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MicroRNA Signatures: An Emerging Forensic Landscape for Body Fluids based Age Estimation

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Abstract

Background

Age estimation is an important aspect of forensic investigations, particularly in cases where biological evidence is recovered from an unknown individual. Conventional age estimation methods based on skeletal and dental analysis are not applicable when, only trace biological materials such as blood, saliva or semen are available at a crime scene. So, molecular biomarkers have emerged as reliable and reproducible tools for forensic age estimation. Among these, microRNAs (miRNAs) have emerged as a possible biomarker due to their small size, stability, tissue-specific expression, and involvement in aging-related biological pathways.

Main Text:

This review summarizes the current understanding of the biological mechanisms underlying aging, miRNA biogenesis, and the forensic relevance of miRNA biomarkers for age estimation. It critically evaluates studies investigating age-associated miRNA expression in various biological samples, including blood, saliva, semen, menstrual blood, and vaginal secretions. In addition, the review discusses analytical approaches used for miRNA profiling, particularly RT-qPCR-based methods, and examines predictive models developed for chronological age estimation. The strengths of miRNAs, including their resistance to degradation, high analytical sensitivity, and compatibility with forensic workflows.

Conclusion

MicroRNAs represent a promising class of molecular biomarkers for forensic age estimation due to their stability, tissue specificity, and age-associated expression patterns. However, further validation using larger and more diverse populations, together with standardized methodologies and robust predictive models, is essential before miRNA-based approaches can be routinely implemented in forensic practice.

BACKGROUND

Aging is a complex process defined by a gradual loss of a physiological integrity leading to a decrease in function and increasing vulnerability to death. As cells and molecules age they become damaged and more vulnerable to disease and death. The process of cellular senescence is one of the most crucial characteristics of ageing. The key cellular pathways that underlie normal ageing include epigenetic stress, proteotoxic stress, oxidative stress, telomere damage, and DNA (Deoxyribonucleic acid) damage (Noren Hooten et al., 2013; Bao et al., 2014; Childs et al., 2015; Kumar et al., 2017;). Forensic age estimation is the process to identify unknown deceased and trace donors. This can help law enforcement and investigators by limiting the area for search and by relieving the worry of possible suspects (Zhou et al., 2022).

Information regarding an individuals age can assist law enforcement agencies limit the number of potential suspects and identify the missing person as well as help the investigators to reconstruct the events at a crime scene. Traditionally, age estimation has been based on morphological and radiological assessment of the skeletal and dental characteristics. Although these methods are effective for the analysis of deceased individuals with complete skeletal remains, they cannot be used for the analysis of trace biological evidences like blood, saliva or semen stains often seen at crime scenes (Ritz-Timme et al., 2000; Cunha et al., 2009). Many traditional morphological techniques, including bone and tooth measurements have been adapted to new approaches using molecular changes related to age, including the amino acid racemization of teeth, as well as skin and cartilage tissues. However, these methods have limitations in cases where a skeleton is not present. Molecular methods can obtain a donors age from various biological samples including body fluids from crime scenes. (Koh et al., 2017; Kumar et al., 2017; Zubakov et al., 2016; Tiplamaz et al., 2018; Matteussi et al., 2022; Zhou et al., 2022). (Table 1)

Table 1
Comparison of Traditional and Molecular Ages Estimation Models

Method	Sample Type	Principle	Advantages	Limitations	Accuracy
Morphological	Bone, Teeth	Skeletal development	Simple	Requires remains	Moderate
Histological	Bone	Osteon counting	Useful in adults	Destructive	Moderate
Telomere Length	Blood	Telomere shortening	Molecular marker	High variability	Low
DNA Methylation	Biological Samples	CpG methylation	High accuracy	Costly	High
miRNA Profiling	Biological Samples	Age-related expression changes	Stable in degraded samples	Limited validation	Emerging

In recent years, molecular methods have been developed for age estimation to overcome these limitations. Of these, DNA-based approaches, in particular DNA methylation profiling, have yielded promising results. (Bekaert et al., 2015; Jung et al., 2019). One of the first DNA methylation (DNAm) based epigenetic age prediction models was developed by Hannum et al. in 2013, marking a major advancement in molecular age estimation. While providing high accuracy, DNAm has several limitations, including the need for relatively large amounts of DNA and complicated bioinformatics analysis, and has emerged as a most promising method for predicting age in forensics with an uncertainty mean absolute deviation of about 3–5 years in the predicted age (Hannum et al., 2013; Park et al., 2016; Naue et al., 2017; Koop et al., 2021). The methylation assays are somewhat costly, technically challenging, and frequently need bisulfite treatment, which may result in degradation of DNA and loss of sample. Further, methylation is regulated by environmental factors and disease conditions (Zbieć-Piekarska et al., 2015; Naue et al., 2018). All these challenges have encouraged the search for alternative molecular markers that are more stable, less sample intensive and are compatible with degraded forensic samples.

Main Body

MicroRNAs are small (18–22 nucleotide) non-coding RNAs that bind to target sequences in mRNA, causing post-transcriptional changes in the stability or translational efficiency of mRNA. MicroRNAs are important regulators of gene expression involved in various cellular processes such as differentiation, proliferation, apoptosis and replicative senescence. The small RNA molecules, called miRNAs, are thought to regulate about 60% of all human genes, highlighting their potential role in many aspects of biology (Bartel & Chen, 2004; Bates et al., 2009; Garzon et al., 2009; Leung & Sharp, 2010; Gorospe & Abdelmohsen, 2011; Noren Hooten et al., 2013). They are essential for many biological processes, such as development, cell differentiation, metabolism and ageing (Kumar et al., 2017). Lee et al. (1993) were the first to discover Lin – 4, an RNA molecule that could function as a miRNA. Whereas the first forensic application of miRNAs was by Hanson et al. in 2009 suggested miRNA as biomarkers for forensic body fluid identification. (Lee et al., 1993; Hanson et al., 2009). Importantly, miRNAs are very stable, short lenght, and are bound to protein complexes and extracellular vesicles to protect them from enzymatic degradation (Mitchell et al., 2008; Etheridge et al., 2011). This stability ensures that they can be detected in dried and environmentally stained biological stains, and are especially useful in forensic analysis (Hanson et al., 2009; Zubakov et al., 2010) (Fig. 1).

