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EXTRACELLULAR MICRORNAS IN FORENSIC SCIENCES: potential biomarkers for body fluid identification

Dissertação de Candidatura ao grau de Mestre em Medicina Legal submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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Informação Técnica

TÍTULO:

Extracellular microRNAs in Forensic Sciences: potential biomarkers for body fluid identification

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Aos meus pais…

"*One sometimes finds what one is not looking for*." – Alexander Fleming

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Abbreviations

A

AGO	Argonaute
ALS	Alternative Light Source
ATP	Adenosine triphosphate

C

cDNA	Complementary deoxyribonucleic acid
C. elegans	*Caenorhabditis elegans*
Ct	Cycle threshold

D

DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid

F

FSH	Follicle stimulating hormone

H

HCC	Hepatocellular carcinoma
HDL	High-density lipoprotein

L

LH	Luteinizing hormone

M

MetS	Metabolic Syndrome
miR	microRNA
miRNA	microRNA
mL	Milliliter
mRNA	Messenger ribonucleic acid

N

ncRNAs	Non-coding RNAs

P

PACT	Protein activator of PKR
PCR	Polymerase chain reaction
pre-miRNA	miRNA precursor
pri-miRNA	Primitive miRNA

Q

qPCR	Real-time PCR

R

RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNase	Ribonuclease
RNU	Small-nucleolar RNA
rpm	Revolutions per minute

T

TRBP	Transactivation-responsive RNA-binding protein

U

UQCRFS1	Ubiquinol-cytochrome *c* reductase
UTR	Untranslated region
UV	Ultraviolet
μL	Microliter

V

VEGF	Vascular endothelial growth factor

X

XPO5	Exportin-5

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Abstract

Abstract

Introduction: One of the challenges that the forensic community faces is how to find a reliable biomarker and turning it into an accurate tool for a rapid identification of body fluids. Driven by their important forensic applications, body fluid identification methods have been extensively developed in recent years. Two of the most common types of body fluids found in forensic scenes are blood and urine; however, most of the current techniques used to identify these fluids aren't very accurate and have a low sensitivity that often lead to false positive results. Consequently, finding an accurate identification tool for these fluids would be particularly useful. Recently, it was found that the intrinsically short fragment and tissue-specific expression of microRNAs (miRNAs) make them ideal biomarkers for forensic body fluid identification. MiRNAs are small endogenous, 19 to 24 nucleotides in length, single-stranded, non-protein coding regulatory RNA molecules that modulate cellular mRNA and protein levels by inhibiting protein translation or destabilizing target transcripts. To date, no miRNA was detected as a biomarker for blood or urine identification. Therefore, the main objective of the present study is to identify a specific miRNA profile useful for plasma and urine sample's identification.

Material and Methods: Following a review of the literature, we selected the miR-801, miR-369-3p and miR-323b-5p to be evaluated as forensic biomarkers. The miRNA expression profiling study was conducted in plasma and urine samples from twenty-two healthy individuals (age 46.6 ± 17.78 years) using relative quantification by real-time PCR.

Results: All the miRNAs tested were detected in both fluids, with different detection frequencies. We observed that miR-369-3p presented higher expression levels in plasma when compared to urine samples (fold-change: 37.52; $P< 0.001$). However, there were no statistically significant differences in the expression levels of miR-323b-5p and miR-801 between plasma and urine samples. No significant statistical difference was detected, for either fluid, in the levels of the miR-801 and miR-323b-5p according to gender or age. MiR-369-3p also exhibited no significant statistical difference in expression levels between the male and female samples; however, it was the only miRNA with a significant statistical difference between the two age groups tested, as the older group (≥ 48 years) presented a higher miR-369-3p relative expression than the younger individuals (Urine: P=0.018; Plasma: P=0.037).

Discussion: The up-regulation of miR-369-3p observed in older individuals (in plasma and urine) could influence UQCRFS1 regulation and consequently, influence the aging process. Nonetheless, since miR-369-3p was only detected in two urine samples from male individuals versus fourteen urine samples from women, it could indicate that this miRNA may play a more relevant role in women; thus, we also suggest that the hormonal changes caused by menopause could explain the statistical difference between the two age groups in urine samples. Moreover, this was the first time that miR-801 was detected in plasma samples, and miR-323b-5p in urine samples; hence, we suggest further studies with a larger number of samples to replicate these results.

Conclusion: We can hypothesize that miR-369-3p could be a potential molecular biomarker, allowing the distinction of two of the most frequently found body fluids in forensic scenarios. Nevertheless, we suggest more studies to be carried out, with a higher number of samples, in order to replicate these results. This miRNA also shows potential as an age biomarker; although, we propose future studies to be performed, using individuals with larger age ranges and gender heterogeneity to test this hypothesis. MiRNA detection in body fluids is an emerging field in the world of biomarkers that could represent a novel approach to the identification of forensically relevant biological stains.

Sinopse

Sinopse

Introdução: Um dos maiores desafios que a comunidade forense enfrenta é encontrar um biomarcador fiável e transformá-lo numa ferramenta útil na identificação rápida de fluidos corporais. Devido à importância das suas aplicações forenses, os métodos de identificação de fluidos corporais têm sido bastante desenvolvidos nos últimos anos; porém, a maioria dos métodos de identificação usados atualmente apresentam uma baixa especificidade e sensibilidade, o que pode conduzir a resultados falsos-positivos. Um dos tipos de fluidos biológicos mais frequentemente encontrados em cenas de crime, em especial em cenas de crimes violentos, é o sangue. A maioria das técnicas atuais para a identificação de sangue recorrem a biomarcadores de proteínas que podem ter modificações ao nível da pós-tradução, afetando assim a precisão da identificação. A urina, frequentemente encontrada em casos de agressão sexual, assédio e má conduta, é um fluido biológico bastante difícil de detetar devido à baixa sensibilidade dos testes disponíveis, que podem levar a resultados falsos positivos. Deste modo, existe a necessidade de se encontrar um teste de confirmação universal, que possa ser aplicado a uma mancha ou fluido desconhecido, sendo capaz de identificar corretamente a presença de sangue ou urina. Recentemente, verificou-se que o curto tamanho e expressão específica nos tecidos dos microRNAs (miRNAs) torna-os biomarcadores ideais para a identificação de fluidos corporais com interesse forense. Os miRNAs pertencem a uma pequena classe de moléculas de RNA não codificante, com 19 a 24 nucleótidos de tamanho, possuem cadeia simples e regulam a expressão génica por inibição da tradução ou destabilizando transcritos alvo, geralmente na região não traduzida 3' (UTR), através de complementaridade parcial da sequência. Conhecer os padrões de expressão dos miRNAs em diferentes fluidos pode representar um novo método para a identificação de fluidos biológicos com relevância médico-legal e, tem ainda o potencial para ser particularmente útil na análise de amostras comprometidas ou degradadas, que são frequentemente encontradas em cenários forenses. Até à data, não foi detetado nenhum miRNA como biomarcador para a identificação de plasma e de urina, o que revela uma lacuna que a análise forense de perfis de miRNAs precisa de colmatar. Como tal, o principal objetivo do presente estudo é identificar um perfil de miRNAs útil para a identificação de amostras biológicas de plasma e urina.

Material & Métodos: Uma revisão sistemática da literatura revelou três miRNAs que mostram o potencial para serem biomarcadores forenses: o miR-801 e o miR-369-3p (para análise em amostras de plasma) e o miR-323b-5p (para análise em amostras de urina). O presente foi realizado a partir de amostras de plasma e urina provenientes de vinte e dois indivíduos adultos saudáveis da população portuguesa (46,6 ± 17,78 anos). Para quantificar a expressão de miRNAs, reações de PCR em tempo real foram realizadas. O tratamento estatístico dos resultados foi realizado usando o programa IBM®SPSS®Statistics (versão 22.0).

Resultados: Todos os miRNAs testados foram detetados em ambos os fluidos, apresentando diferentes frequências de deteção. Observou-se que miR-369-3p apresentou maiores níveis de expressão no plasma quando comparado com amostras de urina (*Fold-change*= 37,52; P< 0,001). Não se verificaram diferenças estatisticamente significativas nos níveis de expressão do miR-323b-5p e do miR-801 entre as amostras de plasma e urina. Nenhuma diferença estatisticamente significativa foi detetada, em ambos os fluidos, quanto aos níveis do miR-801 e miR-323b-5p em relação à idade ou ao género. O mir-369-3p também não apresentou nenhuma diferença estatisticamente significativa entre as amostras masculinas e femininas; no entanto, foi o único miRNA a apresentar uma diferença estatisticamente significativa entre os dois grupos etários testados, uma vez que o grupo mais velho (≥ 48 anos) apresentou uma maior expressão relativa de miR-369-3p em relação aos indivíduos mais jovens (Urina: P=0,018; Plasma: P= 0,037).

Discussão: Os níveis elevados de miR-369-3p observados nos indivíduos mais velhos (no plasma e na urina) podem influenciar a regulação do gene UQCRFS1 e, consequentemente influenciar o processo de envelhecimento. No entanto, o miR-369-3p foi apenas detetado em duas amostras de urina de indivíduos do género masculino *versus* catorze amostras de urina de mulheres, o que poderá indicar que este miRNA desempenhará um papel mais relevante em mulheres. Deste modo, sugere-se que as alterações hormonais causadas pela menopausa poderão também explicar a diferença estatística entre as duas faixas etárias, nas amostras de urina. O presente estudo foi o primeiro a detetar o miR-801 em amostras de plasma e o miR-323b-5p em amostras de urina, pelo que se sugere a realização de novos estudos, com um número maior de amostras biológicas, para validar os presentes resultados.

