Received: 28 October 2023 | Revised: 12 March 2024 | Accepted: 26 March 2024

DOI: 10.1002/pmic.202200335

Proteomics
Proteomics and Systems Biology

REVIEW

From flesh to bones: Multi-omics approaches in forensic science

Noemi Procopio | **Andrea Bonicelli**

Research Centre for Field Archaeology and Experimental Taphonomy, School of Law and Policing, University of Central Lancashire, Preston, UK

Correspondence
Noemi Procopio, Research Centre for Field Archaeology and Experimental Taphonomy, School of Law and Policing, University of Central Lancashire, Preston, PR1 2HE, UK.
Email: nprocopio@uclan.ac.uk

Funding information
UK Research and Innovation, Grant/Award Number: MR/S032878/1

Abstract

Recent advancements in omics techniques have revolutionised the study of biological systems, enabling the generation of high-throughput biomolecular data. These innovations have found diverse applications, ranging from personalised medicine to forensic sciences. While the investigation of multiple aspects of cells, tissues or entire organisms through the integration of various omics approaches (such as genomics, epigenomics, metagenomics, transcriptomics, proteomics and metabolomics) has already been established in fields like biomedicine and cancer biology, its full potential in forensic sciences remains only partially explored. In this review, we have presented a comprehensive overview of state-of-the-art analytical platforms employed in omics research, with specific emphasis on their application in the forensic field for the identification of the cadaver and the cause of death. Moreover, we have conducted a critical analysis of the computational integration of omics approaches, and highlighted the latest advancements in employing multi-omics techniques for forensic investigations.

KEYWORDS
age-at-death, bioinformatics, forensic sciences, manner/cause of death, multi-omics, omics, personal identification, *post-mortem* interval

Abbreviations: 5mC, 5-methylcytosine; AAD, age-at-death; ADD, accumulated degrees days; ADH, accumulated degree hours; alphaHL, staphylococcal alpha-hemolysin; AM, *antemortem*; AMI, acute myocardial ischaemia; AR-CpG, age-related CpG; ARF, Anthropology Research Facility; BCE, before the common era; bp, base pairs; BrS, Brugada syndrome; BS, bisulphite sequenciandrealsobaric tags for relative and absolute quantitation; CE, capillary electrophoresis; CID, collision-induced dissociation; circRNA, circular RNA; CLR, continuous long read; CPVT, catecholaminergic polymorphic ventricular tachycardia; DDA, data-dependent acquisition; DIA, data-independent acquisition; DNBSEQ, DNA nanoball sequencing; dNTP, deoxynucleotide triphosphate; DVI, disaster victim identification; ECD, electron capture dissociation; ELISA, enzyme-linked immunosorbent assay; emPCR, emulsion PCR; ESI, electrospray ionization; FARF, Forensic Anthropology Research Facility; FTI-CR, Fourier transform ion cyclotron resonance; GA, genetic algorithm; GC, gas chromatography; gDNA, genomic DNA; GTEx, genotype-tissue expression; GVP, genetically variant peptide; HCD, higher-energy collisional dissociation; HCM, hypertrophic cardiomyopathy; HPLC/UPLC, high and ultra performance liquid chromatography; HR, high resolution; KNN, k-nearest neighbour; k-NN, k nearest neighbors; LC, liquid chromatography LC; LIT, linear-ion trap; LQTS, long QT syndrome; LVTA, lethal ventricular tachyarrhythmia; MAE, mean absolute error; (MAS)-NM, magic angle spinning; M/COD, manner/cause of detah; MDA, multiple displacement amplification; MFS, Marfan syndrome; miRNA, microRNA; MLP, multi-layer perceptron; MLR, multivariate linear regression; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MRM, multiple reaction monitoring; mtDNA, mitochondrial DNA; NGS, next generation sequencing; NMR, nuclear agnetic resonance; NN, neural network; ONT, Oxford Nanopore Technology; OPLS-DA, orthogonal partial least squares discriminant analysis; OSC-PLS, orthogonal signal correction-PLS; PCA, principal component analysis; PCR, polymerase chain reaction; PLS, partial least squares; PLS-DA, partial least squares discriminant analysis; PM, *post-mortem*; PMI, *post-mortem* interval; PMSI, *post-mortem* submersion interval; PPi, pyrophosphate; PRM, parallel reaction monitoring; QA, quality assurance; QC, quality control; QQQ, triple quadrupole; Q-TOF, quadrupole-time of flight; RF, random forest; RFLP, terminal restriction fragment length polymorphism; RMSE, root-mean-square error; RNA-seq, RNA sequencing; SBL, sequencing by ligation; SBS, sequencing by synthesis; SCD, sudden cardiac death; SGD, stochastic gradient descent; SIDS, infant death syndrome; SILAC, stable-isotope labelling by amino acids in cell culture; SIRM, stable isotope resolved metabolomics; SMRT, single molecule, real-time; SNP, single-nucleotide polymorphism; SQTS, short QT syndrome; STAFS, Southeast Texas Applied Forensic Science; stLFR, single-tube long fragment read; STR, short tandem repeats; SVM, support vector machine; SVMs, support vector machines; TADR, thoracic aortic dissection or rupture; VH, vitreous humor; VIP, variable importance in the projection; VUS, variants of uncertain significance; WES, whole exome sequencing; WGA, whole genome amplification; WGS, whole genome sequencing.