There have been several studies that have shown age-related changes in miRNA expression profile. The expression of age-associated miRNAs has been identified in blood, serum and plasma, and their levels vary predictably with age in other tissue types as well (Serna et al., 2012; Noren Hooten et al., 2013; Kumar et al., 2017). Based on these findings, age prediction models based on miRNA expression have been developed, which are based on quantitative real-time polymerase chain reaction (RT-qPCR) and next-generation sequencing (NGS) approaches and statistical and machine-learning algorithms (Fang et al., 2020; Zhou et al., 2022). The mean absolute errors (MAE) reported are around 4–8 years, which is comparable to DNA methylation-based methods and indicate that miRNA profiling has the potential to produce reasonably accurate age estimates (Fang et al., 2023; Fang et al., 2020).

Semen, vaginal secretions, blood, saliva and menstrual blood are commonly recovered at a crime scene in forensic casework. The ability to estimate the age of the donor from these fluids would represent a significant advancement in forensic intelligence. One of the reasons that miRNAs act as promising biomarker because they can be isolated from the same biological samples as DNA profiles, and DNA and RNA can be co-extracted and minimize sample consumption (Courts & Madea, 2010; Zubakov et al., 2010). Furthermore, the assays based on miRNAs are suitable for standard molecular biology laboratory equipment and instrumentation, which are currently available in forensic laboratories (Hanson et al., 2009; Wang et al., 2022).

Although several benefits have been demonstrated, the forensic use of miRNA-based age estimation is still in the early stages. The inter-individual variability, disease-specific and lifestyle-specific effects, population-specific differences and an absence of standardized reference miRNAs for normalization represent challenges (Kumar et al., 2017; Fang et al., 2020). In addition, most of the previous research

has been conducted on blood samples and only a small number of studies investigated other body fluids like saliva which is of great importance in bite-mark and sex offense cases (Zhou et al., 2022). In conclusion, a comprehensive review and integration of existing understanding of miRNA-based age estimation in biological fluids are required to assess the current potential and limitations of these methods and their future prospects.

The main objective of this review is to offer a detailed overview of the biological basis of the miRNA expression in the context of aging, summarize the findings from previous studies regarding the age estimation based on the expression of miRNA in biological fluids, critically discuss the methods and procedures, and discuss the challenges and future perspectives for the application of miRNA in the forensic world. This review combines the knowledge of molecular biology, forensic science and bioinformatics to conclude that miRNAs are a promising class of biomarkers for age estimation in biological residues encountered in forensic investigations.

METHODS

Literature Search Strategy

A comprehensive literature search was conducted to identify studies relevant to microRNA (miRNA)-based forensic age estimation from biological fluids. The search was performed across three major electronic databases: PubMed, and Google Scholar. The search strategy combined key terms using Boolean operators, including combinations such as (“microRNA” OR “miRNA” OR “miRNA markers”) AND (“age estimation” OR “age prediction” OR “chronological age”) AND (“forensic science” OR “biological fluids” OR “blood” OR “saliva” OR “semen” OR “vaginal secretion” OR “menstrual blood”). Reference lists of relevant articles and review papers were also manually screened to identify additional studies that may not have appeared in the initial database search.

ELIGIBILITY CRITERIA

Inclusion criteria

For this scoping review, only studies that fulfilled the following criteria were considered: (a) Studies that investigated the expression of miRNAs and chronological or biological age, (b) Studies that included human biological fluids (blood, saliva, semen, vaginal secretions, and menstrual blood), (c) Studies that evaluated the potential use of forensic applications of miRNAs in age estimation, and studies that provided biological evidence for age-associated expression of miRNAs, (d) Studies that used molecular methods to assess miRNA expression, including reverse transcription quantitative polymerase chain reaction (RT-qPCR), microarray analysis, next-generation sequencing (NGS), or massively parallel sequencing, and (e) Studies published in peer-reviewed journals. There was no restriction on publication date or geographical location to capture as much as possible the relevant literature

Exclusion criteria

Studies were excluded if they were: (a) Editorials and conference abstracts, letters to the editor, thesis and case reports non-empirical publications, including commentaries, opinion pieces, (b) Studies that focused solely on DNA methylation, telomere length, proteins, mRNA, circRNA, or other biomarkers without information or studies on the expression of miRNAs in the body fluid, unless they provided important methodological information regarding miRNA stability or forensic applicability,(c) Studies that lacked adequate methodological detail and data related to age-relevant outcomes.

Biology of Aging and the Role of microRNAs

A. Biological Processes Underlying Human Aging

Aging is a biological process with multiple factors with gradual loss of physiological functions and higher risk of disease and death. At the cellular level, aging is correlated with the genomic instability, shortening of the telomeres, changes in the epigenome, dysfunction of mitochondria, and failure in protein homeostasis (López-Otín et al., 2013). All of these changes collectively lead to a state of irreversible cell cycle arrest, which is associated with changes in gene expression and secretory patterns, called cellular senescence (Campisi & d'Adda di Fagagna, 2007).

Chronic low-grade inflammation (also called “inflammaging”) and oxidative stress are among the key processes of aging that lead to the accumulation of molecular damage (Franceschi et al., 2000). These changes in gene regulation over time cause alterations in tissue homeostasis and regenerative capacity, therefore, involving DNA damage, defective DNA repair mechanisms, and epigenetic drift (Horvath, 2013). Such changes in the molecular level are not only seen in tissues but also in circulating biomolecules like blood and other body fluids RNA species (Noren Hooten et al., 2010).

Histologically, molecular changes related to aging offer a tool for estimating chronological age in biological samples from a forensic perspective. Morphological features cannot be detected from small amounts of biological material, whereas molecular markers can therefore, molecular markers can be used in trace evidence analysis (Ritz-Timme et al., 2000). The regulatory RNAs, including microRNAs, have been especially investigated as a class of molecules because of their role in cellular aging pathways.

