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Review article

Forensic transcriptome analysis using massively parallel sequencing

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ABSTRACT

The application of transcriptome analyses in forensic genetics has experienced tremendous growth and development in the past decade. The earliest studies and main applications were body fluid and tissue identification, using targeted RNA transcripts and a reverse transcription endpoint PCR method. A number of markers have been identified for the forensically most relevant body fluids and tissues and the method has been successfully used in casework. The introduction of Massively Parallel Sequencing (MPS) opened up new perspectives and opportunities to advance the field. Contrary to genomic DNA where two copies of an autosomal DNA segment are present in a cell, abundant RNA species are expressed in high copy numbers. Even whole transcriptome sequencing (RNA-Seq) of forensically relevant body fluids and of postmortem material was shown to be possible. This review gives an overview on forensic transcriptome analyses and applications. The methods cover whole transcriptome as well as targeted MPS approaches. High resolution forensic transcriptome analyses using MPS are being applied to body fluid/ tissue identification, determination of the age of stains and the age of the donor, the estimation of the post-mortem interval and to post mortem death investigations.

1. Introduction: transcriptomics and potential forensic utility

In 2008, when RNA analysis had barely just arrived on the scene in forensic genetics, Bauer published a review on “RNA in forensic science” [1]. He anticipated the high potential of RNA analyses for solving forensic questions, e.g. for body fluid identification, wound age determination, estimation of the post-mortem interval (PMI), estimation of the age of stains and determination of the cause of death. Since then, many RNA-based studies have been published on these topics, especially on body fluid identification applications. Determining the cellular (pheno)type from which the DNA originated can be important in supporting sexual versus social intercourse, for example, by distinguishing menstrual blood from peripheral blood on suspected perpetrators in sexual assault cases [2]. Molecular methods for cellular phenotype determination (i.e. body fluid and tissue identification) include classical biochemical methods, mainly singleplex antigen-antibody reactions [3], as well as more specific, multiplex methods based upon the transcriptome [4,5], the epigenome [6,7], the proteome [8] and even the microbiome [9]. While all of these "-omes" contain tissue specific biomarkers, the transcriptome is of particular interest, since most of the information in the genome (including sequence variation) is reproduced in multiple copies of RNA.

Due to a decrease in operational costs and an increase in throughput in the last two decades, Massively Parallel Sequencing (MPS) has revolutionized research and diagnostics [10]. Nowadays, whole genomes or transcriptomes can be studied. The transcriptome as used in this review is the complete set of RNA transcripts (either coding or non-coding) in a specific type of cell, cell population or tissue. Alongside, approaches for the targeted analyses of subgenomic DNA regions and specific RNA biomarkers exist. The huge amount of data being generated requires suitable analysis pipelines and sophisticated knowledge of bioinformatics. Although, the degraded nature and low abundance of nucleic acid species render forensic samples a genuine challenge for any MPS experiment, several studies showed promising results when analyzing these kind of samples [11,12]. Also whole transcriptome sequencing (RNA-Seq) was successfully applied to forensic samples [13–15].

What have fundamental studies revealed about the human transcriptome [16–19]? Although the approximately 21,000 known protein coding genes constitute ~2% of the genome, > 80% of the genome may be transcribed into different RNA species (although not in a single

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tissue) [17,20]. Primary transcription is recognized as the major driver of cellular specificity and therefore the transcriptome is an indicator of cellular phenotype [21]. It is this fact that primarily makes RNA typing potentially useful in forensic genomics. Importantly, genes vary more across tissues (47% of total variance in gene expression) than individuals (4% of total variance) [21]. Approximately 12% of genes are preferentially enriched in a single tissue (i.e. > 5x compared to other tissues) and 10% of transcripts in a tissue come from expression-elevated genes (differential expression in several tissues compared to most) [19]. Only a small percentage of genes are exclusively expressed in a single tissue (< 200 total, i.e. < 1%) [21]. On the other hand, almost half of all genes (44%) are expressed in all tissues (i.e. housekeeping genes) [21,22]. Additional tissue specific variation is obtained due to the fact that ~50% genes express tissue dependent isoforms because of alternative transcription start and termination sites, although not so much variation as initially believed due to alternative splicing [21].

Among the different types of RNA, only a small proportion is transcribed into protein coding messenger RNA (mRNA) whereas the vast majority of the genome is transcribed into non-coding RNAs (ncRNAs) that do not encode information about proteins. ncRNAs can be sub-classified by length into long non-coding RNAs (lncRNAs) and small non-coding RNAs (sncRNAs). lncRNAs comprise a highly heterogeneous group of long transcripts (> 200 nucleotides) with a wide range of structures and subcellular locations, which are involved in post-transcriptional modifications, splicing and epigenetics [23]. sncRNAs are between 18 and 200 nucleotides in length and have diverse roles which - in conjunction with other molecules - involve gene regulation through RNA interference, RNA modification or spliceosomal involvement [24]. MicroRNAs (miRNA) are a particular type of small (approximately 22 nucleotides) single-stranded non-coding RNA molecules with regulatory functions [25]. miRNAs participate in many physiological and pathological processes, such as proliferation, differentiation and cancer development [26]. Piwi-interacting RNAs (piRNAs) form RNA-protein complexes through interactions with Argonaute proteins and are mostly involved in the epigenetic and post-transcriptional silencing of transposable elements [27]. Circulating extracellular RNA (exRNAs) are another type of sncRNAs and have been identified in the blood circulation and in several body fluids [28]. Small nuclear RNAs (snRNAs) are involved in the assembly of the spliceosome and the splicing of pre-mRNA [29]. Small nucleolar RNAs (snoRNAs) are located in the nucleus and process/modify rRNA [30]. Circular RNAs (circRNA) are not linear but form a loop, where the 3'-end is covalently linked to the 5'-end. They originate from a special type of pre-mRNA splicing (back-splicing) and are expressed in a tissue and cell specific manner [31,32].