1 | INTRODUCTION

One of the fundamental concepts in forensic anthropology is that 'every body tells a story'. This notion, deeply ingrained not only in forensic literature but also embedded in common knowledge, underscores the significance of studying human remains to enable the reconstruction of past events and to contribute to the investigation of unresolved cases. The art of studying the cadaver to solve criminal cases has old roots and dates back to 82 BCE, when, in the ancient Rome, Cicero used forensic evidence from the exhumed body of Sextus Roscius's father to demonstrate the innocence of the son, charged of patricide. The use of scientific examinations to establish innocence or guilt in legal matters summarizes the main concept of forensics also in modern days, which is defined as 'the application of scientific principles and techniques to matters of criminal justice especially as relating to the collection, examination, and analysis of physical evidence' (Merriam-Webster.com Medical Dictionary, Merriam-Webster, accessed on 4 September 2023). Personal identification and the establishment of the cause of death are two key points in any investigation involving human remains, as they form the basis for understanding the circumstances surrounding a person's death and contribute to the pursuit of justice. Traditionally, the role of the forensic anthropologist has been to assist with sex, age, ethnicity, and stature determination, as well as to study skeletal traumas associated with the cause of death, starting from remains in various decomposition stages and states (*e.g.*, fleshed, decomposing, skeletal, burnt) [1] with the final aim to identify the remains. This process involves comparing *antemortem* information of a person reported missing with the results of a *post-mortem* examination of an unidentified individual. A positive identification is confirmed when the *antemortem* and *post-mortem* data match completely, with no explainable discrepancies. The evidence must be substantial enough to establish that both sets of data pertain to the same individual [2]. The use of biometrics, the measurement of physical aspects of the human body, has become the gold standard in forensic investigations. Amongst those, fingerprint identification, which assesses variations in the typical ridge patterns (*minutiae*) unique to each person, continues to be widely used in forensic settings, in conjunction with DNA analysis. Another tool largely employed in personal identification is facial recognition, driven by the extensive deployment of closed-circuit television systems and personal digital devices that facilitated the creation of vast databases that serve as reference matches, leading to significant advancements in facial recognition digital technology. Finally, gait analysis has become extremely popular in personal identification for identifying living individuals from a significant distance. It is out of doubt that the combination of more than one of these approaches could lead to more accurate identifications in various scenarios [3]. Biometrics traits, as all disciplines applied to the forensic settings, need to adhere to seven essential characteristics: universality, distinctiveness, permanence, collectability, performance, acceptability and resistance to circumvention [4]. However, in case of advanced body decomposition, these features may not be reliable or accessible, necessitating the consideration of additional traits for personal identification. The use of orthopedic surgical implants can provide accurate personal identification and was found to be extremely effective during disaster victim identification (DVI) [5]. When recording orthopedic devices, serial number and comprehensive details on the manufacturer as logo, name and company number are essential information to be collected. Moreover, these have to match patients' medical records that have to be legally disclaimed by the next of keen [5]. The main limitation is the lack of durability of implants or medical records resulting, frequently, in only partial identifications.

Traditional forensic anthropology approaches aim to create the biological profile of the individual by reconstructing age, sex, stature, and ancestry of unknown skeletal remains to aid positive identifications [6]. Despite not leading automatically to positive identifications, these contribute to the goal of either confirming a deceased person's identity or eliminating the possibility of certain remains belonging to a specific missing individual.