B. Biogenesis of MicroRNAs and their Regulatory Function

MicroRNAs (miRNAs) are small, non-coding endogenous RNA molecules that control gene expression at the post-transcriptional level. The biogenesis of miRNAs starts in the nucleus with the transcription of genes that encode miRNAs by the main enzyme of RNA polymerase II to give rise to long primary transcripts referred as primary miRNAs (pri-miRNAs). These pri-miRNAs have a characteristic stem-loop structure, and are processed by the microprocessor complex, consisting of the RNase III enzyme Drosha

and its cofactor DGCR8, to form precursor miRNAs (pre-miRNAs) with an average length of 70 nucleotides. The pre-miRNAs are then exported from the nucleus to the cytoplasm by Exportin-5. The pre-miRNA structure is then cleaved by RNase III enzyme Dicer to produce a miRNA duplex of ~ 22-nucleotide length, consisting of two strands. The guide strand or mature miRNA is selectively loaded into the RNA-induced silencing complex (RISC) while the passenger strand is usually degraded. When the miRNA-RISC complex binds to the complementary sequences in the 3' untranslated region (3'-UTR) of target messenger RNAs (mRNAs), the process of translational repression or mRNA degradation follows, depending on the level of sequence complementarity. Through this mechanism miRNAs can control a variety of biological functions such as cell growth, cell differentiation, cell death, metabolism, immune function, and ageing. The role of miRNAs as biological regulators and potential forensic biomarkers has been demonstrated in numerous pathological conditions, such as cancer, cardiovascular disorders, neurodegenerative diseases, and age-related physiological decline (Bartel, 2004; Ha & Kim, 2014; O'Brien et al., 2018) (Fig. 2).

Importantly, miRNAs are also involved in pathways related to aging and longevity. Multiple miRNAs regulate major signalling pathways including the insulin/IGF-1 pathway, p53 mediated stress response, and inflammatory signalling, which have all been shown to affect lifespan and aging (Noren Hooten et al., 2010). Age-related changes in the miRNA expression have been detected in blood, serum and plasma. Some miRNAs have been found to be gradually up- or down-regulated as a function of age, indicating their potential as biomarkers of biological or chronological aging (Noren Hooten et al., 2010; Smith-Vikos & Slack, 2012).

C. Stability of miRNAs and Forensic importance

A major advantage of miRNAs over longer RNA molecules like mRNA is their stability. miRNAs are RNase resistant and are detectable in extreme environmental conditions, including dried stains and prolonged storage (Hanson et al., 2009; Courts & Madea, 2010). They are considered to be stable due to their short length and their binding to a protective protein complex or their incorporation into extracellular vesicles, like exosomes (Mitchell et al., 2008; Weber et al., 2010).

There are several studies that have shown miRNAs are detectable in forensic samples that have been exposed to adverse environmental conditions such as prolonged storage, repeated freeze-thaw cycles, exposure to ultraviolet radiation, moisture, and high temperatures (Zubakov et al., 2010; Hanson & Ballantyne, 2013). This stability is particularly important in cases where forensic specimens are often recovered after days, weeks, or even months after deposition, where degradation of DNA and mRNA may compromise conventional molecular analyses. (Fig. 3).

The forensic utility of miRNAs extends beyond their stability. The expression of miRNAs has been demonstrated in several studies to be tissue- and body-fluid specific, which makes it possible to distinguish these fluids from a crime scene. Different miRNA has been successfully extracted and identified from various forensic samples such as blood, saliva, semen, vaginal secretion, menstrual

blood, and used to identify the source of unknown biological stains using a single molecular assay (Zubakov et al., 2010; Wang et al., 2013; Hanson & Ballantyne, 2013). They have been demonstrated to express differently in body fluids and have been used to identify body fluids and, if possible, age, based on the same RNA extract (Zubakov et al., 2010; Courts et al., 2014). The dual function makes miRNAs especially appealing useful for forensic intelligence applications. Furthermore, the profiling of the miRNA can be achieved relatively by using laboratory procedures including RNA extraction, reverse transcription, and quantitative PCR (Hanson et al., 2009; Vidaki & Kayser, 2018).

D. Molecular indicators of aging – MicroRNAs

Aging involves the continuous loss of tissue and organ function over time, as well as many transcription regulators, such as microRNAs (miRNAs). miRNAs are short, non-coding RNA molecules that control the expression of mRNA molecules and are known to have an important role in post-transcriptional silencing (Matsuyama & Suzuki, 2019). miRNAs are involved in aging as a transcriptional regulator and some of them have very high-correlated expression levels with age (Inukai & Slack, 2013).

There have been reports of age-dependent downregulation of some miRNAs that regulate cell cycle progression and DNA repair, and others are related to inflammatory and stress response pathways, that have been reported to be upregulated with age (Noren Hooten et al., 2010; Hooten et al., 2013; Wang et al., 2015). The advantages of miRNAs as age markers from a forensic analysis, that they require small samples, are not degraded, and are suitable for body fluid analysis. However, the expression of miRNAs can also be affected by sex, ethnicity, disease and environmental factors, for which it is important to select appropriate markers and use powerful statistical modelling (Vidaki & Kayser, 2017).

There were various data showing that miRNAs participate in age-related modulation of physiological and pathophysiological aging processes, such as the study by Zhang et al. (2015) who identified the significantly down-regulated aging-associated miRNAs in serum miRNA profiles of adults (40–70 years old), including miR-29b, miR-106b, miR-130b, miR-142-5p, and miR-340 and three upregulated miRNAs miR-92a, miR-222, and miR-375) with age (Zhang et al., 2015). Therefore, some forensic scientists proposed the potential forensic application of miRNAs in estimating the age of a donor from biological samples. Using seven machine learning models, Fang et al. (2020) developed age prediction models for bloodstains using six age-related miRNAs (miR-98-3p, miR-324-3p, miR-32-3p, miR-330-5p, miR-374c-5p and miR-342-3p). The mean absolute error (MAE) results were between 5.52 years and 7.46 years in male and female samples, respectively (Fang et al., 2020). Therefore, it is easy to understand the biological link between the aging process and regulation of miRNAs to develop a reliable forensic age estimation assay.