Conclusão: Coloca-se a hipótese que o miR-369-3p poderá ser um potencial biomarcador molecular, permitindo a distinção de dois dos fluidos biológicos mais frequentemente encontrados em cenários forenses. No entanto, propõe-se ainda que este estudo seja replicado com uma amostragem maior. Este miRNA também demonstra potencial como um biomarcador de idade. Porém, propomos que estudos futuros sejam executados, com uma maior heterogeneidade a nível de género e de idades para testar esta hipótese. A deteção de miRNAs em fluidos biológicos é um campo emergente no mundo dos biomarcadores e pode representar uma nova abordagem para a identificação de manchas biológicas com interesse forense.

Introduction

1. Introduction

1.1. When DNA is not enough

One of the challenges that the forensic community faces is how to find a reliable biomarker and turning it into an accurate tool for rapid identification of body fluids [1]. Driven by their important forensic applications, body fluid identification methods have been extensively developed in recent years [2].

For the past 30 years, advances in forensic deoxyribonucleic acid (DNA) technology have revolutionized the criminal justice system worldwide [3]. The demonstrated scientific accuracy of DNA testing for forensic purposes quickly led jurisdictions to accept expert testimony regarding DNA matches between suspects and crime scene evidence, in the late 1980s [3]. Wielding the power to exonerate the innocent and apprehend the guilty, the use of DNA genotyping technology has become an indispensable resource for prosecutors and law enforcement officials, as well as for defense lawyers representing those falsely accused, or wrongfully convicted of crimes they did not commit [3].

DNA profiling allows for the identification of individuals through their respective DNA signatures; nevertheless, it does not identify the type or source of the evidence [4]. What if there isn't enough genetic material to obtain a complete DNA profile? Or what if the genetic material is degraded? Based on the theory that each type of body tissue has a distinctive ribonucleic acid (RNA) signature, messenger RNA (mRNA) profiling has appeared as an advantageous procedure to identify relevant human body fluids [4]. However, it is well know that despite the achievement of mRNA profiling, its susceptibility to degradation by physical or chemical factors has always been problematic [4]. Recently, it was found that the intrinsically short fragment and tissue-specific expression of microRNAs (miRNAs) make them ideal biomarkers for forensic body fluid identification [1, 5].

In a breakthrough investigation Victor Ambros and his colleagues, Rosalind Lee and Rhonda Feinbaum, discovered that lin-4, a gene known to control the timing of *Caenorhabditis elegans* (*C. elegans*) larval development, does not code for a protein but produces a pair of small RNAs [6, 7].

Since then, miRNAs have been shown to intervene in the regulation of several key cellular functions and have been implicated in many diseases [8]. Within the past few years, extracellular circulating miRNAs have been detected in a variety of biologic fluids

[4, 5, 8-13]. These miRNAs have many of the properties of an ideal biomarker, including stability in nuclease-rich body fluids, unique sequences, and tissue-specific expression [8].

MiRNA detection in body fluids is an emerging field in the world of biomarkers, and since more than 1800 transcribed miRNAs have been identified in the human genome, miRNAs could very well represent a novel approach to the identification of forensic biological stains, which may prove to be particularly useful in the analysis of compromised and degraded samples that are frequently encountered in forensic casework [10, 14, 15].

1.2. Body fluid stain identification in Forensic Sciences

Body fluid samples found during a forensic investigation are amongst the most important evidences recovered from crime scenes, providing forensic pathologists and researchers with key information that allows the identification of victims and suspects, as well as to exonerate an innocent individual. Moreover, they are also excellent indicators of the sequence of events and can even help to corroborate testimonial evidences [1, 2, 4, 16, 17].

Sometimes just knowing the origin of a fluid could be enough to influence the outcome of a case [18]. However, this is not always an easy task, as most body fluids' physical appearance and molecular properties (e.g. DNA, mRNA) alter rapidly with time and environmental conditions, and many body fluid stains are either invisible to the naked eye or similar in appearance to other fluids or substances [2, 4]. Even when the identity of a stain may seem obvious to a forensic investigator, an absolute and accurate confirmation is necessary in order for that evidence to be accepted in court [2]. This is especially important in cases of a possible occurrence of mixtures from different body fluids. On the other hand, it is also important to take into account that a stain can contain multiple body fluids from more than one donor [2].

For more than a century, numerous types of body fluid identification methods have been developed; nevertheless, the most conventional serology-based methods for body fluid identification are prone to various limitations. Nowadays, several platforms are used for the detection and identification of biological traces, giving particular emphasis to presumptive and confirmatory tests [2, 4, 16]. When a potential body fluid is discovered at a crime scene, multiple presumptive tests are initially used, carried out in one body fluid at a time, to give some indication as to the identity of the substance, and then further confirmatory tests are conducted to confirm the presence of the fluid or identify the source of origin [10, 16]. As a result, these methods have an high cost, not only in terms of the time and labor required for their completion, but also in terms of the amount of sample consumed during each test [10]. In general, presumptive and confirmatory tests also have a low specificity and sensitivity, and can lead to the destruction of the analyzed sample or to the instability of the biomolecules assessed [2, 16, 19].

Due to the many limitations these tests display, in recent years, there have been great advances in the search for an accurate method for the rapid identification of body fluids [2, 16, 19]. The use of a molecular genetics-based approach, using the detection of specific miRNA profiles in body fluids, has been proposed in order to supplant conventional body fluid identification methods, and has shown promising results. These results are extremely important for the field of forensic sciences, as they could allow for the identification of the source of the biological material, which might be crucial to the investigation and prosecution of a case [1, 2, 5, 16].

1.3. microRNAs

MicroRNAs are small endogenous, 19 to 24 nucleotides in length, single-stranded, non-protein coding regulatory RNA molecules that modulate cellular mRNA and protein levels by inhibiting protein translation or destabilizing target transcripts, usually at the 3′ untranslated region (UTR), through partial sequence complementation [4, 20-23]. These molecules play an important role in a wide range of physiologic and pathologic processes, having been implicated in different areas, such as immune response, neural development, DNA repair, apoptosis and oxidative stress response, among others [4, 22-25]. As the field of miRNA research keeps expanding, it has become quite clear that miRNAs regulate thousands of human genes, which translates in more than one third of the genome’s protein-coding regions [26, 27]. Furthermore, a great number of diseases have been associated with abnormal miRNA expression profiles: cancer, systemic lupus erythematosus, psoriasis, Chron's disease, diabetes, ulcerative colitis or Duchenne muscular dystrophy, to name a few [24].

Nowadays, miRNA-mediated regulation is acknowledged to represent a new instance of regulatory control over gene expression programs in organisms; nevertheless, for years, the central dogma of genetics stated that RNA played the role of messenger between the gene and the final proteins encoded by said gene, and non-coding RNAs (ncRNAs) were ignored in the field of genome sequencing [22]. Even before the discovery of miRNAs, it was known that a large part of the genome is not translated into proteins. This so called ‘junk’ DNA was thought to be evolutionary debris with no real function. Recently, the major advances in this research area have established miRNAs as powerful regulators of gene expression [24]. Genome-wide transcriptional analysis has estimated that most transcribed mammalian genomic sequences are ncRNAs [24]. The importance of miRNA function is further suggested by the extreme evolutionary conservation of both individual miRNA sequences and the miRNA processing machinery. In addition, the number of miRNAs in the genome appears to be correlated with the complexity of the developmental program, with mammals having the largest number of miRNAs [12, 24].

MiRNAs were first identified in *C. elegans* in the early 1990s and, ever since, have been reported in a wide variety of organisms: from single-cell algae to humans, suggesting that miRNA-mediated biological function is an ancient and critical cellular regulatory mechanism [6, 7, 24]. To date, over 1800 human miRNA sequences have been identified; however, genomic computational analysis indicates that more than 50,000 miRNAs may exist in the human genome and each may have multiple targets based on similar sequences in the 3'-UTR of mRNA [15, 20, 21, 24]. In fact, it has been estimated that more than 60% of mammalian mRNAs are targeted by at least one miRNA [24].

miRNAs are transcribed in much the same way as protein-coding genes. The majority of miRNAs are transcribed by RNA polymerase II, though a minor fraction of miRNAs, that lie within repetitive elements in the genome, are transcribed by RNA polymerase III [12]. These primary miRNA transcripts (pri-miRNAs) are often several hundred nucleotides long and are modified similarly to protein-coding transcripts by the addition of a 5' cap and a 3' poly-A tail [12, 24]. Pri-miRNAs are cleaved into a hairpin-shaped 70–100 nucleotide precursor (pre-miRNA) by Drosha (a nuclear ribonuclease - RNase - III), which functions in a complex that contains a double-stranded (ds) RNA-binding protein, DGCR8 (DiGeorge syndrome chromosomal region 8, known as Pasha in *C. elegans* and *Drosophila*). Drosha is stabilized through its interaction with DGCR8, which unlike Drosha, can directly and stably interact with pri-miRNAs. Pre-miRNAs are exported to the cytoplasm by Exportin-5 (XPO5), where Dicer (another RNase III) in association with a dsRNA-binding protein partner called TRBP (transactivation-responsive RNA-binding protein) and PACT (protein activator of PKR – in humans) processes pre-miRNAs into a 19–24 nucleotide miRNA duplex, by removing the terminal loop [20, 28]. Typically, one strand of this mature miRNA duplex, termed the guide strand, associates with the RNA-induced silencing complex (RISC). While it is generally believed that upon incorporation into the RISC complex the other strand (the passenger strand) is unwound from the guide strand and degraded, there is also evidence that, in some cases, both strands of the miRNA duplex are functional. MiRNA-RISC complexes interact with mRNA targets through partial sequence complementation, typically within the 3' untranslated region of target mRNAs. It is thought that the extent of base pairing between the miRNA and its mRNA target determines whether the mRNA is degraded or translationally repressed [12, 24]. The strand of the miRNA complex that remains stably bound to RISC represents the mature miRNA, and drives RISC to the target mRNAs (Figure 1) [20].