Why is transcriptomics useful in forensic genetics? Despite its thermodynamic instability compared to DNA, RNA of sufficient quality and quantity for analysis is recoverable from evidentiary items typically found in forensic biology casework [33–38]. It is possible to co-extract DNA and RNA [4,33,39]. Since the RNA is converted to DNA (cDNA) the same downstream chemical analysis as DNA can be conducted using the same analytical platforms. Contrary to genomic DNA where two copies of an autosomal DNA segment are present in a cell, multiple genome segments are copied into RNA species hundreds or thousands of times in a cell, a copy number somewhat akin to mtDNA. This increase in copy number may help ameliorate the reduction in RNA target number due to normal RNA degradation that takes place during the drying phase after the physiological fluid has been deposited ex vivo. Importantly for future investigations, genomic information is also directly encoded and reproduced in RNA transcripts. Thus, the emerging MPS technology permits genomic-encoded RNA data to be accessed. This digital gene expression technology permits the direct counting of transcripts and variants encountered in a sample and facilitates quantitative analysis.

2. Cellular phenotyping: body fluid and organ tissue identification

2.1. Body fluid identification

Identifying the origin of a biological trace is important for the contextualization of different stains and therefore may provide crucial information to law enforcing authorities. Body fluids are routinely identified by presumptive tests (chemical, immunological and protein catalytic activity tests) as well as spectroscopic methods and microscopy [3,40]. Unfortunately, these tests suffer from different drawbacks such as cross-reactivity with other substances or tissues, they are not available for all forensically relevant body fluids (e.g. vaginal secretion) and the differentiation between related body fluids such as blood and menstrual blood is difficult. Today it is possible with RNA profiling to identify with a high degree of certainty the presence of most of the commonly encountered forensically relevant body fluids and tissues in dried stains, including blood, semen, saliva, vaginal secretions, menstrual blood and skin. Traditionally PCR/CE methods were applied [41–44], but increasingly in the past few years MPS methods have being established. In a proof-of-principle study Zubakov et al. presented an approach for the simultaneous analysis of forensic STRs, amelogenin, and forensic mRNAs based on parallel targeted DNA/RNA sequencing using the Ion Torrent PGM [45]. The assay included 9 autosomal STRs, the AMELX/AMELY system for sex identification and 12 mRNA markers, using separate workflows for RNA and DNA analysis. Unambiguous mRNA-based tissue identification was achieved in all samples from all forensically relevant tissues tested, and STR sequencing analysis of the tissue sample donors was 100% concordant with conventional STR profiling. Consequently, the feasibility of simultaneously sequencing various nucleic acid markers of different types (i.e. STRs, amelogenin insertion/deletion, and mRNAs) for different forensic purposes (i.e. individual, sex, and tissue identification) was demonstrated. Lin et al. used MPS data from fresh and degraded body fluids to identify regions of high read coverage within target transcripts, representing particularly stable sequences, designated as "transcript stable regions" or "StaRs" [46]. They propose a new concept whereby primers targeted to StaRs are able to consistently and specifically amplify a wide range of RNA biomarkers in various body fluids of varying degradation levels. Hanson et al. developed a targeted RNA sequencing assay for the identification of body fluids [47]. The assay, which was designed for Illumina MiSeq/FGx platforms, contains 33 markers for the identification of blood, menstrual blood, semen, saliva, vaginal secretion and skin. The sensitivity and specificity of the assay was verified, and it was successfully applied to single source and mixed stains. Two classification methods, the percentage of reads in a sample that are due to each of the 6 body fluids/tissues tested and inter-sample differential gene expression revealed by agglomerative hierarchical clustering, were investigated to permit inference of the body fluid/ tissue. Dorum et al. used this MiSeq data set to build a probabilistic model based on partial least squares (PLS) followed by linear discriminant analysis (LDA) that predicts the origin of a stain [48]. The model incorporates quantitative information (MPS read counts) rather than just presence/absence of markers and resulted in improved predictions. The model was also successfully applied to mixed body fluid samples to identify the individual components in the mixture. A collaborative exercise within the EUROFORGEN/EDNAP laboratories was organized to test the efficacy of targeted mRNA sequencing to identify body fluids [49]. The above-mentioned Illumina MiSeq/FGx assay and an in-house assay for the Ion Torrent PGM/S5 platform (containing 29 markers) were evaluated. There was some inter-laboratory variability in read counts, but overall the results of the laboratories were similar. The in-house PGM/S5 workflow seemed to be less reliable with low input samples. Recently, an MPS assay on the Ion S5 system with optimized primer sets for body fluid identification was introduced [50]. The presented prototype assays suggest that the analysis of mRNA by targeting body fluid/ tissue specific amplicons is a promising tool for body fluid identification. The corresponding results will serve as a basis for improvements regarding marker selection, library preparation and sequencing.

Due to their tissue-specific expression, miRNAs are also suited for body fluid identification [51–55]. Several body fluid specific miRNA markers were identified using conventional methods, but most of the

candidate miRNAs were not concordant among the different studies [56]. Seashols-Williams et al. sequenced the whole miRNome from blood, feces, menstrual blood, saliva, semen, urine and sweat [57]. Several body fluid specific miRNA markers as well as markers for data normalization have been identified. Wang et al. investigated around 2600 miRNAs in blood and semen samples [58]. In the end, 6 blood- and 19 saliva-specific miRNA markers were identified. Based on whole miRNome data, Dorum et al. used a PLS-DA model do predict the origin of different forensically relevant body fluids (blood, menstrual blood, saliva, semen, skin and vaginal secretion) [59]. With 100 miRNA markers a prediction accuracy of 0.98 could be obtained, with 9 markers a prediction accuracy of 0.93 was achieved.

piRNAs were also investigated as candidate markers for body fluid identification [60]. Wang et al. sequenced small RNA libraries from blood, menstrual blood, saliva, semen, skin and vaginal secretion. Out of 376 candidate piRNA markers, 37 piRNAs were sufficient to discriminate most biological samples using a PLS-DA model. Five piRNAs were further evaluated in a TaqMan RT-qPCR assay. Three of the piRNAs can distinguish between blood and menstrual blood, while two piRNAs are useful for the discrimination of saliva and vaginal secretion.