Biological fluids used for miRNAs Age Estimation

BLOOD

Blood is the most frequently encountered biological fluid at crime scenes and making them a primary target for molecular age estimation. MicroRNAs (miRNAs) are small non-coding RNAs which are stable, resistant to environmental degradation and are emerging biomarkers for age prediction in recent years. Blood contains both intracellular and extracellular miRNAs, derived from various cell types such as leukocytes, erythrocytes, and circulating vesicles. Blood is an excellent biological fluid and changes systematically with age and thus is an ideal for developing predictive age models based on miRNA expression profiles. These miRNAs remain stable even in dried blood stains which are commonly encountered at crime scene. (Zubakov et al., 2010; Fang et al., 2020). The study by Serna et al. (2012) provided the initial evidence for the link between miRNAs and aging they investigated the expression profile of miRNA in centenarians, octogenarians and younger people, finding the several age-associated miRNAs, such as miR-21, miR-130 and miR-494, are significantly upregulated in centenarians, suggesting that miRNA expression correlates with the process of aging (Serna et al., 2012).

In a similar study, Noren Hooten et al. (2013) compared peripheral blood mononuclear cells and identified miRNAs such as miR-181a-5p, miR-151a-3p, miR-1248 and miR-3607-5p were altered with chronological age, also indicating a measurable chronological age dependency in blood-derived miRNAs (Noren Hooten et al., 2013). These studies were not forensic age prediction studies, but they provided a scientific basis for the future forensic studies. Fang et al. (2020) reported the first pilot study using bloodstain samples from 20–69 year old subjects through the use of massively parallel sequencing, RT-qPCR validation and machine learning analysis for direct age estimation of the bloodstains. Six miRNAs were identified as being significantly age-associated miR-98-3p, miR-374c-5p, miR-324-3p, miR-342-3p, miR-32-3p, and miR-330-5p and predictive models were developed using AdaBoost and Random Forest algorithms yielding mean absolute errors of around 5–7 years. More importantly, it was found that this study showed that even the old bloodstains are detectable for the presence of the age-related miRNA signature which has forensic value in real case works (Fang et al., 2020). Zhou et al. (2022) further developed the miRNA and circular RNA (circRNA) biomarkers for forensic age estimation by validating these markers using RT-qPCR and applying machine learning techniques from human blood markers (Zhou et al., 2022).

Recently, Fang et al. (2023) further advanced forensic age estimation with the addition of piRNA (PIWI Interacting RNAs) and miRNA markers for degraded bloodstain samples to achieve increased robustness and predictive power (Fang et al., 2023).In general, these studies confirm that blood-based miRNA profiling is among the most promising molecular methods for forensic age estimation as it has reasonably high accuracy, biological relevance, and potential to apply machine learning-based predictive modelling. However, there are some drawbacks such as inter-individual variation, population expression differences, environmental degradation effects, small sample size, lack of universally recognized endogenous controls and lack of validation. In addition, current models use discovery methods based on sequencing, which can be very expensive and prohibitively costly for general purpose forensic laboratories. The future research should therefore focus on the validation studies on a large-scale level in multi-populations, development of standardized RT-qPCR protocols, optimization of normalization strategies, introduction of other complementary markers such as circRNAs, piRNAs, and methylation

markers, as well as using advanced machine learning algorithms to increase the accuracy of prediction and forensic applicability (Serna et al., 2012; Noren Hooten et al., 2013; Fang et al., 2020; Zhou et al., 2022; Fang et al., 2023).

SALIVA

Saliva has emerged as a highly valuable biological fluid in forensic investigations due to its non-invasive collection, frequent presence at crime scenes, and relatively stable molecular content. Saliva is a special body fluid produced by multiple salivary glands and in recent years, with the development of molecular biology, a number of biomarkers have been identified, including RNA, DNA, and proteins (Du et al., 2023). These properties make salivary miRNAs useful for forensic applications in degraded trace biological samples such as dried saliva deposition on cigarette buds, bite marks, envelopes, and drinking vessels (Zubakov et al., 2010).Salivary miRNA signatures will not only reflect systemic physiological aging but also provide a complex molecular fingerprint for oral epithelial turnover, immune response and interaction with the oral microbiome, which may be of great value in forensic age estimation (Michael et al., 2010; Bahn et al., 2015). Saliva residue may be recovered from the bite marks on the victim's skin or traces that are found in more intimate areas, which can be used in reconstructing the sequence of events and to help identify the type of crime (e.g., a sex assault) (Bond & Hammond, 2008). There have been recent attempts to estimate the age of saliva stains largely based on the DNA methylation. However, current methods for DNA methylation do have certain limitations, such as they are complex and have limited accuracy, thus the search for other markers to improve the ability for age estimation in saliva for forensic applications (Jung et al., 2019; Fokias et al., 2023).

Saliva-based age estimation, however, remains substantially less developed. The pilot study by Guan et al. (2024) is one of the first studies to focus specifically on salivary miRNAs and the use of massively parallel sequencing to discover salivary candidate markers and machine learning approaches to classify age groups. Of these two miRNAs, hsa-miR-142-3p and hsa-miR-486-3p were up regulated with age whereas three miRNAs, hsa-miR-128-3p, hsa-miR-125a-5p and hsa-miR-125b-5p were down regulated with age in this study. The age-related miRNAs found in this study also have great potential in clinical research. This pilot study was performed to develop the age classification model in saliva using miRNAs for the first time. The study however had a small participant size, no external validation, and no targeted verification using RT-qPCR, suggesting the need for larger and more ethnically diverse studies for external validation (Guan et al., 2024) Further evidence of the reliability of salivary miRNAs is the fact that salivary miRNA panels have been found to be highly reproducible and sensitive in oral disease diagnostics, and that the salivary miRNAs are analytically stable in the presence of variable conditions in biomarker studies for the diagnosis of oral squamous cell carcinoma and systemic disease (Park et al., 2009; Balakittnen et al., 2024). The forensic value of salivary miRNA age estimation is that it can be collected non-invasively, it is appropriate for trace evidence, is resilient to degradation in the environment, and may be applicable to current forensic DNA phenotyping protocols (Kayser, 2015). Currently, however, there are several major limitations to the forensic application, such as the lack of large-scale validation studies, variability in oral health status, smoking habits, diet, microbiome

composition, and medication use, while there is no standard protocol for sample preservation, RNA extraction, endogenous normalization, and inter-population validation (Ambroa-Conde et al., 2022).