Age estimation is a critical demographic feature that must be evaluated during the analysis of unidentified skeletal remains. However, traditional methods for assessing age are subject to significant limitations. The expertise of the individual conducting the analyses remains a crucial factor in accurately evaluating anthropological characteristics through morphological methods. In their research, Baccino et al. [7] examined the accuracy of age-at-death (AAD) estimations using various approaches, including the Suchey-Brooks [8] method for pubic symphysis, the İşcan et al. [9] method for sternal end cartilage calcification of the fourth rib, the Lamendin et al. [10] single root translucency technique and the Kerley et al. [11] histomorphometric analysis of the femur. This study was conducted on a French population, and two observers (a forensic pathologist and a forensic anthropologist) demonstrated comparable age estimation abilities. However, the histological method displayed the highest level of inaccuracy, likely due to different levels of experience or expertise of the observers. Furthermore, the pubic symphysis method, while showing relatively low inter-observer inaccuracy, exhibited notable differences in terms of observer variability. The study also evaluated a multifactorial approach, which generally outperformed individual methods used in isolation [7]. Similarly, Garvin and Passalacqua [12] investigated the preferences of 145 forensic anthropologists regarding age estimation methods. Irrespective of the experience level, the Suchey–Brooks method remained the preferred choice, followed by cranial sutures and dental wear. A noteworthy outcome of this study was the majority of anthropologists indicating a case-specific approach to selecting age estimation methods [12]. Overall, the findings underscore the pivotal role of experience, especially to guide method selection, in morphological age estimation. This suggests that while the nature of the case partly influences the choice, experience remains a central factor. This inherent non-quantifiable bias must not be overlooked in terms of witness admissibility. Another technical concern pertains to the fact that most methods are developed for specific reference populations, potentially hindering their application on samples from unknown populations. Konigsberg and Frankenberg [13] warned against applying methods designed for one population to another, as this can lead to increased error rates, a phenomenon observed with traditional methodologies [14, 15]. This issue could also extend to

inter-population variations influenced by factors such as income, diet and physical activity. Moreover, endogenous, and exogenous factors contribute to significant inaccuracy in advanced age individuals, making these methods unsuitable for forensic applications. Advancements in medical sciences and biomedical engineering have yielded quantitative methodologies for studying physiological aging and pathological processes. These approaches offer standardized experimental procedures and statistical analyses that provide quantifiable error measures, giving confidence in reliability but still require validation to be applied to forensic settings [16, 17].

Similarly, for sex estimation, the three main approaches used in forensic examinations of skeletal remains are quantitative and qualitative morphological examinations, morphometric evaluations and quantitative metric methods that can be performed both on dry bones or via digital imaging [18]. Morphological methods, despite being the most commonly applied in forensic settings, have been considered heavily affected by the operator subjectivity and therefore they are not sufficiently repeatable, as previously mentioned for age estimation methods [19]. These mostly rely on the visual assessment of certain traits of skull and pelvis, with the latter being the most reliable for forensic examination [20]. Tested on an Albanian population, Durić et al. [20] highlighted the importance of the anthropologist's experience in obtaining accurate sex estimations. In contrast, morphometric and metric approaches for age estimations are far easier to replicate and provide associated error and probabilistic estimations. Furthermore, the implementation of these techniques with radiology and digital imaging allows the creation of digital databases and the possibility to share and repeat the assessments in a completely non-invasive manner [21]. The main limitation for the use of morphometric and metric approaches for sex estimation is the considerable loss in accuracy in highly fragmented and phonemically weathered remains [22]. Furthermore, it is important to note that also these techniques are strongly influenced by the population they were developed for. In situations involving unidentified human remains where ancestry information is unavailable, there is a risk of misclassification [23].