2.2. Organ tissue identification

Determination of the organ source of tissues from crime scenes may aid in shootings and other investigations. The specific challenges with organ tissue samples from crime scenes are limited biological material, putrefaction, or damage due to crushing or dehydration. Traditionally, tissues have been identified using immunological or histological methods [61–65]. Recently, assays for tissue identification based on mRNA or miRNA have been developed, based on PCR/CE-methods [66–69]. In addition, a male (RPS4Y1) and female (lncRNA XIST) RNA marker to identify the sex of the donor was introduced [70]. Lindenbergh et al. developed an endpoint multiplex RT-PCR assay containing 14 organ tissue-specific markers (two per organ), a general muscle, blood and 18S-rRNA markers to analyze different samples such as neat organ and blood as well as mixed and mock casework samples. The assay showed good sensitivity and specificity and was able to identify components of mixtures [66]. In a follow up study, species specificity was confirmed by analyzing various human tissues (brain, lung, liver, skeletal muscle, heart, kidney, and skin tissue), different human body fluids (blood, menstrual blood, nasal mucosa, saliva, semen, vaginal mucosa) and animal samples (cat, dog, guinea pig, rabbit, cow, chicken, pig, sheep) [68]. Based on literature and data base searches, Lux et al. identified selected mRNA (C1orf61) and miRNAs (miR-124a, miR-124*) as candidate markers for the identification of brain tissue [67]. These markers were successfully tested on ballistic trace samples recovered from the inside of guns that were used for suicidal or homicidal contact shots. Sauer et al. evaluated 15 potential miRNA markers for the identification of brain, skeletal muscle/ general muscle, kidney, lung, skin, heart and liver using RT-qPCR [69]. They found five robust miRNA markers for the identification of brain, heart and skeletal muscle, liver and skin, but no reliable markers for lung and kidney. The qPCR-method is applicable to realistic forensic samples e.g. mixtures, aged and degraded material as well as traces generated by mock stabbings and experimental shootings at ballistic models.

In recent years, several studies employing MPS for organ tissue identification have been published. Based on literature and database searches, Hanson and Ballantyne developed an MPS assay, testing for the presence of 10 organs/tissues (adipose, brain, heart, intestine, kidney, lung, liver, skeletal muscle, stomach, trachea) by using 46 mRNA markers. Commercially available organ RNA (adipose, brain, colon, heart, intestine, kidney, lung, liver, skeletal muscle, spinal cord, stomach, trachea) and collected body fluid samples (blood, buccal, menstrual blood, semen, vaginal secretion) were used to test the assay [71]. The assay proved to be very specific, capable of detecting mixtures and showing low cross-reactivity with non-target body fluids or tissues. In follow up studies, the assay was successfully applied to various types of cadaver samples, tissue samples with different PMI as well as mock and bona fide casework samples [72,73].

3. Assigning body fluids to donors in mixed body fluid stains

Although it is now theoretically possible to definitively identify the most relevant body fluids in a non-physically separable mixture, it is not yet possible to directly link specific body fluids to individual DNA profiles in the mixture. The latter is needed to evaluate source versus subsource level propositions as part of a robust and accurate mixture deconvolution and DNA interpretation process. However, it is not straightforward to combine evidence from STRs and RNA/ presumptive tests to associate donors and body fluids, unless gender-specific body fluids are present. One plausible approach for associating body fluids and donors would be to compare mixture ratios in DNA and RNA; however, Harteveld [74] and Ingold [75] discourage this approach based on their CE and MPS results. Several publications propose the use of Bayesian networks to combine different types of evidence [76–78]. These are all based on presumptive tests to identify body fluids. A problem with the conditional probabilities based on presumptive tests that is needed in such a Bayesian network is that they to some extent may be case and laboratory specific, and so their use should be carefully considered for each case.

The presence of coding region SNPs (cSNPs) in body fluid specific mRNA transcripts enable the direct assignment of a body fluid to a specific individual in the mixture and exclude other individuals from contributing that body fluid. In a preliminary study Ingold et al. investigated 35 cSNPs in a targeted mRNA MPS assay for the Illumina MiSeq platform [79]. The cSNPs were specifically chosen for each forensically relevant body fluid with the aim of being highly discriminating between European individuals. In addition, a DNA MPS assay was introduced for the detection of the 35 selected cSNPs in genomic DNA (gDNA), to confirm the discriminatory power of these markers and, for comparison purposes, to determine the cSNP genotypes of the mixture contributors. In a proof-of-concept study by the same authors this cSNP panel was tested and evaluated more extensively [75]. This set of 35 cSNPs seems promising with regard to linking a body fluid to a donor, although more cSNPs are required to increase the discrimination power. Several scenarios were evaluated, including when the donors had contributed different body fluids as well as when they had contributed the same body fluid. The cSNP assay can also be used for body fluid identification, although the performance was not as good as a previously described body fluid specific 33 mRNA transcript assay [47]. Several of the cSNPs are located on the same gene, which may introduce linkage disequilibrium (LD). The implication of LD is that allele frequencies on different cSNPs cannot be regarded as independent in match probability calculations, and we need to handle haplotypes instead. This involves merging the cSNPs found to be in LD into one polymorphic unit. For cSNPs in the same amplimer, phased haplotypes can also be retrieved, which means they can be regarded as a microhaplotype. In addition, this cSNP panel was tested in a collaborative exercise within the EUROFORGEN and EDNAP laboratories [80]. Recently, an MPS assay on the Ion S5 system comprising a set of 21 cSNPs in body fluid specific mRNA transcripts (7 blood, 8 semen, 6 saliva) was reported [50]. The assay can identify all forensically relevant body fluids and skin as well as differentiating blood, semen and saliva transcripts from different individuals. The data from these prototype assays demonstrate that cSNPs can potentially directly link body fluids/ tissues with specific donors in mixed body fluid stains. However, additional markers are needed to increase the discrimination power in each body fluid/ tissue category. The assay performance varied among the cSNP markers which can result in low coverage. Still, even at high coverage, genotype calling can be difficult due to pseudo-homozygous phenomena with some of the cSNP markers. Therefore, the interpretation of cSNP data remains challenging and more work in this area is needed.