SEMEN

Semen is one of the most forensically important biological fluids it is often found in crime scenes, and contains molecular donor-specific information that can be very helpful in sexual assault cases. MicroRNAs (miRNAs) represent a new and promising class of molecular indicators for forensic applications, as they are extremely resistant to environmental damage, remain stable even in the presence of enzymes, are highly tissue-specific, and are short, consisting of only 18–22 nucleotides (Zubakov et al., 2010). These properties of semen-derived miRNAs make them as an ideal material for forensic molecular analysis. Although significant advances have been achieved in the use of miRNA markers for semen identification, but their application for chronological age estimation remains unexplored. Hanson et al. (2009) compared the expression of miRNA in forensic stains and found that miR-135b and miR-10b could be detected with no change in expression, suggesting their forensic applicability (Hanson et al., 2009). The initial study by Zubakov et al. (2010) conducted large-scale screening of 718 human miRNAs in various forensic body fluids and found a number of semen-enriched markers including miR-135a, miR-10a, miR-507, miR-943, and miR-891a, showed high detection sensitivity and strong specificity through quantitative reverse transcription polymerase chain reaction (RT-qPCR). Importantly, these markers were still present after long environmental exposure, which was indicative of their forensic robustness (Zubakov et al., 2010). In further research, Wang et al. (2013) confirmed the usefulness of semen-specific miRNAs (miR-888 and miR-891a) in the forensic body fluid discrimination by TaqMan qPCR assays and provided suitable normalization strategies for forensic RNA analysis. To expand this work, Zhang et al. (2018) examined if the discriminatory power of semen miRNAs remained consistent in semen samples with poor semen quality and found that some previously identified semen miRNAs markers were still detectable in these samples (Zhang et al., 2018).

However, none of these studies explored the age-associated differential expression of semen-derived miRNAs or constructed predictive models for semen-derived miRNA expression that could be used for age estimation. Since aging is associated with progressive alterations in reproductive gene regulation, oxidative stress pathways, mitochondrial function, and cellular senescence, age-dependent miRNA expression changes in semen are likely to exist but remain insufficiently characterized for forensic application. The major factors that hinder implementation are the lack of dedicated age-estimation studies, the absence of age associated semen-specific miRNA panels, reproductive diseases, environmental exposures, and the lack of a standardized protocol for semen RNA extraction and normalization for forensic age estimation. Systematic sequencing of semen samples from a wide range of ages in the future should be used to identify candidate age-associated miRNAs with broad high-throughput sequencing, with targeted RT-qPCR validation, construction of predictive models using machine learning, and cross-population validation. Moreover, the ability to combine semen-derived miRNAs with complementary molecular markers, such as piRNAs, circRNAs, and DNA methylation

markers, could increase the accuracy for future molecular forensic age prediction studies (Zubakov et al., 2010; Wang et al., 2013; Fujimoto et al., 2019; Fang et al., 2020).

VAGINAL SECREATION AND MENSTRUAL BLOOD

Vaginal secretions and menstrual blood are among the most critical biological matrices encountered in forensic casework, particularly in sexual assault investigations, intimate partner violence cases, and reproductive evidence analysis, where accurate biological fluid characterization can provide crucial investigative leads (Zubakov et al., 2010). Studies focusing on chronological ageing based on these matrices are missing but considerable studies have been performed which have defined well established miRNA signatures for their forensic identification, laying the molecular groundwork for future studies of ageing. Hanson et al. (2009) compared the expression of miRNAs in forensic stains and found that miR-124a, miR-372 and miR- 195 were found to be stable in vaginal secretion while miR-451 and miR-412 were seen to be stable in menstrual blood, which make them potentially applicable in forensics (Hanson et al., 2009). The pioneering study by Zubakov et al. (2010) evaluated 718 human miRNAs across multiple body fluids using microarray studies and identified miR-214, miR-412, miR-124a, miR-372, miR-373, and miR-888 as significant markers associated with menstrual blood and vaginal secretions, with revealing that miR-214 was the most reproducible miRNA marker in menstrual blood and miR-124a was the most specific miRNA marker in vaginal epithelial fluid (Zubakov et al., 2010). Expanding this work, Wang et al. (2013) validated miR-214 as a forensic marker for menstrual blood and miR-124a as a forensic marker for vaginal secretions, and they showed that miR-214 and miR-124a were suitable markers for distinguishing these fluids from peripheral blood, semen and saliva. They reported high accuracy of classification using their discriminant analysis especially using several markers together (Wang et al., 2013).

Luo et al. (2015) also found that miR-141-3p and miR-497- 5p in menstrual blood and miR- 3134 and miR- 31-3p in vaginal secretions (Luo et al., 2015). Although great advances have been made in the identification of body fluids, there are no studies that have directly examined age-associated differential expression of miRNAs in the vaginal and menstrual blood for chronological age estimation. There are, however, several significant disadvantages to the current attempts to use them for age estimation in legal cases, such as the complete absence of studies that focus on age- associated markers, use of hormonal contraceptives, gynaecological disorders and reproductive history, and the fluctuations in the microbiome, as well as limited standardization of RNA extraction, selection of endogenous controls, and normalization protocols. Future research should perform comprehensive high-throughput sequencing of vaginal secretion and menstrual blood samples covering a wide range to identify age associated miRNAs and validate these findings using RT-qPCR, build up predictive models using machine learning, assess the degradation in these samples and incorporate other complementary biopsy markers like DNA methylation, circRNAs and pi-RNAs to improve robustness (Hanson et al., 2009; Zubakov et al., 2010; Wang et al., 2013; Luo et al., 2015).

miRNA- based working model for Forensic Age Estimation from Biological Fluids

Identification based on miRNA approach may be processed for different biological samples, but most investigations based on miRNA markers follow a similar methodological framework. The process typically begins with the collection of biological samples from donors representing different age groups, followed by extraction of total RNA containing small RNAs. Candidate age-associated miRNAs are identified either through high-throughput screening methods such as microarray analysis or massively parallel sequencing, or through literature-based selection of previously reported age-related miRNAs. The selected miRNAs are then quantified using reverse transcription quantitative polymerase chain reaction (RT-qPCR). To minimize technical variation, expression values are normalized against endogenous reference miRNAs or small RNAs. Statistical analyses are subsequently performed to identify significant correlations between miRNA expression levels and chronological age. Finally, predictive models are developed using regression analysis or machine learning algorithms and validated using independent sample sets to assess their forensic applicability (Fang et al., 2020; Zhou et al., 2022; Guan et al., 2024) (Fig. 4).