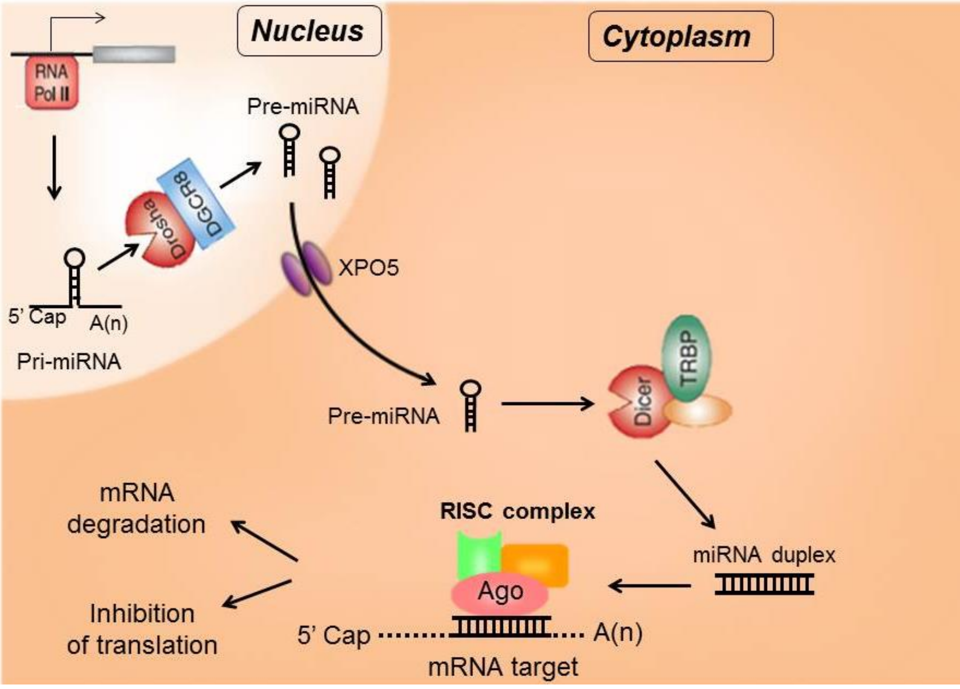

Figure 1 – The biogenesis of miRNAs. Primary miRNA (pri-miRNA) transcripts are transcribed by RNA polymerase II and processed by the microprocessor complex (DGCR8/Drosha) into precursor stem-loop miRNAs in the nucleus. Precursor miRNAs (pre-miRNAs) are exported into the cytoplasm by Exportin-5 (XPO5) and are cleaved by Dicer to produce mature miRNAs. Mature miRNAs recognize their respective target mRNAs and mediate post-transcriptional repression of their targets through translational repression, deadenylation or enhanced mRNA decay. (Adapted from Yates *et al.* [28]).

When compared to protein-based biomarkers, miRNAs offer several advantages: the complexity of miRNAs is low, the miRNAs are stable in various body fluids and, the expression of some miRNAs are restricted to specific tissues or biological stages [12, 21]. The levels of miRNAs can also be easily measured by various commonly used laboratory methods, for instance assorted signal amplification strategies [5, 12, 18, 21, 29].

While a vast majority of miRNAs are found intracellularly, a significant number of miRNAs have been observed outside of cells, including in various body fluids [12, 24]. These miRNAs are stable and show distinct expression profiles in different types of fluids [12, 24]. Given the instability of most RNA molecules in the extracellular environment, the presence and apparent stability of miRNAs is interesting. Urine and other body fluids are known to contain RNases, which suggests that secreted miRNAs are likely packaged in some manner, to protect them against RNase digestion [24]. The small size of mature miRNAs and their tight relationship with the RISC complex may lead to a lesser susceptibility to moisture, ultraviolet (UV) light, temperature, suboptimal environmental pH and other factors that often degrade mRNA beyond its usability, hindering the work of forensic investigators [4, 11, 22].

One question that often arises in the forensic field is whether miRNAs can be considered good biomarkers for body fluids. An ideal biomarker should fit a number of criteria depending on how the biomarker is to be used. It needs to be available to be analyzed through non-invasive methods; have a long half-life in samples; be unalterable by physical or chemical factors; it should be specific to a tissue; but most of all, it should be able to be analyzed by a fast, simple, accurate, reproducible and economical method [4, 12].

Following the discovery of extracellular miRNAs in plasma and serum, a series of studies have consistently revealed the presence of miRNAs in other body fluids (namely saliva, urine, breast milk, seminal plasma, tears, amniotic fluid, colostrum, bronchial lavage, cerebrospinal fluid, peritoneal fluid, and pleural fluid) [4, 8, 9]. However, there is no common opinion regarding the origin, biological function or specific expression patterns of extracellular miRNAs. Turchinovich and co-workers state this could be the consequence of extracellular miRNAs not being a homogeneous population and existing in differently packaged forms [8].

According to Silva and colleagues, the lack of homogeneity and the non-reproducibility of results from different groups trying to determine if miRNAs can be considered a good biomarker for body fluids, highlights the necessity for a standardization for miRNA biomarker assessment [4].

A study performed by Weber and co-workers showed that several miRNAs are common to different biological fluids; however, in their study, plasma and urine shared the fewest number of commonly detectable miRNA species [9]. The study suggests that a majority of the circulating miRNAs were either picked up by the kidneys or destroyed in the urine; nevertheless, the pathway taken by urine through the urinary system may also contribute to this difference [9]. To date, no miRNA was detected as a biomarker for urine or plasma identification, which are very important human fluids in forensic sciences [4, 9].

1.3.1. Extracellular microRNAs

To this date, the origin and function of circulating miRNAs is still not systematically elucidated [8, 9, 30, 31]. However, according Mlcochova and co-workers, the cell-to-cell communication hypothesis, based on the export of miRNAs, seems to be the most satisfying explanation for the existence of extracellular miRNAs [31]. This group also describes two potential secretory mechanisms that may justify the presence of miRNAs in urine. The first, a passive process influenced by stress factors, causing a rupture in the cell and leaking the miRNAs to the extracellular space. The second pathway is the active secretion of miRNAs through microvesicles. Generally, microvesicles include microparticles and exosomes [31]. Turchinovich and colleagues also state that extracellular miRNAs can be encapsulated in microvesicles [8]. According to this group, three types of membranous vesicles can contain extracellular miRNAs, namely apoptotic bodies, shedding vesicles and exosomes [8]. Furthermore, Turchinovich's group mentions that extracellular miRNAs can also be vesicle free and associated with either AGO (Argonaute) proteins or be incorporated into high-density lipoprotein (HDL) particles, although it is not clear whether the miRNAs are incorporated inside these particles or adsorbed on their surface (figure 2) [8].

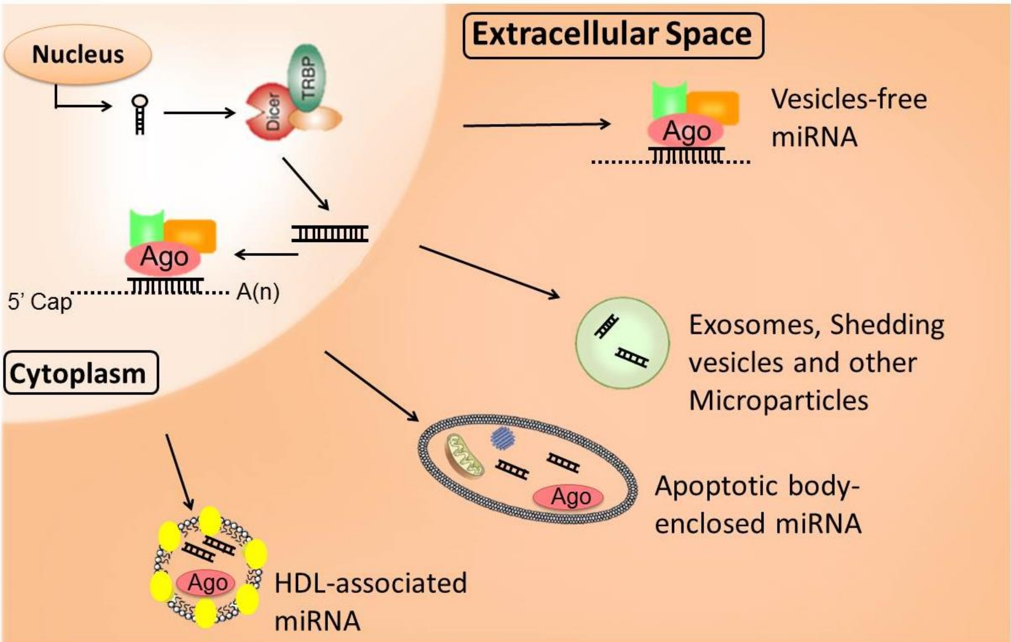

Figure 2 – Possible secretory mechanisms of extracellular miRNAs and modes of extracellular miRNA packaging. HDL: high-density lipoprotein particles. Ago: Argonaute protein. (Adapted from Turchinovich *et al.* and Mlcochova *et al.* [8, 31]).