For this reason, metric and morphometric analysis can also be employed for the estimation of ancestry. It is important to emphasize that in a forensic setting, evaluating an individual's ancestry or geographic provenance is not about social classification with the scope of discrimination, as often debated, but a valuable tool in the identification process, aiding in the refinement of potential matches from a pool of missing persons [24]. For this purpose, the application of metric methods has become the gold standard in forensic investigations with the skull being recognized as the most accurate among all skeletal elements [24]. Additionally, significant effort has been invested in developing automated computational systems that employ user-friendly discriminant formulas. These systems are designed to be replicable and offer clear indications of posterior probability and estimation error. FORDISC [25], for example, contains 34 cranial and 39 postcranial measurements for ancestry estimation based on vast data sets that have shown 91% accuracy when applied to forensic caseworks. Another approach used for ancestry estimation is the assessment of morphological variations of specific cranial, facial, or palatal regions through a present or absent scoring system [24]. This method is less reliable than the earlier approach due to inherent variations among individuals within the same ethnic group and the influence of the operator's experience. A recent attempt in automating this morphological examination has resulted in *rASUDAS: a new Web-Based Application for Estimating Ancestry from Tooth Morphology* that includes the analysis of 21 independent dental traits from 30,000 individuals from seven biogeographic regions and that showed a correct classification 51.8% of the times [26]. Despite their potential, all these methods require continuous validation. Finally, geometric morphometric methods are relatively recent approaches that offer a remarkable benefit for the estimation by employing diverse forms of data to measure shape and size discrepancies, consequently reducing the influence of observer subjectivity [24]. Another great advantage is the possibility to apply these methods on digital material such us computed tomography or magnetic resonance imaging, thus eliminating invasiveness of the sampling.

In recent years, the remarkable growth of omics technologies has revolutionized various fields, enabling high-throughput analysis of biological data in genomics, proteomics, metabolomics and other areas, presenting vast opportunities in personalized medicine [27, 28], biomarkers and drug discovery [29–31], disease research [32–34] and regenerative medicine [35]. However, forensic sciences have only recently started to embrace omics approaches in research contexts [36], and even less so into routine use for caseworks. Reasons for this include awaiting either affordable instrumentation or demonstration of superior scientific validity and cost-effectiveness compared to existing methods, as well as for the establishment of their scientific reliability and robustness, pivotal for the admissibility of evidence in court [37]. As a result of the ruling from Daubert v. Merrell Dow Pharmaceuticals Inc., General Electric Co. v. Joiner and Kumho Tire Co. v. Carmichaelwere, to ensure the reliability and the relevance of a scientific testimony a method must be presented to the scientific community and must provide clear indications of its accuracy, methodological rationale, and application [38]. Furthermore, other important aspects are the accuracy level and the repeatability of the methodology, as well as the possibility to apply it to any material of similar nature [38]. While the use of omics in judicial caseworks requires ongoing efforts to meet admissibility requirements, forensic research in omics disciplines aimed at generating intelligence data is already demonstrating the potential of these approaches in the field. Omics and multi-omics approaches represent the future of modern forensic research in many disciplines and have the potential to reshape the landscape of forensic science by meeting the standards required for whiteness admissibility. This review will focus on the state-of-the-art platforms available for omics analyses, on the application of omics analyses in forensic sciences and specifically for the cause of death, for the identification of the dead, and on the current research existing in the field of multi-omics in forensics.

2 | ANALYTICAL PLATFORMS FOR OMICS RESEARCH

Omics disciplines involve the comprehensive (or global) analysis of different biomolecular types in a particular biological system on a large scale. By acquiring the suffix -omics, disciplines such as genomics, transcriptomics, proteomics and metabolomics (also known as the 'four big omics' and articulated in this order to follow the central dogma of molecular biology) refer to the collective study of the totality of specific biomolecular types within biological systems, like cells, tissues or entire organisms. The development of cost-efficient, high throughput technological platforms such as genetic sequencers and mass spectrometers set the bases for the study of omics disciplines also known as 'technology based omics'. In this chapter, we will discuss the most important platforms for each omics discipline with particular emphasis on the use of such technology in forensics.

2.1 | Genomics

After Dr Alec J. Jeffreys introduced the technique of DNA fingerprinting in 1986 for the case of Colin Pitchfork and the double murder he committed, DNA evidence rapidly became the 'queen's evidence', due to its probative power. DNA is currently considered the gold standard for human identification; its recovery is prioritized in crime scene investigations, and forensic genetics techniques aimed at analyzing DNA are constantly growing and improving to maximize the information that can be obtained from biological sources of interest.

Similarly to what happened in forensics, DNA has been the first target of interest also in omics research, when high-throughput platforms were developed to investigate in deep the inter-individual variations embedded in the genetic code and the phenotype associated with it [39]. Genomics is aimed at studying the whole genome, and it differs from genetics as the latter aims at investigating only specific portions of the genome or single genes [32].