Recent non-MPS approaches to the use of cSNPs for assigning body fluids to specific DNA donors have been reported. Wang et al. explored the consistency of cSNP analysis results from DNA and RNA data using a single base extension (SBE) method (SNaPshot) [81]. Six blood-specific cSNPs were analyzed at the DNA and RNA level, using gDNA and cDNA specific primers. The study showed a high consistency of cSNP analysis results between DNA and RNA. Liu et al. selected five blood-specific mRNA biomarkers (SPTB, CD3G, AMICA1, ANK1, and GYPA) that encompass 16 cSNPs, to identify individuals in mixture samples composed of two body fluids [82]. They also used a multiplex PCR and SBE (SNaPshot) approach. The assay showed good sensitivity in detecting trace amounts of peripheral blood mixed with other body fluids (1:100) and a combined discrimination power (CDP) of 0.99929 in the Chinese population. Although an SBE approach is easier to apply and cost-effective, this method will not circumvent the challenges with cSNP data interpretation, as outlined above.

4. Determination of the age of stains (time since deposition, TsD)

Information on the age of a trace, or more precisely, the time since deposition (TsD) is crucial for evaluating the relevance of evidence in the investigative process. For example, if the trace has been deposited at the estimated time point of the crime, it has a high relevance while the relevance of older/younger traces is low, because deposition might not be related to the crime itself. Several techniques for TsD determination have been exploited, most of which track changes in the optical characteristics of blood [83,84]. However, these methods are limited to colored stains and are not easily transferred to white or nearly colorless traces such as saliva, semen or vaginal secretion. Novel TsD technologies commonly involve the analysis of the time-dependent degradation of biomolecules such as DNA or RNA. These biomolecules degrade over time and show differing persistence rates. Initial studies investigating the degradation of RNA were mostly performed in bloodstains but also in saliva, semen and plucked hair. It was suggested that the ratio of two amplicons originating from housekeeping genes (e.g. ACTB, GAPDH, 18S-rRNA) with differing degradation rates could be used to assess the age of a stain [85–89].

Lin et al. used a whole transcriptome sequencing (RNA-Seq) approach for characterizing the differential RNA degradation in different body fluids [13]. RNA was analyzed from fresh and aged (up to six weeks old) blood, menstrual blood, oral mucosa/saliva and vaginal secretion samples. To evaluate the quality and degradation level of the RNA, RNA integrity numbers (RIN) were assessed (10 indicates an intact sample, and 1 a fully degraded sample [90]). They found that RIN values of blood decreased (8.2–2.4) with increasing age, while oral mucosa, menstrual blood and vaginal secretion showed constantly low RIN values (4–1) throughout the six-week study period. All but one blood sample showed RIN values < 8, which is below the recommended RIN values for RNA sequencing. However, Lin et al. demonstrated that RNA sequencing can be performed with forensic samples, which are degraded as well as of low quality. 85% of all samples showed a Qscore of > 30, indicating high sequencing quality. The authors state that for RNA-Seq a high RNA input seems to be more important than high RIN values, since many samples with low RIN values generated high sequencing output. About 90% of blood and fresh menstrual blood reads could be aligned to the human reference genome, in contrast to 26–88% of oral mucosa and 5.6–59% of vaginal section reads. Since the oral cavity as well as the genital region are microbiota-rich environments, this finding would be expected [91–93]. Besides, they detected and confirmed known body fluid specific mRNA markers in samples of varying ages and presumably in differing states of degradation.

Weinbrecht et al. used RNA-Seq to analyze and characterize the time dependent degradation of blood, saliva, semen and vaginal secretion [14]. Samples were collected from two donors per body fluid and aged at room temperature (protected from light) for up to 1 year. 80–90% of blood and semen reads were of human origin. In contrast, only 5–10% of saliva and vaginal secretion reads were of human and 90% were of bacterial origin. Global transcript abundance declined over time in blood, semen and vaginal secretion samples. However, specific transcripts disappeared with differing rates. The greater the initial transcript abundance was, the longer a transcript could be detected. But the rate at which common transcripts disappeared varied between body fluids. There was no correlation between the length of a transcript and the degradation rate. Also, secondary structures seemed not no influence the persistence rate. They also observed that in dried stains the 5′ end of an mRNA transcript degrades faster than the 3′ end [94]. This differential degradation pattern can be followed with a qPCR assay that quantifies ~90 bp amplicons produced from the 5′ and 3′ ends of 4 transcripts chosen from the transcriptome of blood. They reported that the age of blood stains could herewith be accurately estimated within 2–4 weeks for stains less than 6 months of age and within 4–6 weeks for stains 6 months to 1 year old.

Salzmann et al. sequenced total RNA from fresh and aged (up to 9 months) blood, menstrual blood, saliva, semen, skin and vaginal secretion [15]. In their experimental setup, rRNA depletion (commonly used in mRNA-Seq studies to improve coverage) had a negative influence on the sequencing quality and also the downstream analyses. RNA degradation was assessed with transcript integrity numbers (TIN). TINs assign a score ranging from 0 to 100 to each transcript, where 0 indicates that no fraction of the transcript could be reconstructed by the sequencing reads and 100 represents a fully intact transcript. Aged samples showed in general a higher level of RNA degradation than fresh ones. In addition, transcriptome profiling was capable of identifying source-specific signatures from human RNA.

Results of these studies contribute to our understanding of mRNA degradation in forensically relevant body fluid stains, in ways that may lead to developing a tool to estimate the age of a stain at a crime scene. This knowledge is also relevant for choosing RNA markers that exhibit a relatively high degree of stability and are therefore suited for particular applications (e.g. reliable body fluid identification). Also, targeting stable amplified regions (StaRs) for primer design may improve the amplification of target transcripts in degraded body fluid samples [46].

5. Determination of the donor age

Establishing the age of an unknown person can provide important information in police investigations, for example to narrow down the pool of suspects or for the identification of unknown human remains. A distinction is made between chronological and biological age [95,96]. The chronological age is the time elapsed since birth. The biological age is the age based on a cellular level, which is influenced by individual factors such as chronic diseases, epigenetics, lifestyle and environmental impacts. In a forensic context, age estimation is commonly performed with morphological analysis based on the radiological examination of dental and skeletal developmental stages [97]. However, these approaches are not applicable for retrieving the age of an unknown person from biological samples or traces obtained at crime scenes.