Apoptotic bodies are particles that can contain various cellular organelles (including mitochondria and nuclei), ranging between 1 and 4 µm, and remain after programmed cell death, being byproducts of apoptosis [8]. It should be noted that some authors do not acknowledge miRNAs entrapped in apoptotic bodies as circulating miRNAs, as the size of these particles is comparable to that of cell debris or blood platelets and, most protocols commonly used in extracellular miRNA research involve the removal of both cell debris and apoptotic bodies [8, 32].

Shedding vesicles are heterogeneous in size (between 0.1 and 1 µm), and are formed by outward budding and fission of the plasma membrane, thus being limited by a lipid bilayer [8, 30]. They belong to the same class of microvesicles as exosomes, although they differ in size, content and mechanism of generation [8].

Exosomes are membrane-bound nanovesicles of endosomal origin, released by cells upon fusion of multivesicular bodies with the plasma membrane [33-35]. Exosomes are present in a wide number of biological fluids, including blood and urine, ranging between 30 and 100 nm in diameter and have a limiting lipid bilayer, transmembrane proteins and a hydrophilic core containing proteins, DNA, mRNAs and miRNAs [33-37]. Exosomes can act as extracellular vehicles by which cells communicate through the delivery of their functional cargo to recipient cells, with many important biological, physiological and pathological implications [34-36].

1.3.2. Blood and urinary microenvironment: microRNA content

One of the most common types of body fluids found in crime scenes, particularly those of a violent crime, is blood [2].

Blood can be considered a complex liquid tissue comprising cells and extracellular fluid and provides the major link between cells and tissues in an organism [38]. Blood samples are relatively easy to obtain, technically and psychologically easy to process, and are mostly considered homogeneous [39].

Most of the current blood biomarkers, besides not being specific for the identification this fluid, are based on the levels of specific proteins in the blood, such as troponin associated with cardiovascular conditions, carcinoembryonic antigen for various cancers, prostate specific antigen for prostate cancer, and aminotransferases (alanine aminotranferase and aspartate aminotransferase) for liver function [12]. According to Wang and co-workers, these biomarkers are also inadequate in specificity and sensitivity for definitive disease diagnosis [21]. Furthermore, protein-based biomarkers may have different post-translational modifications, which can affect the accuracy of measurement; therefore, there is a need for the discovery of new blood biomarkers [12].

MiRNA species are relatively homogenous and several reports state that miRNAs have a stable expression in plasma [5, 9, 10, 12, 40-42].

If a blood sample, removed from the circulatory system via venipuncture, is withdrawn in the presence of an anticoagulant and centrifuged to remove cellular elements, a plasma sample is obtained. [25, 39].

In a study by Wang and co-workers, using serum and corresponding plasma samples from the same individuals, it was observed higher miRNA concentrations in serum samples compared to the corresponding plasma samples [21]. The difference between serum and plasma miRNA concentration showed some associations with miRNA from platelets, which may indicate that the coagulation process may affect the spectrum of extracellular miRNAs in blood [21]. The group suggests that there are a number of factors that might affect the measurement of circulating miRNA concentration and that caution must be taken when comparing miRNA data generated from different sample types or measurement platforms [21]. In Wang's study, as the serum and plasma were collected at the same time from the same individuals, the higher miRNA concentration in serum suggests that additional miRNA was released from cells during the coagulation process. Cell lysis, especially from the highly abundant red blood cells, during the coagulation process is one of the plausible explanations for this concentration difference between serum and plasma. Wang and colleagues also added that the miRNA concentration difference between serum and plasma showed some associations with the miRNA spectrum in platelets and white blood cells, instead of the most abundant cell population, red blood cells, in the blood [21]. The miRNA concentration difference between serum and plasma is consistent with the idea of miRNA 'trafficking" between cellular compartments and the extracellular environment [21, 43]. It is known that during the coagulation process, blood cells are exposed to a "stressful environment" which may "stimulate" the release of certain miRNAs and other RNAs [21, 41]. Wang's miRNA study yielded a similar conclusion and suggests that plasma may be the sample of choice in studying circulating miRNAs, since miRNAs released during the coagulation process may change the true repertoire of circulating miRNAs [21].

When compared with mRNA and other cellular RNAs, miRNAs have shown a high degree of stability, in urine, for reasons that are yet unknown [44]. The harsh urinary environment, particularly the urea content and the levels of ammonia and pH (normal values range from pH 4.6 to pH 8.0) might interfere and ultimately destroy miRNAs in urine; however, to the best of our knowledge no studies have been reported concerning this subject.

Even though urine contains abundant nucleases, several studies have reported miRNAs to be identifiable in urine, with their stability attributed to nuclease resistance due to their small size, being bound to proteins and/or microvesicular containment [44].

Nucleases can be regarded as molecular scissors, which cleave phosphodiester bonds between the sugars and the phosphate moieties of DNA. They contain conserved minimal motifs, which usually consist of acidic and basic residues forming the active site. These active site residues coordinate catalytically essential divalent cations, such as magnesium, calcium, manganese or zinc, as a cofactor. Cleavage reactions occur either at the end or within DNA and thus DNA nucleases are categorized as exonucleases and endonucleases, respectively. Exonucleases can be further classified as 5' end processing or 3' end processing enzymes, according to their polarity of consecutive cleavage [45]. Human extracellular RNases, together with other members of the mammalian RNase superfamily, can be classified into four different enzyme types on the basis of their structural, catalytic and/or biological properties. Their occurrence and main distinctive features have been described and their catalytic differences (action on single- and double-stranded RNAs, dependence of enzyme activity on pH, ionic strength and cations, and hydrolysis of cyclic nucleotides) have been comparatively analyzed and discussed by Sorrentino and colleagues [46]. Many members of this superfamily have important biological actions including neurotoxicity, angiogenic activity, immunosuppressivity and antitumor activity. Some nondigestive extracellular RNases also serve as cytotoxic agents in host defense in higher plants and mammals and their potential use as therapeutic agents for human disorders has been suggested [46, 47].

It is known that microvesicles are impermeable to RNases, a hypothesis which might explain the stability of miRNAs in urine, since this fluid has a very high level of these enzymes [8, 31]. However, not all miRNAs detected in urine samples are protected within microvesicles and, this phenomenon seems to be common among body fluids. Studies performed by Turchinovich and colleagues, using blood samples revealed that a majority of the nuclease-resistant extracellular miRNAs, in plasma and cell culture media, were floating outside exosomes and bound to the Ago2 protein (protein of the Argonaute family) [8, 32]. To further confirm the binding of extracellular miRNAs to Ago2 protein in blood plasma and conditioned media (the medium in which the cells were being cultured), Turchinovich's group subjected anti-Ago2 and control immunoprecipitates to relative quantification. This study showed that miRNAs were detected in anti-Ago2 immunoprecipitates while control immunoprecipitates (containing no anti-Ago2) were almost totally free of miRNAs [32]. During intracellular miRNA synthesis, all mature miRNAs become associated with one of the four Ago proteins; therefore, it is not entirely surprising that extracellular miRNAs could be coupled with the same proteins. The remarkable stability of the Ago2 protein could explain the stability of associated miRNAs, especially when compared with mRNAs, thus allowing for a superior discriminatory potential in nuclease and protease rich environments [4, 32].

Proteomic analysis of miRNA-containing supernatants from cells cultured *in vitro* revealed that a number of other RNA binding proteins, such as nucleophosmin-1 and ribosomal protein L10a and L5, were found bound to miRNA outside of any vesicles. Researchers state that the physical interaction of miRNAs with these protein complexes, in the extracellular space, significantly protects them from extracellular nucleases [48].

In order to test the possibility that urinary miRNAs are associated with urinary proteins or whether they exist as an integral component of proteins on the exterior of exosomes, Mall and colleagues, assessed the effect of trypsin digestion on the quantity of urinary miRNAs [44]. This study showed that there was no change in the levels of the miRNAs assessed; thus, according to the authors, the unusual stability of urinary miRNAs is not due to its association with exosome-bound structures or macromolecules in urine [44]. Moreover, Mlcochova and co-workers also suggest that miRNAs might be associated and stabilized by miRNA binding complexes, such as RISC or chemical modifications including methylation, adenylation or uridylation might increase miRNAs stability [31].

1.4. How to Identify Body Fluid Stains in crime scenes?

1.4.1. Blood

As previously mentioned, blood is one of the most common body fluid encountered at crime scenes [2]. The simplest test that crime scene investigators use to detect bloodstains that are not clearly visible is an alternate light source (ALS), such as UV light [2]. This method is especially helpful when the stain is on a dark background. An ALS can direct attention to a latent stain at a crime scene, and then further presumptive tests can be used to form more conclusions about any body fluids that are present. However, these light sources must be used with caution, since certain UV wavelengths can damage the DNA enough that none is detected during polymerase chain reaction (PCR) – short tandem repeat quantification and amplification [2, 49].

The luminol test is another presumptive blood test that investigators quite often use at a crime scene [50]. It is based on the ability of hemoglobin and its derivatives to enhance the oxidation of luminol, in the presence of an alkaline solution, and involves spraying a suspected area with an aqueous solution of luminol and an oxidant [2]. It is known to be the most sensitive of the current presumptive tests used today, and there are also several formulations available that have advantages and disadvantages regarding sensitivity, intensity and duration of illumination, and effect on subsequent DNA analysis [2, 51, 52].

