| | 7 | 1 | 1 | ,130 | 1 | ,971 | 2,298 | 0 | ,029 | 9,347 | 2,423 |
| | 8 | 1 | 1 | ,374 | 1 | ,928 | ,792 | 0 | ,072 | 5,911 | 1,796 |
| | 9 | 1 | 1 | ,625 | 1 | ,874 | ,239 | 0 | ,126 | 4,121 | 1,395 |
| | 10 | 1 | 1 | ,030 | 1 | ,989 | 4,715 | 0 | ,011 | 13,785 | 3,078 |
| | 11 | 1 | 1 | ,811 | 1 | ,694 | ,057 | 0 | ,306 | 1,698 | ,668 |
| | 12 | 1 | 0** | ,378 | 1 | ,927 | ,778 | 1 | ,073 | 5,874 | -1,517 |
| | 13 | 1 | 1 | ,680 | 1 | ,635 | ,170 | 0 | ,365 | 1,276 | ,495 |
| | 14 | 1 | 1 | ,889 | 1 | ,803 | ,020 | 0 | ,197 | 2,826 | 1,046 |
| | 15 | 1 | 0** | ,445 | 1 | ,502 | ,584 | 1 | ,498 | ,604 | ,130 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 16 | 1 | 0** | ,836 | 1 | ,819 | ,043 | 1 | ,181 | 3,055 | -,841 |
| 17 | 1 | 0** | ,295 | 1 | ,943 | 1,096 | 1 | ,057 | 6,700 | -1,682 |
| 18 | 1 | 1 | ,446 | 1 | ,504 | ,580 | 0 | ,496 | ,608 | ,145 |
| 19 | 1 | 0** | ,871 | 1 | ,719 | ,026 | 1 | ,281 | 1,903 | -,473 |
| 20 | 1 | 0** | ,690 | 1 | ,639 | ,159 | 1 | ,361 | 1,305 | -,235 |
| 21 | 1 | 1 | ,687 | 1 | ,859 | ,162 | 0 | ,141 | 3,781 | 1,310 |
| 22 | 0 | 0 | ,658 | 1 | ,867 | ,196 | 1 | ,133 | 3,938 | -1,078 |
| 23 | 0 | 0 | ,506 | 1 | ,901 | ,442 | 1 | ,099 | 4,869 | -1,300 |
| 24 | 0 | 0 | ,746 | 1 | ,844 | ,105 | 1 | ,156 | 3,481 | -,959 |
| 25 | 0 | 0 | ,965 | 1 | ,778 | ,002 | 1 | ,222 | 2,515 | -,679 |
| 26 | 0 | 0 | ,733 | 1 | ,847 | ,116 | 1 | ,153 | 3,545 | -,976 |
| 27 | 0 | 0 | ,585 | 1 | ,586 | ,298 | 1 | ,414 | ,992 | -,089 |
| 28 | 0 | 0 | ,714 | 1 | ,852 | ,134 | 1 | ,148 | 3,638 | -1,001 |
| 29 | 0 | 0 | ,471 | 1 | ,909 | ,520 | 1 | ,091 | 5,121 | -1,356 |
| 30 | 0 | 0 | ,763 | 1 | ,839 | ,091 | 1 | ,161 | 3,395 | -,936 |
| 31 | 0 | 0 | ,324 | 1 | ,938 | ,975 | 1 | ,062 | 6,395 | -1,622 |
| 32 | 0 | 0 | ,693 | 1 | ,641 | ,156 | 1 | ,359 | 1,315 | -,240 |
| 33 | 0 | 1** | ,522 | 1 | ,550 | ,410 | 0 | ,450 | ,813 | ,267 |
| 34 | 0 | 1** | ,645 | 1 | ,617 | ,213 | 0 | ,383 | 1,167 | ,446 |
| 35 | 0 | 0 | ,718 | 1 | ,851 | ,130 | 1 | ,149 | 3,619 | -,996 |
| 36 | 0 | 0 | ,486 | 1 | ,906 | ,486 | 1 | ,094 | 5,010 | -1,332 |
| 37 | 0 | 0 | ,990 | 1 | ,763 | ,000 | 1 | ,237 | 2,336 | -,622 |
| 38 | 0 | 0 | ,576 | 1 | ,886 | ,313 | 1 | ,114 | 4,413 | -1,194 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 39 | 0 | 0 | ,283 | 1 | ,945 | 1,154 | 1 | ,055 | 6,841 | -1,709 |
| | 40 | 0 | 0 | ,610 | 1 | ,878 | ,260 | 1 | ,122 | 4,210 | -1,145 |
| | 41 | 0 | 0 | ,551 | 1 | ,892 | ,355 | 1 | ,108 | 4,569 | -1,231 |