Human exceptional longevity represents an extreme phenotype and studies have shown that centenarians exhibit a remarkable compression of morbidity and a resistance to otherwise lethal illnesses occurring earlier in life, providing important insights into the underlying molecular mechanisms of aging [98]. In general, aging is the gradual decline of physiological functions leading to age-dependent fitness loss, diseases and eventually mortality [99]. The process of aging is characterized, and likely influenced, by gradual alterations of biomolecules such as telomere repeat length, mitochondrial DNA mutations, accumulation of advanced glycation end products (AGEs), decline in signal joint T-cell receptor rearrangement excision circles (sjTRECs), aspartic acid racemization (AAR), epigenetic modifications and gene expression changes during the human lifespan [95,100–106]. Among these age-associated biomarkers, epigenetic aging clocks represent the most accurate

models for age prediction so far and have therefore attracted attention in the forensic field [107–109]. However, the required quantity of high-quality DNA for DNA methylation analysis is often not available in forensic trace material. Therefore, prediction models based on age-related changes in the transcriptome seems to be a promising alternative for the age estimation of the person who contributed a forensic sample.

Numerous whole-genome studies have focused on changes in the transcript levels in people of different age groups and have identified subsets of genes that show age-related changes in expression in mitochondrial, metabolic and immune function-related pathways [110–112]. One of the first studies focusing on age-related transcriptome changes was performed in blood samples of 154 healthy individuals between 23 and 77 years of age and identified 16 age-dependent transcripts mostly involved in emerging or recessing functions of immune cells associated with premature senescence [113]. In 2015, Peters et al. carried out one of the largest age-associated transcriptome studies based on the meta-analysis of 8847 human peripheral blood samples from six independent cohorts [114]. In this study, 1497 genes were reported as differently expressed in relation to chronological age with a mean absolute deviation (MAD) of 7.8 years. Most of the RNA markers were involved in known aging processes including dysregulation of transcription and translation, immune senescence, ribosome biogenesis and mitochondrial decline. In addition, differences between the transcriptome age and chronological age were linked to clinical features such as blood pressure, blood glucose and cholesterol levels. In 2016, Zubakov et al. generated different age prediction models by considering not only age-related RNA markers, but also other biomarkers of age such as DNA methylation, telomere length and sjTRECs in blood samples of 350 healthy male individuals of a wide range of ages [115]. The most accurate prediction model was achieved with a subset of 6 biomarkers, namely 5 methylation markers and 1 mRNA marker with a MAD of 4.5 years. Another transcriptome aging clock was published in 2018 which was trained on human dermal fibroblast profiles from 133 participants from 1 to 94 years old [116]. The model was based on a machine learning approach that could predict chronological age with a MAD of 7.7 years. A deep neuronal network was used for age prediction in a large study of 6465 blood samples of individuals from 17 combined datasets obtained from specific ethnic populations with the best-performing prediction model achieving a mean absolute error (MAE) of 5.94 [117]. With this aging clock model, the authors were able to show that ethnically diverse aging clocks have the potential to predict chronological age with high accuracy.

Beside mRNA based transcriptome aging clocks, a few studies also focused on different types of noncoding RNA molecules and their implications in human aging. Recent studies have suggested that changes in miRNA expression levels occur with human cellular senescence, whereby most of them are downregulated with increasing human age [118–120]. This loss of miRNA function during the aging process may be due to transcriptional repression, deletion, mutation, epigenetic silencing or aberrant miRNA processing [121]. Huan et al. explored age-related miRNA expression in blood samples of 5221 adults and identified 127 miRNAs that were differentially expressed by age whereas most miRNAs were under-expressed in older individuals [122]. A study by Fang et al. focused on 220 blood samples from Han Chinese descendants and established an miRNA age prediction model based on six age-related miRNAs with a MAE of 5.52 and 7.46 years in male and female bloodstain samples, respectively [123].

Several studies have shown that lncRNAs are linked to processes important for various aging-associated diseases, including cancer or neurodegenerative diseases [124,125]. A study in senescent human fibroblast cells identified several lncRNAs that display differential expression levels as compared with the corresponding young cells [126]. Another study reported age-associated lncRNA expression patterns in 29 human tissues [127]. These lncRNA markers are involved in immune system processes, signal transduction and transcription and most of them are highly tissue specific.

Dluzen et al. identified in serum samples of a small cohort of 13 young (30–32 years) and 10 old (80–85 years) African American women age-related differences in different types of circulating extracellular RNAs (exRNAs) species [128]. Among these different RNA types, they observed higher levels of mitochondrial transfer RNAs and mitochondrial ribosomal RNAs in older individuals which is consistent with a progressively impaired mitochondrial function with increasing age.

In general, human aging is a complex process. Many gene expression markers vary considerably between different body sites due to their functional involvement in gene regulation, which will require cell type specific prediction models. The challenges of molecular age prediction models include delineating the relationship between the chronological versus the biological age. Multi-omics methods such as combining DNA methylation, mRNA and miRNA markers together could improve the accuracy of age prediction models [129]. Finally, predictive biomarkers and analysis methods will need to be validated prior to implementation into the forensic field.

6. Estimation of the post-mortem interval (PMI)

Post-mortem interval (PMI) refers to the time interval between physiological death and the examination of the deceased person and is an important part of forensic death investigations. Currently used methods to determine PMI include biochemical, physical, physicochemical, microbiological, entomological and botanical investigations [130]. In standard forensic practice, short-time PMI, i.e. within the first 24 h post-mortem, is usually estimated by assessing gross post-mortem physical changes such as the body temperature, muscular and neuro-muscular reactivity and post-mortem lividity [131]. The long-time PMI (days to years) is evaluated based on the decomposition stage, entomological analysis (fly larvae growth) and determination of bone radioisotope concentration. However, these methods are often inaccurate and influenced by individual characteristics such as age, gender, physiological and pathological states of the deceased. Therefore, more precise methods are needed for the determination of PMI. One viable approach is the time-dependent degradation of biological markers such as DNA, RNA and proteins [132,139,171].