CONCLUSION

MicroRNAs have emerged as a promising molecular biomarker for forensic age estimation due to their high stability, tissue specificity, and age-dependent expression patterns. Age determination is an important factor in many forensic scenarios such as the identification of unknown human remains, disaster victim identification, estimation of age in missing persons. However, in certain circumstances, traditional morphological techniques are constrained by the state of the sample, or lack of, while miRNA-based techniques are capable of identifying biological evidence from crime scenes and other forensic settings. Recent studies indicate that multiple miRNAs are significantly associated with chronological age, and can be included in predictive models using sequence-based methods, RT-qPCR and machine learning algorithms.

But miRNA analysis will probably be best used in combination with other markers, not as a single marker. The use of miRNAs in conjunction with other age-related markers like DNA methylation markers, telomere length, mRNA expression profiles, protein-based markers, and advanced statistical or AI models could enhance the accuracy, robustness, and forensic utility of the system. Although promising results have been reported, the forensic application of miRNA age estimation is still in its infancy, due to limited sample sizes, population-specific variability, absence of standardized procedures, and limited external validation. Therefore, future research is needed on large populations in different ethnic backgrounds, development of validated multi-biomarker panels, incorporation of complementary molecular tools and validation of standardized workflows for routine forensic cases processing. As a result of the ongoing progress in molecular biology, bioinformatics and forensic genomics, the use of miRNA for age estimation could become a valuable forensic tool for resolving age and human identification cases.

Abbreviations

DNA
Deoxyribonucleic Acid
RNA
Ribonucleic Acid
miRNA
MicroRNA
DNAm
DNA Methylation
RISC
RNA-Induced Silencing Complex
PCR
Polymerase Chain Reaction
NGS
Next Generation Sequencing
RT
qPCR-Reverse Transcription Quantitative PCR
pri
miRNA-Primary miRNA
pre
miRNA-Precursor miRNA
circ miRNA
Circular miRNA
MAE
Mean Absolute Error
Fig
Figure

Declarations

Author Contribution

Study conception and design: MP, KV, BJDrafting of manuscript: MP, KV, BJCritical revision: MP, KV, BJ.All authors have read and approved the final manuscript.