A very popular presumptive catalytic method is the phenolphthalein test which is also known as the Kastle-Meyer test [2]. Phenolphthalein will cause an alkaline solution to turn pink after its oxidation by peroxide, when blood is present. Although it is not as sensitive as luminol, using it on other body fluids does not yield a positive result [2, 19].

1.4.2. Urine

Urine is a difficult body fluid to detect due to the low sensitivity of the tests available and false positive results [2]. An accurate identification of this fluid can specially be useful in sexual assault, harassment, and misconduct cases.

As is the case with other fluids, urine will fluoresce when exposed to UV light; thus this is not a specific identification method for urine identification. Additionally, urine is especially hard to locate as nature of this fluid causes it to spread out so the stains tend to be more diluted than in other body fluids, which makes them fluoresce much less [2]. Odor could be an indicator, but it will cover an entire item and not localize itself to the stained area [2]. Other basic microscopic methods have been established, relying on the presence of solids in the sample, namely various crystalline materials and epithelial cells characteristic of the urinary tract linings. This only gives useful results in liquid samples not in stains, and as a result has a limited usefulness to forensic cases [2].

Inorganic ions such as chloride, phosphate, and sulfate have also been used to identify urine [2]. These ions are not unique to urine, but phosphate and sulfate are present in much higher concentrations in urine than in other body fluids.

Urea is an organic compound that is also found in higher concentrations in urine [2, 53]. Tests for urea depend on the activity of the enzyme urease as it enzymatically decomposes urea to ammonia and carbon dioxide [53]. The ammonia can then be detected by either using Nessler's reagent as an indicator (the solution becomes deeper yellow in the presence of ammonia) or p-dimethylaminocinnamaldehyde (development of a yellowish-green color) [53]. The radial gel diffusion test can also be used to detect the presence of urine, and this technique uses urea and a bromothymol blue indicator [2, 53]. The ammonia produced during urease activity alters the pH (making it alkaline), changing the color of the indicator in the agar from yellow to blue [54]. This method is both qualitative and quantitative, with the diameter of the reaction circle being proportional to the square root of the urea concentration; nonetheless, semen and sweat stains lead to slight false-positive results, due to their urea content [2, 55].

1.5. microRNAs detected in blood and urine samples: potential new biomarkers for fluid identification

In recent years, researchers have been trying to establish a link between specific miRNA patterns and body fluid identification, as it is known that miRNAs are differentially expressed in these fluids [9, 10, 42, 56, 57].

A systematic review of the literature revealed three miRNAs that show the potential to be forensic biomarkers: miR-801, miR-369-3p and miR-323b-5p.

According to a study performed by Weber and co-workers, that analyzed the presence of miRNAs in twelve body fluids (plasma, saliva, tears, urine, amniotic fluid, colostrum, breast milk, bronchial lavage, cerebrospinal fluid, peritoneal fluid, pleural fluid and seminal fluid), the presence of miR-801 was only detected in plasma samples [9]. Moreover, Schwarzenbach and colleagues, revealed that the expression levels of miR-801 were elevated in plasma samples of breast cancer patients in comparison to healthy individuals [58]. However, these authors also found the presence of this miRNA in plasma samples of healthy individuals. According to Zhou and co-workers, miR-801 levels in plasma allow the differentiation between healthy individuals and patients with hepatocellular carcinoma (HCC), since there is an elevated expression of this miRNA in patients with HCC compared to the healthy individuals [59]. Additionally, Madhavan and colleagues also compared the miR-801 plasmatic levels in several individuals, and concluded that patients with metastatic breast cancer had much higher plasma levels of this miRNA than the healthy individuals [60, 61]. The literature provides little information about miR-801. Weber's was the only group, to the best of our knowledge, that has studied this miRNA using only healthy individuals; all other information available is regarding the study of miR-801 in those afflicted with some kind of pathology. This "blank slate" concerning miR-801 makes it a very interesting miRNA to study. Besides, all these studies have detected the presence of miR-801 in the plasma of healthy individuals, which is a strong indicator that this miRNA is expressed in plasma [58-61].

Weber's miRNA research has shown that miR-369-3p was only detected in plasma samples [9]. Moreover, Guo and co-workers showed that miR-369-3p levels in the serum and skin of psoriasis' patients are higher than in healthy subjects, but the authors stress the need for further studies in order to clarify the role of this miRNA in the pathogenesis of psoriasis [62]. Upon reviewing the literature, no articles were found that systematically showed the presence of miR-369-3p in the urine, so it is considered that it may be advantageous to conduct a more detailed analysis of this miRNA.

According to Gildea and colleagues, urinary miRNAs are composed of a combination of both filtered miRNAs (derived from the blood) as well as kidney-derived miRNAs [63]. Several reports have shown that miR-323b-5p regulates the Claudin-16 protein, which is essential for maintaining the permeability of the thick ascending limb of Henle's loop [64, 65]. Studies with Claudin-16 deficient mice, showed calcium and magnesium loss in the kidney as well as nephrocalcinosis; therefore, it can be surmised that miR-323b-5p is a key miRNA in normal kidney function. Moreover, Argyropoulos and co-workers compared urine samples from patients with and without diabetic nephropathy and determined that the expression of miR-323b-5p was higher in those with diabetic nephropathy [66]. This group also suggests that the onset of microalbuminuria is associated with reduced levels miR-323b-5p [66]. Additionally, miR-323b-5p has never been identified in plasma samples which could mean this miRNA has the potential to be a biomarker for urine identification. It is important to highlight that miR-453 is processed from the 5p arm of miR-323b and although the correct designation is miR-323b-5p, sometimes 'miR-453' is still found in the literature [15].

MiR-801, miR-369-3p and miR-323b-5p were thus chosen for the present study, in order to evaluate if they can be forensically relevant miRNAs, as well as to prove that these miRNAs can be used for the identification plasma and urine samples. There is a shortage of studies comparing the miRNA signatures of plasma and urine, body fluids that are so common in forensic scenarios; therefore, knowing the miRNA behavior pattern for each fluid and finding suitable miRNA biomarkers for them could represent a breakthrough for the field of forensic sciences.

Objectives

2. Objectives

2.1. Main objective

The main objective of the present study is to identify a miRNA profile useful for plasma and urine sample identification.

2.2. Specific objectives

- Systematic review of the literature regarding the origin and expression profiles of extracellular miRNAs in plasma and urine samples;

- Detection and relative quantification of circulating miR-801 and miR-369-3p in plasma samples;

- Detection and relative quantification of circulating miR-323b-5p in urine samples;

- Establishing a miRNA profile associated with plasma and urine identification.

Material and Methods

3. Material and Methods

3.1. Study Population

A miRNA expression profiling study was conducted in twenty-two healthy adult individuals (five males and seventeen females) from the North of Portugal. This study group includes random Caucasian individuals with a mean age of 46.6 ± 17.78 years, a median age of 48.5 and with no major pathological condition.

Peripheral venous blood (6ml) and urine (30 ml) samples were collected from each subject after obtaining a written informed consent, according to the Helsinki Declaration principles.

3.2. Sample processing

Once the biologic samples were collected, they were processed according the following experimental conditions:

In urine samples, the pellet resulting from a 20 minute centrifugation at 3000 rpm, at room temperature, was diluted with 1X PBS (Phosphate buffered saline) and then centrifuged for 15 minutes at 3000 rpm, at room temperature. The pellet was then stabilized with TriPure® Isolation Reagent (Roche Applied Science®) and preserved at -80 ºC, until molecular analysis.

Regarding the plasma samples, after being collected into anticoagulant-treated tubes, the whole blood samples were processed. Initially, the blood samples were submitted to a centrifugation, at 2500 rpm during 5 minutes, at room temperature, to separate the plasma fraction. Following centrifugation, the plasma was then stored at -80 °C, until molecular analysis.

3.3. microRNA extraction

MiRNA extraction was performed for urine and plasma samples, using the GRS microRNA kit (Grisp®) according to manufacturer instructions. Some modifications were performed in the experimental procedures, namely the lysis buffer wasn't added and 14µl of miRNA buffer as well as 70µl of phenol-chloroform were used in urine samples, and 35µl of miRNA buffer and 385µl of phenol-chloroform were added to the plasma samples.

3.4. cDNA synthesis and relative quantification by real-time PCR

After miRNA extraction, RNA concentration and purity were measured for each sample, at 260 nm and 280 nm using the NanoDrop® ND-1000 spectrophotometer. The miRNA samples (5µl) were then used as templates for cDNA synthesis using a Taqman®MicroRNA Reverse Transcription kit (Applied Biosystems®), according to the manufacturer's instructions, and sequence-specific stem-loop primers for miR-801, miR-369-39, miR-323b-5p and RNU48, which was used as an endogenous control.

MiRNA expression was analyzed by quantitative real-time PCR (qPCR). The reactions were carried out on a StepOne™ qPCR Real-Time PCR machine, containing 1X TaqMan® Fast Advanced Master Mix (Applied Biosystems®), with 1X probes to amplify the target miRNAs (Taqman® microRNA Expression Assays, miR-801: 001994, miR-369-3p: 000557, miR-323b-5p: 002318), cDNA sample (2.6µl) and RNU48 (RNU48: 001006, Applied Biosystems®). During qPCR amplification, the samples were subjected to 95 ºC for 20 seconds and then 40 cycles of 95 ºC for 1 second, followed by 60 ºC for 20 seconds. MiRNAs detected in samples above the 40 cycle detection limit were labeled as negative samples, following the Applied Biosystems StepOne™ Real-Time PCR Systems Guideline for standard cycling. The miRNA quantifications were performed in duplicate and negative control lacking cDNA was included in all reactions. The results were confirmed by two independent investigators.

qPCR is the optimal approach for assessing circulating miRNAs, as it is a simple tool to efficiently determine the amount of a gene transcript in a sample [29]. In qPCR, reactions are characterized by the point in time during cycling when amplification of a target is first detected, the cycle threshold (Ct), rather than the amount of target accumulated after a fixed number of cycles [67]. By definition, the Ct indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold [67, 68]. The higher the starting copy number of the nucleic acid target, the earlier a significant increase in fluorescence is observed [68].