In contrast to DNA, the less thermodynamically stable RNA was initially believed to degrade rapidly after death due to ubiquitously present ribonucleases, bacterial processes and environmental influencing factors such as sunlight, humidity or high temperatures [5]. During the last years, several studies have demonstrated that, depending on the circumstances, RNA can remain largely intact even for long time periods and quantification of mRNA degradation could be used for PMI estimation [133]. In 2003, Bauer et al. showed in a pilot study that RNA degradation of the fatty acid synthase-messenger RNA (FASN mRNA) is significantly correlated with the PMI in autopsy cases up to 5 days post-mortem [134]. Further studies focused on quantitative PCR analysis and RNA integrity numbers (RIN) in various tissues and cell types in rats and humans [133,135–137]. They suggested GAPHD, β-actin, 18S rRNA and HIF-1α as promising markers to estimate the PMI up to several days. Other studies reported no correlation between PMI and RNA degradation in human samples with long PMI up to 40 years [138,139]. Since most of the commonly used transcripts degrade over time, Tu et al. focused on the stability of miRNAs and circular RNAs (circRNAs) in mice and reported the following markers as suitable for PMI estimation: miR-122, miR-133a and 18S in heart tissue, LC-Ogdh, circ-AFF1 and miR-122 in liver and miR-133a, circ-AFF1 and LC-LRP6 in skeletal muscle tissue [132]. Additionally, Li et al. explored the relationship between time-dependent level changes of miRNA (miR-1-2) and 18S rRNA in rat cardiac muscle and demonstrated that these markers could be useful for estimating early PMI [140]. Zhang et al. observed that U6, GAPDH and 18S RNA were the most suitable PMI markers in their investigated set of human tissues (heart, brain, kidney and skin) from 40 individuals with different PMI ranging from 1 to 72 h [141]. But they

emphasized that miRNAs should not be chosen as endogenous controls because they are less stable (at least in vivo), than mRNA due to the lack of the 5'-cap structure or 3'-polyA tail. Another study investigated the miRNA expression level in 71 human bones with a PMI of up to 2 years and identified two specific miRNA markers (let-7e and miR-16) which showed a negative correlation between the expression level and increasing PMI [142].

A relatively novel and emerging topic in the forensic field is the investigation of the human thanatotranscriptome to determine cause and time of death [143–146]. The thanatotranscriptome is derived from the Greek word for death (*thanatos*-), and it encompasses all RNA transcripts expressed from the part of the genome that is still functional or that becomes awakened in internal organs of a dead body. After death, apoptosis or programmed cell death is leading to the activation or repression of a plethora of genes and of diverse regulatory factors depending on the absence or presence of stimulated feedback. In a preliminary thanatotranscriptome study, Javan et al. showed that RNA is stable in internal organs of cadavers and that pro-apoptotic genes such as caspases were up-regulated and the expression of genes responsible for anti-apoptosis such as *BCL2* and *BAG3* were down-regulated in human liver samples [145]. Another study investigated mRNA transcript abundances in prostate tissue from human remains and identified several anti-apoptotic genes (*BCL2*, *BFAR*, *BIRC2*) and negative regulator of apoptosis (*XIAP*) that produced significantly elevated fold-changes in a time-dependent manner [146]. In addition, pro-apoptotic genes such as *CASP2*, *DIABLO* and *APAF1* also produced significant up-regulation in relation to time of death, indicating that there is still active gene expression after death.

In addition, outside of the forensic context, several recent studies examined gene expression based on large datasets of post-mortem samples in order to identify global patterns of post-mortem RNA changes. Hunter et al. analysed 71,179 microarray data of zebrafishes and mice and identified 1063 transcripts that were significantly increased in abundance between 48 and 96 h post-mortem [147]. Systematic investigation of various human post-mortem gene expression data sets derived from the Genotype-Tissue Expression (GTEx) database [18] (2016 samples from 15 tissue types), demonstrated that mRNA degradation occurs in a tissue-specific manner and is associated with gene-specific properties [148]. Considering the possibility that post-mortem mRNA degradation may be a non-linear process, the study identified 266 genes which showed a significant difference in expression variance in short-time and long-time PMI. A third study analysed mRNA sequencing data from 7105 humans including 36 tissues from the GTEx project and showed that there are remarkable differences between tissues regarding the transcriptional response to PMI [149]. Some tissues such as the digestive tract exhibited early changes in gene expression after death whereas gene expression levels in other tissues such as the central nervous system, heart and brain remain more stable over several hours. In addition, a machine-learning model was applied to predict the time of death of a recently deceased individual with a few key tissues. Interestingly, the most informative tissues to predict the PMI are relatively accessible ones such as skin and subcutaneous adipose. Although this prediction model was only generated as a proof of concept, the R^2-value for the real vs. predicted tissue PMI was 0.86 and the prediction performance was not impacted by the cause of death.

A major limitation of the above mentioned studies is that they were carried out under controlled conditions such a fixed temperature and were only conducted over relatively short time frames, therefore the effects of changing environmental conditions on RNA degradation were not extensively evaluated and therefore the suitability for real-life cases is accordingly restricted [133]. In general, more sensitive biomarkers are necessary for PMI estimation, especially also with a focus on long-term PMI estimation in cases of skeletal remains [139].

7. Post-mortem death investigations: cardiac causes of death

Analyzing the functional status of cells in post-mortem samples could offer insight into the pathological mechanisms leading to death. Messenger RNA up-regulation can occur rapidly within minutes so that even acute events could be monitored. However, for the quantitative use of mRNA data from post-mortem tissues it is necessary to rule out concomitant post-mortem changes. Here we focus specifically on the determination of cardiac causes of death.

Sudden and unexpected death of a previously healthy infant, adolescent or young adult is a tragic and distressing event for those left behind. Up to 30% of these cases remain unexplained after standard forensic autopsy, with no definite cardiac etiology identified after gross and microscopic inspection of the heart and are therefore termed as sudden infant death syndrome (SIDS) or sudden unexplained death (SUD) [150]. There are often no apparent warning signs during lifetime and sudden death might be the first manifestation of an undetected cardiac disease. In the last years, several genetic studies have demonstrated that post-mortem genetic testing ('molecular autopsy') represents a valuable tool to identify functional pathogenic variants in cardiovascular disease-associated genes in 20–35% of autopsy-negative SUD/SIDS cases [151–154]. However, 70–80% of the SUD cases still remain elusive after genetic testing, and therefore post-mortem RNA expression profiling may provide a supplementary tool to investigate the cause of death in autopsy cases.