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Figures

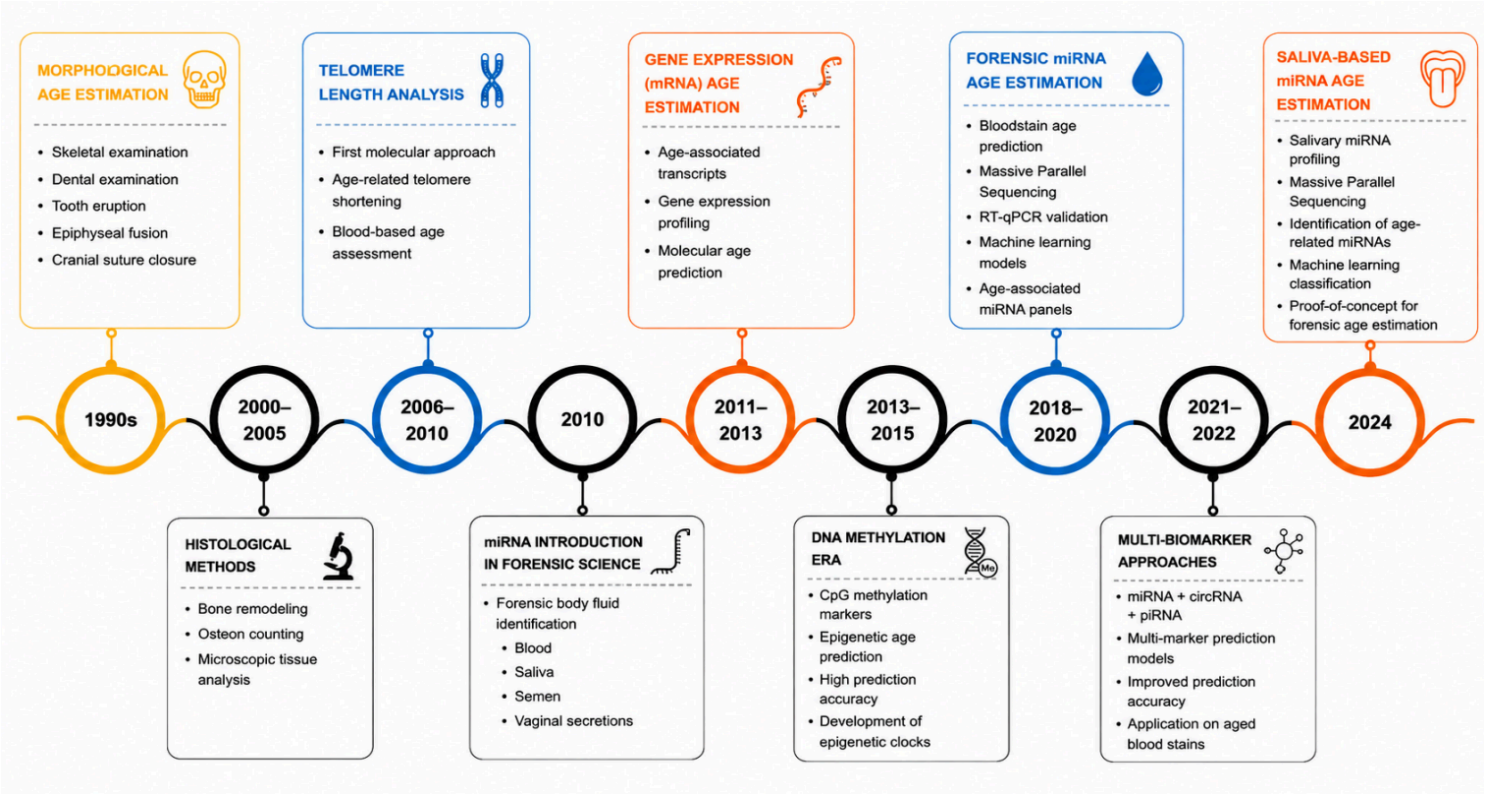


Figure 1

Evolution of Forensic Age Estimation Methods

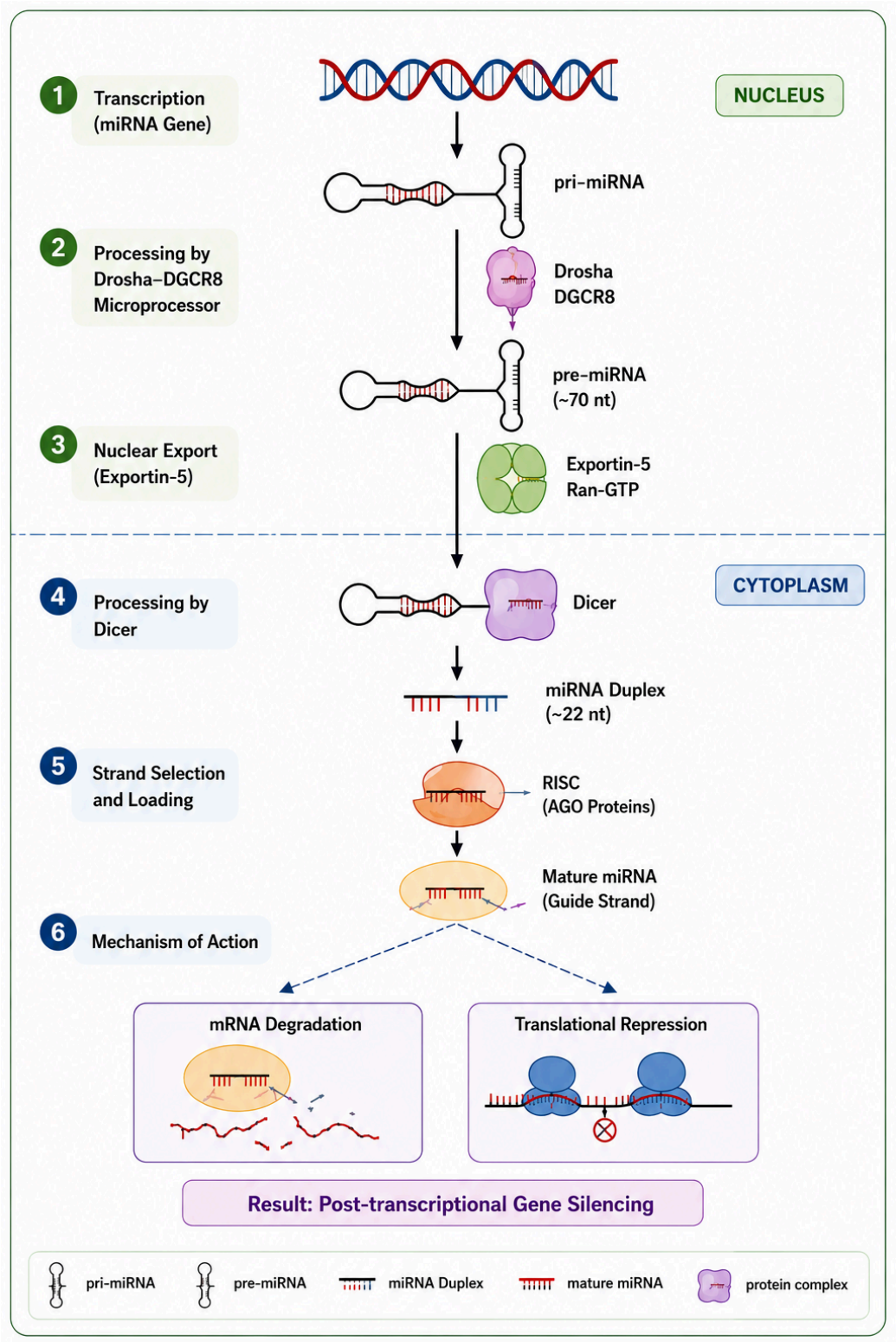


Figure 2

Biogenesis and Mechanism Action of microRNAs