DNA microarray (or expression array) technology was the first high-throughput platform designed to evaluate gene expression levels via the hybridization of cDNAs on capture probes arranged on a coated glass slide [40]. The major step towards genomics, however, was made by the introduction of Sanger sequencing (or first-generation DNA sequencing) in 1977, that allowed the sequencing of the whole genome of the bacteriophage Phi X174 [41].

2.1.1 | Sanger sequencing

Sanger sequencing is a traditional DNA sequencing method that uses chain-terminating nucleotides and electrophoresis to determine the sequence of a DNA strand, enabling the reading of the bases' sequence letter by letter and providing long read lengths (around 1000 bp). Significant achievements obtained with the introduction of Sanger sequencing include the completion of the Human Genome Project in 2003 [42]. Sanger sequencing continues to be the gold standard methodology for various analyses in forensic genetics. In particular, Sanger platforms are used in forensic laboratories for the analysis of short tandem repeats (STR), highly polymorphic regions of repetitive DNA consisting of 2–6 base pairs that vary in length between individuals, and of mitochondrial DNA (mtDNA), particularly valuable for the identification of human remains when nuclear DNA is unavailable or damaged. The primary constraints associated with Sanger technology, notably the high analytical costs and limited throughput, have been effectively addressed through the evolution of enhanced sequencing methodologies known as next generation sequencing (NGS) or massive parallel sequencing (MPS) technologies.

2.1.2 | NGS platforms

NGS platforms can provide increased data outputs and efficiency, lower costs, higher throughput, and variable read lengths. Short-read (35–800 bp) sequencing platforms can be divided in two broad categories: sequencing by synthesis (SBS) and sequencing by ligation (SBL). Both methods involve the preliminary fragmentation and clonal amplification of the DNA template on a solid support, where clones of the same DNA template are clustered in different portions of the surface. The clonal amplification can be achieved mainly via emulsion PCR (emPCR), where clones are generated on beads in water-in-oil emulsion droplets [43] or via bridge amplification, where clones are generated on a solid surface through the creation of 'DNA bridges' that generate spatially separated clonal clusters [44]. The high-throughput capability of NGS platforms compensates for the relatively short read lengths they offer, making them invaluable in addressing several areas within the field of forensics. These applications include DNA profiling, which encompasses paternity testing, missing persons identification, victim identifications and population genetics studies. NGS also plays a pivotal role in *post-mortem* interval (PMI) estimation through metagenomics, AAD estimation, mitochondrial DNA (mtDNA) analysis and the identification of various body fluids. Specific applications include whole exome sequencing (WES), targeted sequencing of specific genes, amplicon sequencing and metagenomics, (see Section 2.3 for more details), RNA-seq, DNA methylation (see Section 2.2 for more details) and microarray-based genotyping platforms/single nucleotide polymorphisms (SNP) arrays. For an extensive review of the NGS platforms and their details, including platform comparisons, we recommend Goodwin et al. [45] and Hu et al. [46].

SBS consists in the use of DNA polymerase to elongate a DNA strand complementary to the single strand template to be sequenced, and can involve three main chemistry approaches: pyrosequencing, sequencing by reversible termination and sequencing by detection of hydrogen ions [47]. Pyrosequencing, the chemistry used by 454 platform from Life Sciences since 2005 and then purchased by Roche in 2007, represented the first step towards the massive parallelization of the sequencing reactions and the reduction of sequencing costs in comparison with Sanger sequencing [48]. Pyrosequencing relies on the emission of light following the incorporation of nucleotides and the release of a pyrophosphate (PPi) during the elongation of the DNA

strand. The intensity of the light produced is directly proportional to the number of nucleotides incorporated at each position on the DNA strand, and the sequence of the DNA is determined by analyzing the specific light peaks corresponding to each incorporated base, allowing for accurate base identification [49]. Read length is between 400 and 500 bp [48]. Roche discontinued this platform in 2016 due to challenges in keeping up with the latest NGS technologies available on the market.