The fluorescent PCR amplification marker chosen for a qPCR reaction is vital, as it will determine the specificity and sensitivity of the assay [29]. In a heterogenous, yet closely related RNA species such as miRNAs, specificity is a most important factor [29].

SYBR® green, an asymmetrical cyanine dye, binds to nucleic acids, especially double stranded DNA molecules, with high affinity but no specificity. As the PCR reaction generates more amplicons, SYBR® green will bind and create a net increase in fluorescence. The main disadvantage of this dye is its lack of specificity, as it may generate false positives through the binding to nonspecific DNA fragments (e.g. primer dimers or multiple dye molecules may bind to one amplicon), increasing the perceived quantity; thus, SYBR®-based chemistries are best to be avoided when performing miRNA quantifications [29, 69].

Fluorophore-tagged probe-based chemistry, such as TaqMan® assays, provides several advantages over SYBR® green or dye-based technologies. TaqMan® probes, which are designed to hybridize to an internal stretch of the amplicon, contain a fluorescent reporter and quencher upon adjacent nucleotides [69]. The close proximity of the fluorescent reporter to its quencher molecule prevents the emission of fluorescence [69]. Since the expression of the appropriate fluorescence is regulated and obtained after amplification of a specific complementary DNA, barely any background fluorescence is obtained, hence offering a better signal to noise ratio [29]. The high level of specificity (specific to target copy accumulation during PCR), sensitivity (low copy detection) and reproducibility make TaqMan® assays the superior choice for the quantification of circulating miRNAs [29]. For this reason, we choose to use it for all qPCR reactions.

3.5. Statistical Analysis

Data analysis was made using StepOne™ Sofware v2.2 (*Applied Biosystems®*) with the same baseline and threshold set for each plate, in order to generate Ct values for all the miRNAs in each sample.

Relative quantification of miRNA expression was performed by the delta Ct (ΔCt) method using the average expression of the reference control gene for normalization of the miRNA abundances across the fluids. All Ct values were used to obtain ΔCt values for each of the miRNAs analyzed, applying the formula:

$$\Delta Ct = Ct_{target\ miRNA} - Ct_{endogenous}$$

Statistical analysis was performed using IBM®SPSS®Statistics software for Windows (Version 22.0). The relative fold change (according to Livak and Schmittgen, as $2^{-\Delta\Delta Ct}$) and the Student's t-test were also calculated in order to evaluate the differences in the expression levels of the normalized miRNAs, where [70]:

$$\overline{\Delta\Delta Ct} = \overline{\Delta Ct}_{Plasma} - \overline{\Delta Ct}_{Urine}$$

Results

4. Results

4.1. microRNA detection frequencies in plasma and urine samples

Figure 3 displays an overview of each miRNA detection frequency, that is, the percentage of samples in which each of the tested miRNAs was found.

In the twenty-two plasma samples analyzed, the miR-801 was found in all samples (100%). However, this miRNA was only detected in 80% of urine samples. Following the same trend, the miR-369-3p was detected in 91% of plasma samples, and its presence was only detected in 20% of urine samples. MiR-323b-5p was detected in 86% of the analyzed plasma samples, whilst its detection frequency in urine samples was of 75%.

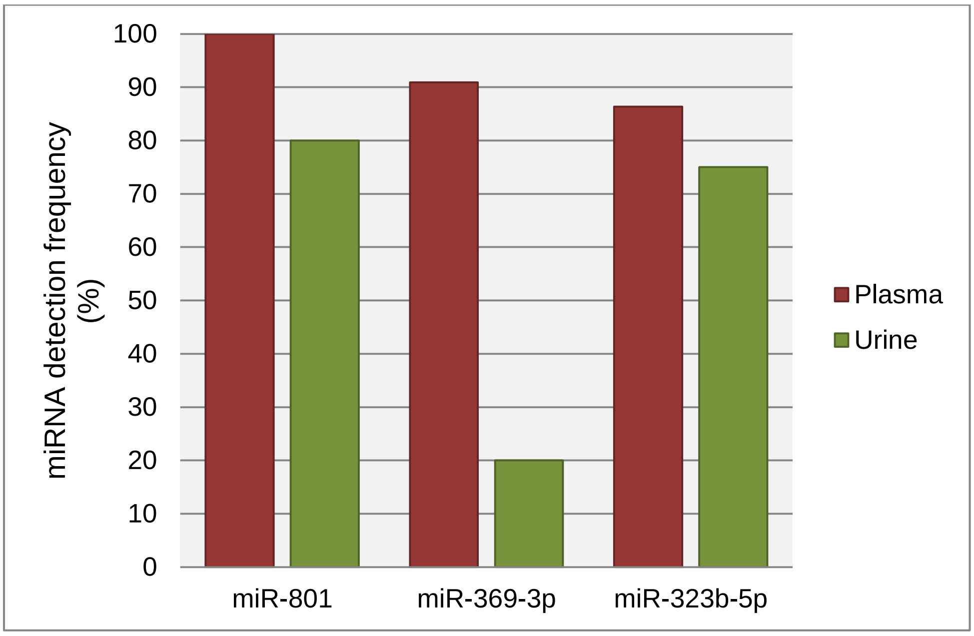

Figure 3 – miRNA detection frequency, in percentage, of miR-801, miR-369-3p and miR-323b-5p, in plasma and urine samples.

4.2. microRNA relative quantification levels in plasma and urine samples

A graph with the relative quantification of each miRNA was performed for each body fluid (Figure 4).

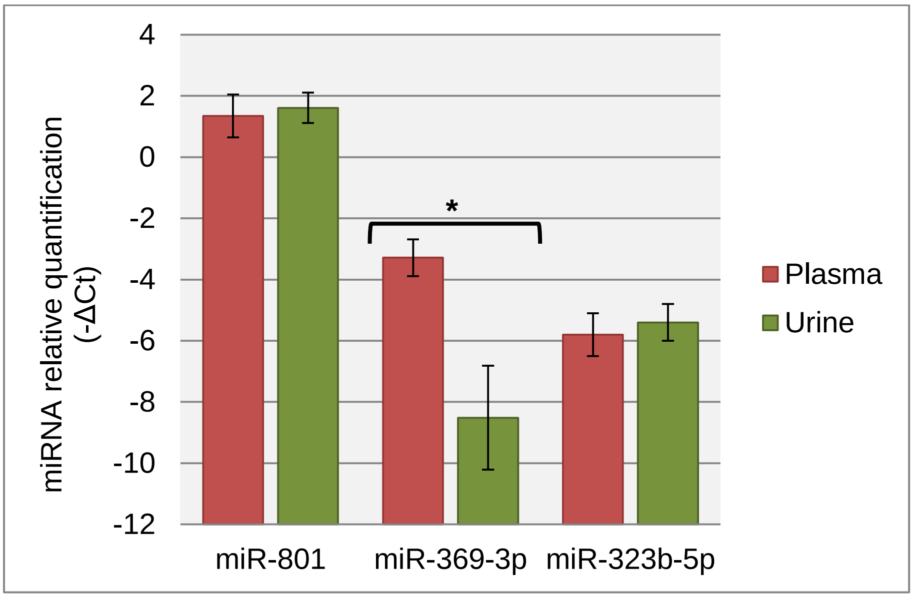

Figure 4 – Relative quantification levels of miR-801, miR-369-3p and miR-323b-5p, found in plasma and urine samples. (Mean ± Standard Error Mean; *$P < 0.001$).

The relative quantification level of miR-801, in plasma and urine samples, is higher than the expression level of the other miRNAs tested. However, the graph shows no statistically significant differences in the miR-801 expression levels between the plasma and urine.

Regarding the miR-369-3p, we observed that this miRNA exhibits a higher relative expression in plasma samples when compared to urine samples ($P < 0.001$).

Concerning the miR-323b-5p, according to our results, we did not observe any differences in the expression levels between the plasma and urine samples.

The relative fold change was also calculated (table 1), as $2^{-\Delta\Delta Ct}$. Table 1 indicates that, from the three miRNAs studied, miR-369-3p is the only with a statistically significant difference in expression between plasma and urine ($P < 0.001$). This miRNA exhibited a 37.52 fold-change in plasma when compared to urine samples.

Table 1 – Fold Change values for miR-801, miR-369-3p and miR-323b-5p.

	miR-801	miR-369-3p	miR-323b-5p
ΔΔCt	0.27	-5.23	0.4
Fold Change* (Plasma - Urine)	0.829	37.52	0.758
P-value**	0.768	<0.001	0.715

$^*2^{-\Delta\Delta Ct}$; ** Student's *t-test*

4.3. microRNA expression profile according to gender

In the present study, we also tested the miR-801, miR-369-3p and miR-323b-5p expression levels according to gender. Figure 5 displays an overview of the miRNAs relative quantification levels within male and female.