| | 42 | 0 | 0 | ,833 | 1 | ,703 | ,044 | 1 | ,297 | 1,771 | -,424 |
| | 43 | 0 | 0 | ,472 | 1 | ,520 | ,517 | 1 | ,480 | ,677 | ,084 |
| | 44 | 0 | 0 | ,674 | 1 | ,632 | ,177 | 1 | ,368 | 1,255 | -,214 |
| | 45 | 0 | 0 | ,932 | 1 | ,789 | ,007 | 1 | ,211 | 2,646 | -,720 |
| | 46 | 0 | 1** | ,482 | 1 | ,526 | ,495 | 0 | ,474 | ,702 | ,203 |
| | 47 | 0 | 1** | ,511 | 1 | ,544 | ,431 | 0 | ,456 | ,783 | ,250 |
| | 48 | 0 | 1** | ,938 | 1 | ,744 | ,006 | 0 | ,256 | 2,144 | ,830 |
| | 49 | 0 | 0 | ,497 | 1 | ,903 | ,461 | 1 | ,097 | 4,929 | -1,313 |
| | 50 | 0 | 0 | ,950 | 1 | ,749 | ,004 | 1 | ,251 | 2,188 | -,573 |
| | 51 | 0 | 1** | ,748 | 1 | ,667 | ,103 | 0 | ,333 | 1,489 | ,586 |
| Cross-validated[b] | 1 | 1 | 1 | ,231 | 4 | ,955 | 5,599 | 0 | ,045 | 11,709 | |
| | 2 | 1 | 1 | ,517 | 4 | ,931 | 3,249 | 0 | ,069 | 8,468 | |
| | 3 | 1 | 1 | ,390 | 4 | ,959 | 4,117 | 0 | ,041 | 10,436 | |
| | 4 | 1 | 1 | ,158 | 4 | ,863 | 6,607 | 0 | ,137 | 10,296 | |
| | 5 | 1 | 1 | ,931 | 4 | ,858 | ,853 | 0 | ,142 | 4,448 | |
| | 6 | 1 | 1 | ,089 | 4 | ,958 | 8,082 | 0 | ,042 | 14,348 | |
| | 7 | 1 | 1 | ,199 | 4 | ,971 | 6,000 | 0 | ,029 | 13,021 | |
| | 8 | 1 | 1 | ,475 | 4 | ,921 | 3,520 | 0 | ,079 | 8,437 | |
| | 9 | 1 | 1 | ,019 | 4 | ,822 | 11,825 | 0 | ,178 | 14,890 | |
| | 10 | 1 | 1 | ,001 | 4 | ,993 | 17,745 | 0 | ,007 | 27,607 | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 11 | 1 | 1 | ,754 | 4 | ,669 | 1,902 | 0 | ,331 | 3,308 | |
| 12 | 1 | 0** | ,787 | 4 | ,965 | 1,721 | 1 | ,035 | 8,363 | |
| 13 | 1 | 1 | ,618 | 4 | ,596 | 2,650 | 0 | ,404 | 3,430 | |
| 14 | 1 | 1 | ,625 | 4 | ,780 | 2,608 | 0 | ,220 | 5,138 | |
| 15 | 1 | 0** | ,868 | 4 | ,524 | 1,258 | 1 | ,476 | 1,449 | |
| 16 | 1 | 0** | ,951 | 4 | ,861 | ,700 | 1 | ,139 | 4,344 | |
| 17 | 1 | 0** | ,766 | 4 | ,976 | 1,835 | 1 | ,024 | 9,215 | |
| 18 | 1 | 0** | ,267 | 4 | ,595 | 5,209 | 1 | ,405 | 5,977 | |
| 19 | 1 | 0** | ,348 | 4 | ,821 | 4,455 | 1 | ,179 | 7,502 | |
| 20 | 1 | 0** | ,490 | 4 | ,722 | 3,421 | 1 | ,278 | 5,332 | |
| 21 | 1 | 1 | ,586 | 4 | ,843 | 2,833 | 0 | ,157 | 6,192 | |
| 22 | 0 | 0 | ,894 | 4 | ,860 | 1,102 | 1 | ,140 | 4,734 | |
| 23 | 0 | 0 | ,905 | 4 | ,897 | 1,031 | 1 | ,103 | 5,364 | |
| 24 | 0 | 0 | ,820 | 4 | ,835 | 1,535 | 1 | ,165 | 4,777 | |
| 25 | 0 | 0 | ,992 | 4 | ,773 | ,265 | 1 | ,227 | 2,710 | |
| 26 | 0 | 0 | ,910 | 4 | ,840 | ,997 | 1 | ,160 | 4,316 | |
| 27 | 0 | 0 | ,691 | 4 | ,559 | 2,244 | 1 | ,441 | 2,718 | |