Myocardial transcriptome analysis in human cardiovascular diseases has shown that the expression pattern can clearly distinguish between arrhythmogenic right ventricular cardiomyopathy (ARVC), dilated cardiomyopathy (DCM) and healthy ventricular myocardium [155]. Another study demonstrated that myocardial mRNA expression profiles are changed in sarcolemma calcium regulation, apoptosis, and adipogenesis in patients with ARVC compared to DCM patients and controls, suggesting that these molecular pathways may play a critical role in the pathogenesis of ARVC [156]. Furthermore, certain mRNA species, namely those encoding hemoglobin A1/2 and B (*HBA1/2* and *HBB)* as well as pyruvate dehydrogenase kinase 4 (*PDK4*) have been reported to exhibit distinct postmortem expression patterns in the left ventricular free wall of sudden cardiac death patients compared to the corresponding tissues from control persons with non-cardiac causes of death [157]. Schiano et al. investigated specific transcriptome changes occurring in cardiac tissues of patients with heart failure compared to healthy patients and were able to identify DCM- and restrictive cardiomyopathy-specific expression signatures for protein-coding genes and describe dysregulation in focal adhesion and oxidative phosphorylation in end-stage heart failure [158]. Andersen et al. performed whole genome sequencing (WGS) and whole transcriptome sequencing (WTS) in sudden cardiac death victims in order to correlate gene expression levels with DNA variations in regulatory non-coding regions of the genome [159]. In their cohort of 13 SADS (sudden arrhythmic death syndrome) and SUDI (sudden unexplained death in infancy) cases, they identified a rare variant in the promoter region of the nexilin F-actin binding protein encoding gene *NEXN* (c.–194A>G), that was found to be associated with decreased expression of *NEXN* and cardiac hypertrophy.

With respect to the role of non-coding RNAs in cardiac physiology and pathology, a number of miRNAs have been identified in the healthy adult heart that are highly expressed in non-diseased cardiac tissue and thus likely play a key role in both normal cardiac maintenance and diseases [160]. Furthermore, HCM (hypertrophic cardiomyopathy) patients with *MYBPC3* mutations demonstrated differentially expressed miRNAs which target mRNAs involved in cardiac hypertrophy and cardiac beta-adrenergic receptor signaling [161]. In addition, several studies have identified heart-specific lncRNAs that are up- or down-regulated during acute myocardial infarction and heart failure, whereas others control hypertrophy and cardiomyocyte death by interfering with miRNAs [162–164].

These findings indicate that RNA analysis of post-mortem tissue could contribute to the determination of the cause of death with regard to cardiovascular diseases. Combined RNA and DNA analysis may help to solve some of the formerly unexplained death cases. This is

Table 1
Collation of MPS-based topic-specific RNA markers.

Application	Organism	Tissue / Body Fluid / Organs	Technique	Marker	RNA species	Reference
Tissue Identification	Human	Brain	Targeted MPS	SNAP25, RTN1, GABRA1, OPALIN, GFAP, NEUROD6	mRNA	[71,72]
Tissue Identification	Human	Lung	Targeted MPS	SFTPB, SFTPD, SFTPA1	mRNA	[71,72]
Tissue Identification	Human	Trachea	Targeted MPS	BPIFB1	mRNA	[71,72]
Tissue Identification	Human	Liver	Targeted MPS	AMBP, F2, SPP2, CFHR2, F9, MBL2, AHSG, C9	mRNA	[71,72]
Tissue Identification	Human	Skeletal muscle	Targeted MPS	TNNI2, MYLK2, ATP2A1, MYH2, NEB, MYLPF	mRNA	[71,72]
Tissue Identification	Human	Heart muscle	Targeted MPS	ITGB1BP3	mRNA	[71,72]
Tissue Identification	Human	Heart	Targeted MPS	MYBPC3, NPPB, NPPA, TNNI3	mRNA	[71,72]
Tissue Identification	Human	Kidney	Targeted MPS	UMOD, SLC12A1, SLC34A1, SLC22A12	mRNA	[71,72]
Tissue Identification	Human	Adipose	Targeted MPS	TUSC5, ADIPOQ, PLIN1	mRNA	[71,72]
Tissue Identification	Human	Intestine	Targeted MPS	FABP6, LCT, CCL25, DEFA5, DEFA6	mRNA	[71,72]
Tissue Identification	Human	Stomach	Targeted MPS	PGA5, PGA3, PGA4, GIF, GKN1	mRNA	[71,72]
Body Fluid Identification	Human	Blood	Targeted MPS	ALAS2, ANK1, SPTB, CD3G, CD93, AMICA1	mRNA	[47]
Body Fluid Identification	Human	Semen	Targeted MPS	PRM1, PRM2, TGM4, SEMG1, SEMG2, KLK3	mRNA	[47]
Body Fluid Identification	Human	Saliva	Targeted MPS	HTN3, HTN1, STATH, PRB3, PRB4, PRH2	mRNA	[47]
Body Fluid Identification	Human	Vaginal secretion	Targeted MPS	CYP2B7P1, DKK4, FAM83D, CYP2A6	mRNA	[47]
Body Fluid Identification	Human	Menstrual blood	Targeted MPS	MMP10, LEFTY2, MMP7, MMP11, SFRP4, MMP3, STC1	mRNA	[47,170]
Body Fluid Identification	Human	Skin	Targeted MPS	LCE1C, CCL27, IL37, SERPINA12, KRT77, COL17A1	mRNA	[47]
Body Fluid Identification	Human	Blood	MPS	miR-200b, miR-486-5p, miR-16-5p, miR-451a, miR-144-3p, miR-126-5p, miR-144-5p	miRNA	[57,58]
Body Fluid Identification	Human	Semen	MPS	miR-891a	miRNA	[57,58]
Body Fluid Identification	Human	Saliva	MPS	miR-26b, miR-203a-3p, miR-205-5p, miR-223-3p, miR-200c-3p, miR-141-3,; miR-375, miR-34a-5p, let-7c-5p, miR-27b-3p, miR-125b-5,; miR-23b-3p, miR-99a-5p, miR-29a-3p, miR-23a-3p, miR-27a-3p, miR-210-3p, miR-24-3p, miR-29b-3p, miR-22-3p	miRNA	[57,58]
Body Fluid Identification	Human	Menstrual blood	MPS	miR1246	miRNA	[57]
Body Fluid Identification	Human	Urine/ feces	MPS	miR-320c, miR-10b-5p	miRNA	[57]
Body Fluid Identification	Human	Blood, semen, saliva, vaginal secretion, menstrual blood	MPS	1034 different markers	miRNA	[59]
Body Fluid Identification	Human	Blood/ menstrual blood	MPS	piR-hsa-27622, piR-hsa-1207, piR-hsa-27493	piRNA	[60]
Body Fluid Identification	Human	Saliva/ vaginal secretion	MPS	piR-hsa-27493 and piR-hsa-26591	piRNA	[60]
cSNPs	Human	Blood	Targeted MPS	AMICA1, ANK1, CD3G, CD93, SPTB	mRNA	[75]
cSNPs	Human	Semen	Targeted MPS	KLK3, SEMG1, SEMG2, TGM4	mRNA	[75]
cSNPs	Human	Saliva	Targeted MPS	MUC7, PRB3	mRNA	[75]
cSNPs	Human	Vaginal secretion	Targeted MPS	CYP2A7, DKK4	mRNA	[75]
cSNPs	Human	Menstrual blood	Targeted MPS	MMP10, MMP7	mRNA	[75]
cSNPs	Human	Skin	Targeted MPS	COL17A1, KRT77, LCE1C	mRNA	[75]
Age of Donor	Human	Blood	Microarray	16 transcripts	mRNA	[113]
Age of Donor	Human	Blood	Gene expression meta-analysis	1497 genes	mRNA	[114]
Age of Donor	Human	Blood	Microarray	NRCAM, ABLIM1, LRRN3, NELL2, SLC16A10, NOG, AK5, CCR7, CFH	mRNA	[115]
Age of Donor	Human	Dermal fibroblasts	RNA-Seq	Ca. 4000 genes	mRNA	[116]
Age of Donor	Human	Blood	Gene-expression meta-analysis	1497 genes	mRNA	[117]
Age of Donor	Human	Blood	RNA-Seq	127 marker	microRNA	[122]
Age of Donor	Human	Blood	RNA-Seq	miR-98-3p, miR-324-3p, miR-32-3p, miR-330-5p, miR-374c-5p, miR-342-3p	microRNA	[123]
Age of Donor	Human	Fibroblast cells	Microarray	lncRNAD3	lncRNA	[126]
Age of Donor	Human	29 different tissues	GTEx data	1264 age-lnRNAs	lncRNA	[127]
Age of Donor	Humans	Serum	RNA-Seq	1154 genes	exRNA	[128]