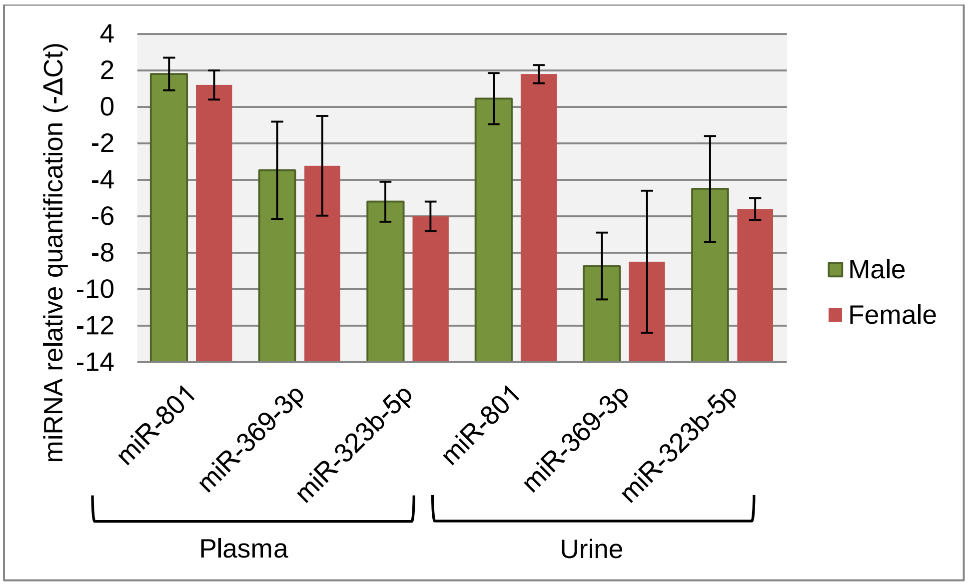

Figure 5 – Relative quantification levels of miR-801, miR-369-3p and miR-323b-5p according to gender. (Mean ± Standard Error Mean).

Analyzing the plasma samples' results, there were not any significant statistical difference in the miRNAs expression levels for either gender (miR-801: *P*= 0.708; miR-369-3p: *P*= 0.857; miR-323b-5p: *P*= 0.595). The same result was found for urine samples, as no significant statistical difference between the male and female samples was observed (miR-801: *P*= 0.405; miR-369-3p: *P*= 0.718; miR-323b-5p: *P*= 0.527). However, it should be mentioned that miR-369-3p was only detected in two urine male samples, whereas it was detected in fourteen female urine samples.

4.4. microRNA expression profile according to age

In order to assess a possible link between age and the miRNAs expression levels for each biological fluid, a graph was obtained (Figure 6).

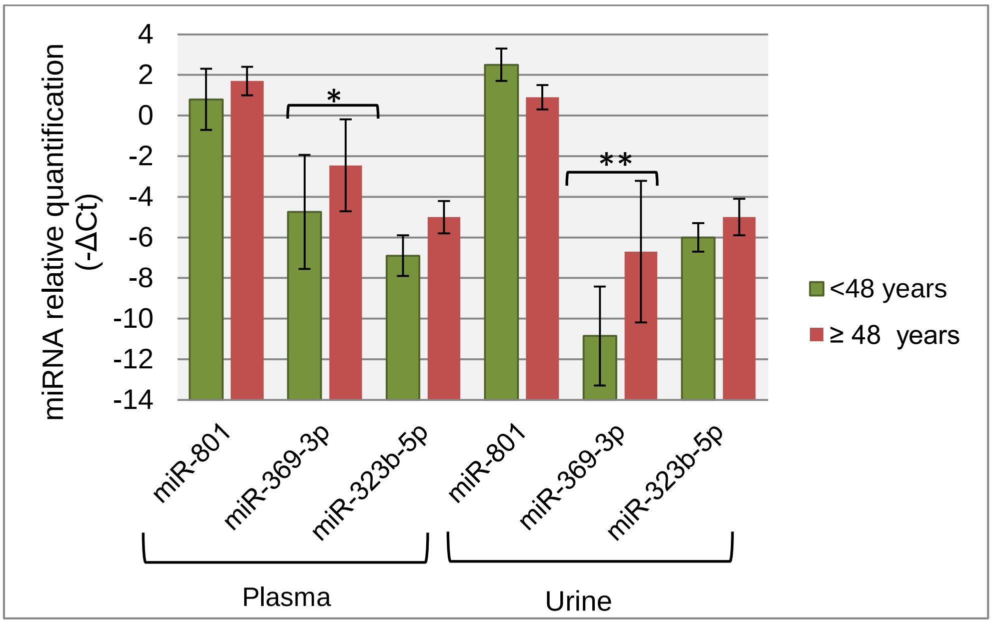

Figure 6 – Relative quantification levels of miR-801, miR-369-3p and miR-323b-5p according to age. (Mean ± Standard Error Mean; *P= 0.037; **P= 0.018).

Taking into account the median age, it allowed for the definition of two age categories: <48 years and ≥ 48 years.

In urine samples, the miR-369-3p showed a significant statistical difference between the two age groups, as the older group showed higher miR-369-3p levels than the younger individuals (P= 0.018). MiR-369-3p also exhibited a higher relative expression in the plasma of the older individuals (≥ 48 years), thus a significant statistical difference was observed for this miRNA in plasma samples (P= 0.037). The relative quantification of the miR-801 and miR-323b-5p, in plasma, revealed no significant statistical differences between the two study groups defined (miR-801: P= 0.522; miR-323b-5p: P=0.161). In urine samples, there was also no significantly statistical differences, between the age groups, observed for miR-801 and miR-323b-5p (miR-801: P= 0.125; miR-323b-5p: P= 0.419).

Discussion

5. Discussion

In the field of forensic sciences, there is a great need to find an accurate and reliable biomarker for the identification of body fluids. Recently, it was found that miRNAs could be the potential answer, providing additional information that might assist with the identification of forensically relevant biological stains; which, in time, could prove to be particularly useful in the analysis of compromised and degraded samples, that are frequently encountered in forensic investigations.

In the present study, when analyzing the relative quantification levels of miR-801, we found there was no statistically significant difference between the two fluids (P= 0.768). We were testing miR-801 to see if it could be a biomarker for plasma as evidences found in the literature stated this miRNA was detected in plasma samples of healthy individuals, and it had not been previously identified in urine samples; therefore, our results contrast with what has been published so far in the literature. A study performed by Weber and co-workers, tested twelve human body fluids from five healthy individuals and found that miR-801 was only present in plasma samples [9]. Our results aren't on par with Weber's observations; nevertheless, it should be taken into account that the number of samples we tested (twenty-two samples) is higher than the one tested by Weber's groups, and this could explain the detection of miR-801 in urine samples [9]. These results also emphasize the importance of a large number of biologic samples in miRNAs profiling studies. To our knowledge, the present study is the first to identify miR-801 in urine samples, so we suggest further studies with a higher number of samples, in order to validate these results.

According to our results, we observed that miR-369-3p exhibits higher expression levels in plasma samples when compared to urine (P<0.001). These findings were in accordance with the literature, since in several studies the miR-369-3p has been previously detected in plasma and serum samples but not in urine [9, 62]. Thus, we can hypothesize that miR-369-3p could be a molecular biomarker, allowing to establish the identity of body fluids in forensic sciences.

Concerning the miR-323b-5p, when comparing its expression levels between the two body fluids, we did not find any statistically significant differences (P= 0.715). MiR-323b-5p has been described in the literature as playing a vital part in maintaining proper kidney function, so it was to be expected for this miRNA to have a higher detection frequency in urine samples, and for its expression levels between the two fluids to be statistically significant [64, 65]. To date, there are no reports of miR-323b-5p being identified in any blood fraction, making the present study the first to identify it.

A systematic review of the literature highlights the existence of miRNAs that are gender specific in an healthy population [4, 21, 71, 72]. For instance, a 2013 study assessed the link between circulating serum miRNAs with metabolic syndrome (MetS) and connected it to gender [4, 72]. This group confirmed a significant change in the miRNA profile, which was overexpressed in the serum of individuals with MetS and more prominent in women [4, 72]. This relationship some miRNAs have with gender points out the need to choose proper miRNAs as biomarkers for body fluids, along with a need to understand more about the expression behavior of extracellular miRNAs.

We observed that in the two fluids analyzed, none of the miRNAs we tested showed any significant statistical differences in their expression levels for either gender. Interestingly, miR-369-3p was only detected in two urine samples from male individuals versus fourteen urine samples from women. Therefore, it is necessary to perform future studies using a higher number of samples to replicate these results, as well as to determine if there could be a link between miR-369-3p and gender.

It is also known that the expression levels of some miRNAs vary with age. In a 2011 study performed by Li and co-workers, the expression of miRNAs in rat liver during aging was studied, and the group observed that miR-34a and miR-93 levels increased in middle and old-age rat liver when compared to young rats [4, 73]. Maes and colleagues also researched the expression levels of miRNAs in rat liver and proved that a gradual increase of miR-669c and miR-709 was observed from the mid-age (18–33 months), whereas miR-93 and miR-214 levels are increased in extremely old (33 months) mice [4, 74]. Other similar studies were done in mice tissues by Hamrick *et al*. and Drummond and co-workers [4, 75, 76].