| 28 | 0 | 0 | ,661 | 4 | ,841 | 2,409 | 1 | ,159 | 5,745 | |
| 29 | 0 | 0 | ,923 | 4 | ,905 | ,913 | 1 | ,095 | 5,425 | |
| 30 | 0 | 0 | ,936 | 4 | ,832 | ,818 | 1 | ,168 | 4,021 | |
| 31 | 0 | 0 | ,618 | 4 | ,935 | 2,650 | 1 | ,065 | 7,995 | |
| 32 | 0 | 0 | ,338 | 4 | ,591 | 4,540 | 1 | ,409 | 5,276 | |
| 33 | 0 | 1** | ,030 | 4 | ,717 | 10,742 | 0 | ,283 | 12,604 | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 34 | 0 | 1** | ,035 | 4 | ,782 | 10,337 | 0 | ,218 | 12,891 | |
| 35 | 0 | 0 | ,268 | 4 | ,832 | 5,198 | 1 | ,168 | 8,397 | |
| 36 | 0 | 0 | ,380 | 4 | ,898 | 4,197 | 1 | ,102 | 8,558 | |
| 37 | 0 | 0 | ,432 | 4 | ,735 | 3,810 | 1 | ,265 | 5,846 | |
| 38 | 0 | 0 | ,454 | 4 | ,876 | 3,662 | 1 | ,124 | 7,580 | |
| 39 | 0 | 0 | ,109 | 4 | ,944 | 7,566 | 1 | ,056 | 13,201 | |
| 40 | 0 | 0 | ,723 | 4 | ,870 | 2,068 | 1 | ,130 | 5,879 | |
| 41 | 0 | 0 | ,584 | 4 | ,884 | 2,846 | 1 | ,116 | 6,908 | |
| 42 | 0 | 0 | ,085 | 4 | ,629 | 8,178 | 1 | ,371 | 9,231 | |
| 43 | 0 | 1** | ,730 | 4 | ,505 | 2,032 | 0 | ,495 | 2,075 | |
| 44 | 0 | 0 | ,416 | 4 | ,588 | 3,925 | 1 | ,412 | 4,633 | |
| 45 | 0 | 0 | ,931 | 4 | ,780 | ,856 | 1 | ,220 | 3,389 | |
| 46 | 0 | 1** | ,658 | 4 | ,563 | 2,424 | 0 | ,437 | 2,932 | |
| 47 | 0 | 1** | ,336 | 4 | ,618 | 4,555 | 0 | ,382 | 5,515 | |
| 48 | 0 | 1** | ,748 | 4 | ,794 | 1,935 | 0 | ,206 | 4,631 | |
| 49 | 0 | 0 | ,380 | 4 | ,896 | 4,198 | 1 | ,104 | 8,498 | |
| 50 | 0 | 0 | ,734 | 4 | ,731 | 2,012 | 1 | ,269 | 4,013 | |
| 51 | 0 | 1** | ,082 | 4 | ,804 | 8,282 | 0 | ,196 | 11,102 | |

For the original data, squared Mahalanobis distance is based on canonical functions.

For the cross-validated data, squared Mahalanobis distance is based on observations.

**. Misclassified case

b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

**Classification Results[a ,c]**

| | | blood | Predicted Group Membership | | Total |
|---|---|---|---|---|---|
| | | | Non-blood | Blood | |
| Original | Count | Non-blood | 24 | 6 | 30 |
| | | Blood | 6 | 15 | 21 |
| | % | Non-blood | 80,0 | 20,0 | 100,0 |
| | | Blood | 28,6 | 71,4 | 100,0 |
| Cross-validated[b] | Count | Non-blood | 23 | 7 | 30 |
| | | Blood | 7 | 14 | 21 |
| | % | Non-blood | 76,7 | 23,3 | 100,0 |
| | | Blood | 33,3 | 66,7 | 100,0 |

a. 76,5% of original grouped cases correctly classified.
b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.
c. 72,5% of cross-validated grouped cases correctly classified.

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