(*continued on next page*)

Table 1 (*continued*)

Application	Organism	Tissue / Body Fluid / Organs	Technique	Marker	RNA species	Reference
PMI	Zebrasfish/ Mice	Brain/ liver	Microarray	1063 genes	mRNA	[147]
PMI	Human	15 different tissues	GTEx data	266 genes	mRNA	[148]
PMI	Human	36 different tissues	GTEx data	> 600 genes (depending on the tissue)	mRNA	[149]

specifically important for direct relatives, since they might carry the same genetic variant and are at risk to develop a similar disease or even die. Therefore, an optimal management of such cases should be based on a multidisciplinary team involving legal pathologists, geneticists, and cardiologists [165]. In case of a confirmed cardiac variant or mechanism there are therapy options for the affected relatives that could save their lives.

8. Future of forensic transcriptomics

As we have shown in this review, transcriptome analysis has increasingly become useful in forensic genetics and reported studies have covered a variety of several topics and applications. Targeted and whole transcriptome analyses have been successfully applied to forensic material, including dried physiological stains and post-mortem tissues. Some promising methods and markers have been tested and evaluated, especially for the identification of body fluids and tissues. Table 1 shows a collation of the MPS-based topic-specific RNA markers that were described or alluded to in the previous sections. This list is not claimed to be comprehensive, but represents more of a momentary snapshot of the general state of the field at this time. The field and technologies develop fast and continued studies in the near future should result in the use and/or discovery of additional RNA biomarkers and types.

Further developments in forensic transcriptomics might include point-of-use, non-PCR based RNA methods for rapid (< 1 h) definitive body fluid identification as a triage for downstream DNA analysis. It is also possible that non-microscopical identification of spermatozoa will be possible due to the presence of sperm specific RNAs (e.g. protamines such as PRM1) [166]. It is likely that the transcriptome will be a constituent part of a (multi)omics/data integration process for evidence analysis that will include the not-isolated biological entities of the genome, transcriptome, methylome, proteome and microbiome. This will include not only mRNA and miRNA, since mRNA is regulated by miRNA binding and both exhibit tissue specific expression, but also other non-coding RNA species. Most genes are expressed in multiple isoforms caused by alternative or cryptic splicing and intronic alternative polyadenylation sites [167]. This additional variation, which is easily revealed via MPS methods, has not yet been applied in forensic transcriptomics. Such studies are expected to improve our ability to resolve specific cellular phenotypes in complex organs and tissues such as the brain and also to increase the number of specific biomarkers available to identify epithelial cell types from skin, the mouth, the vagina and the uterus. Gene expression itself also exhibits genetic variation and expression quantitative trait loci (eQTLs) have been described [168]. This additional variation is yet to, but should as the field matures, be studied in the forensic context as is the influence of transcriptome altered diseases [169] on RNA-based forensic analyses.

It is expected that artificial intelligence (AI), such as machine learning, will play an increasing role in data analysis and interpretation of the transcriptome and other -omes. Socio-economic forces will probably result in the use of whole genome sequencing approaches (Whole Genome (WGS)/ Transcriptome (WTS)/ Exome (WES)) exploiting the concept that one obtains the whole -ome data from which the relevant case dependent biomarker information is subsequently extracted. Since most of the genome is faithfully reflected in transcribed RNA, it is not out with the bounds of possibility that RNA could be used as a complement to DNA typing for the whole gamut of forensic genomic applications including personal identification, providing context to the identification and obtaining a genetic eyewitness of the donor of a physiological stain (i.e. a phenotype that includes ethnicity, sex, age, external visible and behavioral traits, etc.).

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