In our study, miR-801 and miR-323b-5p showed no statistically significant differences, for either fluid, between the two age groups analyzed. However, miR-369-3p showed higher expression levels in plasma and urine samples from older individuals (≥ 48 years). A study performed by Li and co-workers, in which the role of miRNA regulation in mice brain during the normal aging process was evaluated, found that the expression levels of miR-369-3p were higher in older mice (33 months) than in younger ones (10 months), which corroborates our findings [77]. The group also stated that ubiquinol-cytochrome *c* reductase, or Rieske iron-sulfur protein (UQCRFS1) is targeted by miR-369-3p. UQCRFS1 is a key subunit of the cytochrome *bc1* complex of the mitochondrial respiratory chain, and a deficiency in this component may result in a decline of efficient adenosine triphosphate (ATP) production [77, 78]. Since, oxidative phosphorylation is known to decline and suffer loss of efficiency in aging tissues, it could be suggested that the high levels of miR-369-3p are connected to low levels of UQCRFS1, and consequently to older individuals. Most published studies analyzing a connection between miR-369-3p and age, either use animal models or unhealthy individuals; for that reason, it would be interesting to carry out further studies using healthy individuals in order to ascertain if there is, in fact, a connection [77-79].

As previously mentioned, miR-369-3p was only detected in two male urine samples, which can indicate that this miRNA may play a more relevant role in women and that the difference in expression levels observed between the age groups, is mostly due to our female population. For that reason, we suggest that in the urine samples, the difference between the age groups might be due to hormonal changes caused by menopause (average age is 51 years old) [80]. In menopause, the loss of ovarian follicular activity is responsible for low and stable levels of progesterone and estradiol, which leads to a significant increase in the follicle stimulating hormone (FSH) and the luteinizing hormone (LH) levels and the permanent cessation of menstruation, resulting in the end of the natural female reproductive life [81].

In a study by Zhao and co-workers, miRNAs were extracted from endometrial samples of women that underwent luteal phase support using steroid hormones, namely micronized progesterone and 17-beta-estradiol [82]. The results showed that the expression levels of miR-369-3p were associated with vascular endothelial growth factor (VEGF), a predicted target gene, as well as higher levels of micronized progesterone and 17-beta-estradiol [82]. The modulation of VEGF expression levels, in consequence of miR-369-3p regulation, could promote endothelial cell growth and neo-angiogenesis, which is a pivotal process in reproductive function regulating endometrial regeneration, corpus luteum formation, embryogenesis and placentation [82, 83].

Thus, higher expression levels of this miRNA lead to a greater VEGF protein inhibition, affecting endometrial regeneration and the formation of the corpus luteum. If there is no regeneration of the endometrium walls, the shedding of the functional layer during menstruation can’t ensue. Besides, since the corpus luteum is involved in the production of relatively high levels of progesterone and moderate levels of estradiol, if its formation is compromised, so are the levels of these hormones. These abnormal (and low) levels of progesterone and estradiol will lead to the higher levels of LH and FSH, so characteristic of menopause.

When the levels of progesterone and estradiol are very low, like is the case in menopause, VEGF is inhibited by miR-369-3p; therefore, we hypothesize this is the reason miR-369-3p levels are higher in older women. Furthermore, considering that the regeneration and growth of the human endometrium depends on an adequate angiogenic response, once a woman enters menopause, there isn’t a need for this response and, theoretically, the levels of VEGF should decrease. Figure 7, displays the possible connection between VEGF and miR-369-3p and their role in the uterine cycle. The results of a study by Agrawal and colleagues, in which serum VEGF concentrations in the normal menstrual cycle were measured, corroborates our hypothesis [84].

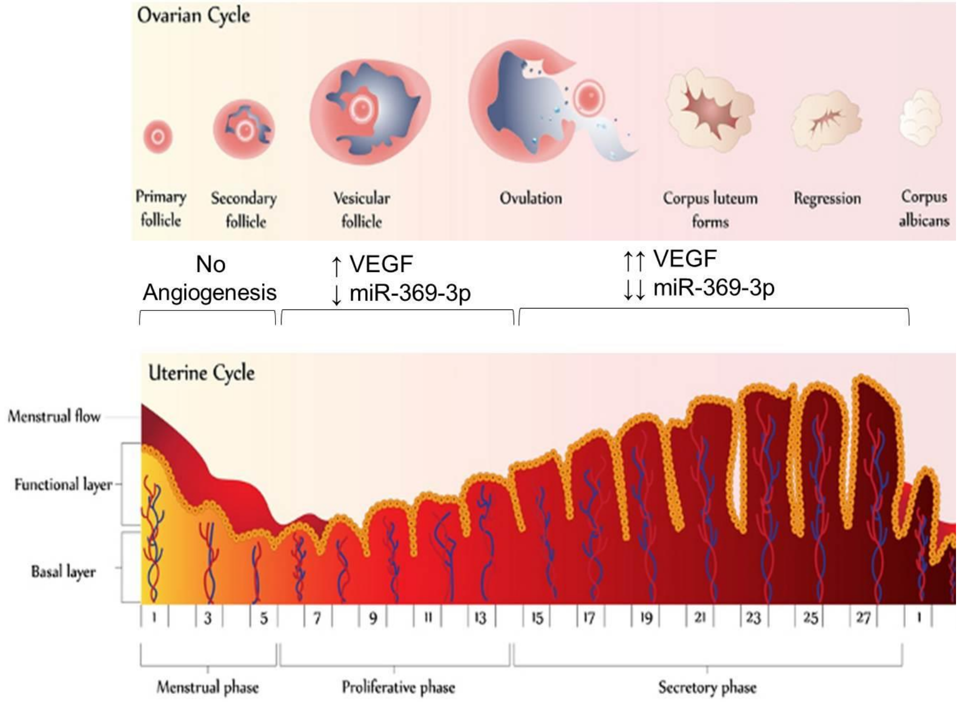

Figure 7 – Potential role of miR-369-3p and VEGF in the uterine cycle. VEGF: vascular endothelial growth factor. (Adapted from http://www.utahfertility.com/infertility-diagnosis-treatment/fertiliy-your-health/phases-of-menstrual-cycle-utah-county/ [85]).

The difference in expression levels, some miRNAs show in body fluids (like miR-369-3p in urine), detected throughout gender and different age groups, could be useful in forensic investigations, as it can help determine an age range or even the gender of the fluid's donor.

Conclusion and Future Perspectives

6. Conclusion and Future Perspectives

Throughout a crime scene investigation, traces of blood and urine are often found, especially in the form of dried stains that might not always contain enough DNA to obtain a DNA profile. In recent years, miRNA analysis has been described as having the potential to help identifying these fluids, mostly due to their intrinsically short size and tissue-specific expression. Nevertheless, even though over 1800 mature miRNAs have been identified in the human genome, it remains necessary to search for suitable miRNA markers for forensically relevant body fluids, like plasma and urine. Using blood and urinary miRNA biomarkers is especially useful since sample collections are fairly non-invasive, they can be collected in a relatively large amount, urine especially, and multiple time-series measurements are easy to obtain.

The present study highlights the importance of miR-369-3p as a potential forensic biomarker, as miR-369-3p showed higher expression levels in samples from older individuals (≥ 48 years). Taking into account our results, we hypothesize miR-369-3p and its influence on UQCRFS1 may be associated with age. It would be interesting to perform new studies evaluating the expression levels of miR-369-3p and its relation with UQCRFS1, in healthy individuals of different ages. As we only detected miR-369-3p in the urine of two male samples, we suggest that the difference in expression levels between age groups could be influenced by the high number of women included in this study. For this reason, we also hypothesize that miR-369-3p could be associated to the hormonal changes that occur during menopause. We propose that carrying out a study evaluating the expression levels of miR-369-3p in pre and post-menopausal women could help validate this miRNA as a urinary age biomarker for female samples.

The difference in expression levels that miR-369-3p showed, in the age groups assessed, can be extremely valuable in forensic research, as it can help to establish the age range for the fluid's donor. To the best of our knowledge, several studies trying to determine an association between miR-369-3p and age are performed using animal models or unhealthy individuals; thus, it would be interesting to carry out further studies, using a higher number of healthy individuals, to replicate the association.

To our knowledge, this was the first study to identify miR-801 in urine samples and miR-323b-5p in plasma, which denotes a need for additional miRNA studies in the field of forensic sciences, and also emphasizes the importance of using a high number of samples. Hence, we also suggest this study to be replicated using a higher number of samples. Moreover, it would be interesting to test different pH levels in urine samples to see what repercussions it has in the miRNAs expression levels. Additional research should also be dedicated towards the discovery of optimum miRNAs for body fluid identification and analysis of potential influences of environmental factors such as humidity, UV radiation and bacterial contamination on miRNA stability *in vitro*.

MiRNAs are extremely resistant to temperature variations, so it would also be interesting to test if extracting and analyzing miRNAs in burn victims, when other forensically relevant molecules are degraded, could be viable.

MiRNA analysis could, one day, become the gold standard of body fluid identification, and it might even follow the footsteps DNA profiling techniques took in the 1980s, which could ultimately lead jurisdictions to accept expert testimony regarding miRNA matches between suspects and crime scene evidences.

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7. References

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Attachments

8. Attachments

Submitted to:
Pharmacogenomics

Extracellular microRNAs, from toxicology screens to urine identification: the future of forensic sciences

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Abstract

Urine is perhaps the most amenable body fluid to perform routine testing, as it can be collected frequently and through non-invasive methods. Recently, in forensic science, microRNA profiling has been explored as an alternate method for body fluid identification. MicroRNAs also attracted great interest in forensic toxicology, since exposure to toxic compounds alters microRNA expression, which is then easily detected in urine.

With this review, we intend to gather information about microRNAs that might have a urinary tract origin and could be potential biomarkers for urine identification. We also highlight the importance of establishing a connection between urinary microRNA expression and illicit substances, ultimately leading to a new drug evaluation method, representing a major breakthrough in the forensic field.