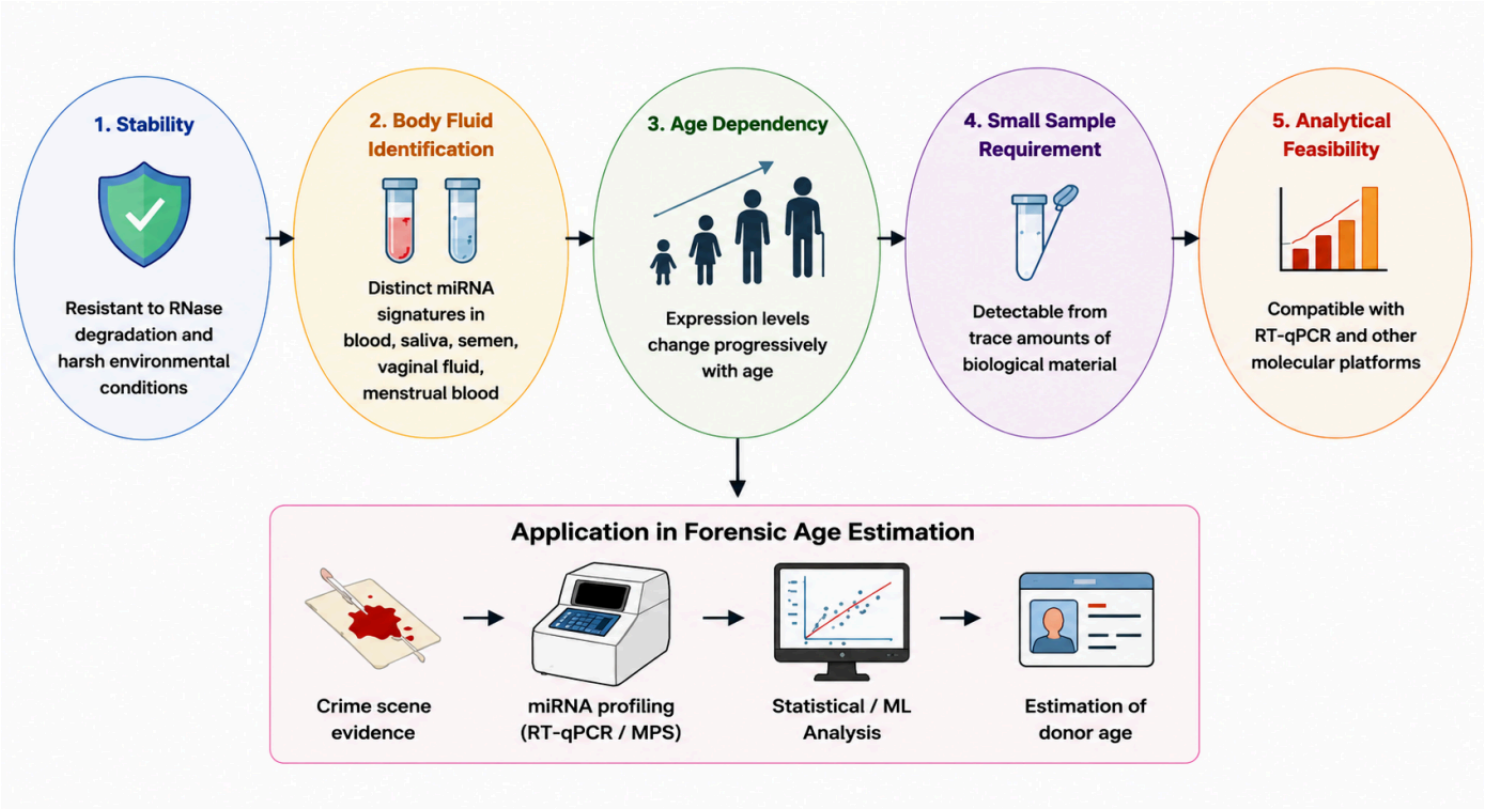


Figure 3

Forensic Application of miRNAs in Age Estimation

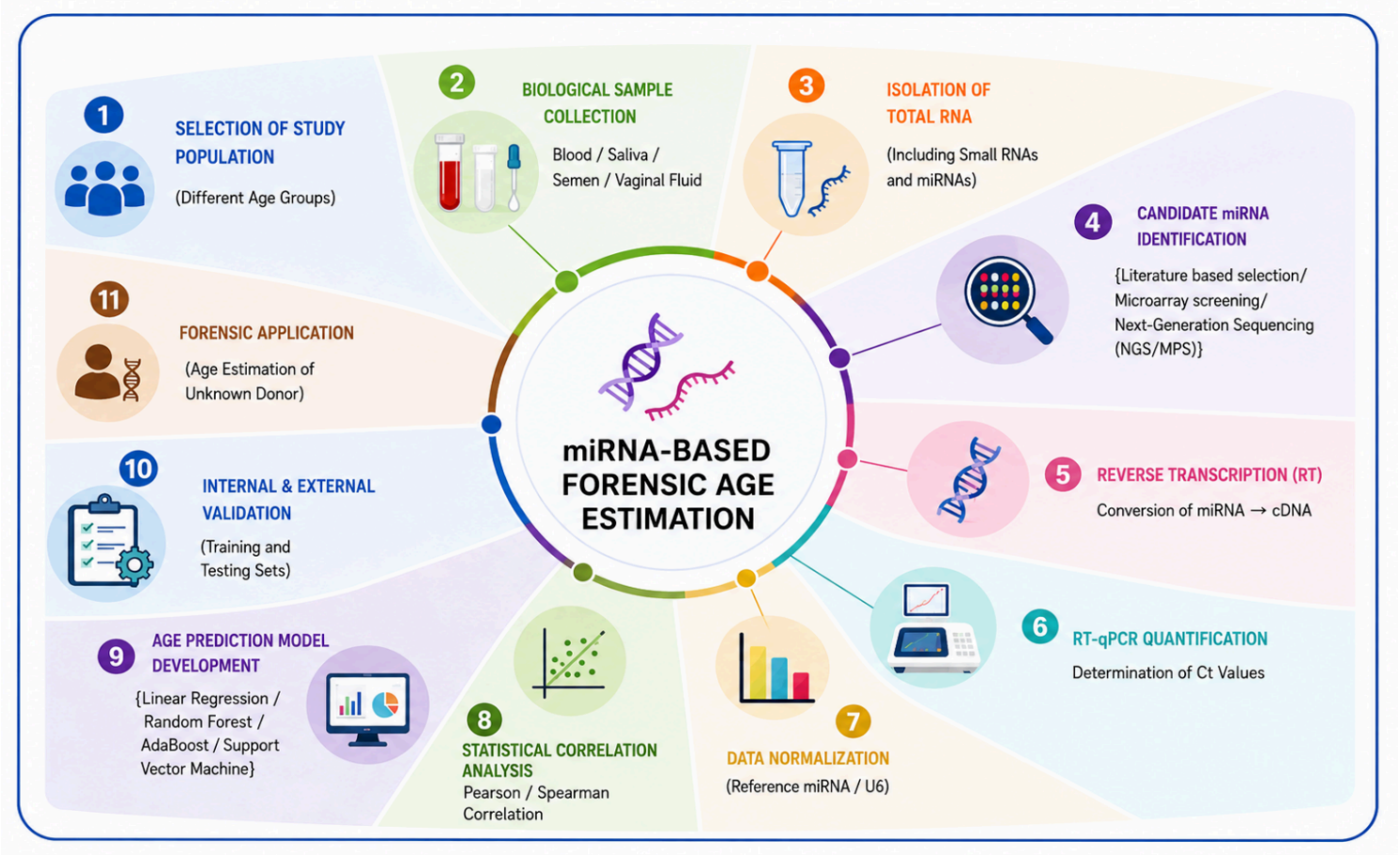


Figure 4

Methodological framework for miRNA – based Forensic Age Estimation from Biological Fluids

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