After the success of 454, Solexa/Illumina [50] set the bases for the creation of platforms that, instead, still represent one of the gold standards for genomics research. Illumina sequencing uses fluorescent 'reversible-terminator' dNTPs that get incorporated in the extending DNA strand; after the addition of a mixture of all four dNTPs and the incorporation of the complementary dNTP in the growing DNA filament, the fluorophore is cleaved away and through laser excitation, the type and number of dNTP incorporated is recorded. The fluorescent tag is then removed enzymatically, dNTPs and DNA polymerase are removed, and sequencing continues on the next position. Read length is 50–600 bp, depending on the platform used (Introducing Illumina Complete Long Read sequencing technology, accessed on 4 September 2023). Minimum DNA input is 1–10 ng for applications like targeted amplicon sequencing and metagenomics, and at least 100 ng for WGS.

More recently, Ion Torrent developed a new platform based on the generation of hydrogen ion signals to detect the incorporation of dNTPs in the growing DNA filament. The concept is similar to pyrosequencing when considering the clonal amplification via emPCR and the potential incorporation of multiple dNTPs in homopolymer regions, however the accuracy of the detection of the number of nucleotides incorporated in such areas using a pH sensor is less precise than those achieved with optical sensors or by sequencing by reversible termination approaches [46]. Read length is between 200 and 600 bp (Ion GeneStudio System Models, accessed on 4 September 2023). Similarly to Illumina platforms, 10 ng is the minimum amount of material recommended for targeted sequencing and 100 ng for whole genome sequencing (WGS).

In contrast with SBS, SBL involves the incremental addition of short DNA probes, each one labelled with specific fluorescent dyes, to a template DNA fragment. The template contains a known sequence on at least one end, where an anchor strand is bound. Complementary oligonucleotides from the pool of labelled probes preferentially ligate to the anchor strand using DNA ligase, releasing a distinct fluorescent signal. By associating specific dyes with positions on the DNA strand, the released signals accurately inform the DNA sequence, making SBL a powerful and high-throughput method for DNA sequencing [51]. Commercial platforms using the SBL technology were those named SOLiD platforms [52] and discontinued in 2013, and those developed by Complete Genomics [47], that were acquired by Illumina in 2013.

2.1.3 | Third-generation sequencing

In addition to the short-read platforms developed after Sanger sequencing, known as second-generation sequencing technologies, the demand for performing WGS and WES on large genomes led to the emergence of third-generation sequencing platforms capable of producing even longer reads. The main difference in comparison with second-generation NGS platforms is that sequencing can be achieved starting from native DNA (e.g., non-amplified) and the read length is $>$ 10 kb. Applications in forensics for long read-length sequencing platforms include de novo genome assembly for studying heterozygosity or structural variations, mtDNA analysis, epigenetic analysis (see Section 2.2 for more details), RNA-seq and metagenomic (see Section 2.3 for more details) analysis.

The two main third-generation technologies available on the market are produced by Pacific Biosciences (PacBio) and by Oxford Nanopore Technology (ONT). PacBio sequencing, also known as single molecule, real-time (SMRT) sequencing, is a third-generation DNA sequencing technology that reads DNA sequences arranged in single-strands and circularized. Each circularized DNA is replicated with a DNA polymerase within tiny wells on a surface, creating multiple sequencing reactions simultaneously. During sequencing, the DNA polymerase incorporates fluorescently labelled nucleotides into the DNA strand, producing a light signal whose color indicates the type of nucleotide incorporated. This process is observed in real-time, allowing for continuous data collection. Light pulses are interpreted as nucleotide sequences, and the resulting sequence obtained from each well is referred to as a 'continuous long read' (CLR). Circular consensus sequences are generated by analyzing multiple reads of the same DNA molecule, to enhance accuracy [53]. By adapting the protocol using the Iso-Seq method, that converts RNA into cDNA, also RNA can be sequenced on this platform. With the extensive read length ($>$ 135 kb) and the real-time data acquisition, PacBio systems such as the PacBio Sequel II allow for WGS and WES analyses, as well as for the analysis of epigenetic modifications such as DNA methylation, in a fast turnaround time (Sequel II System v8.0 and SMRT Link v8.0 Technical Overview, accessed on 4 September 2023). Minimum amount of DNA for human samples is 10 μg, but can reach 5 ng using the Ultra-Low DNA Input Workflow that includes a PCR to detect human genome variants. In all cases, the requirement for DNA quality to exceed 30 kb poses a significant challenge, especially in forensic research, where samples are frequently degraded, and achieving the desired DNA quality can be problematic.

ONT, applied on instruments like Flongle, MinION, MinION Mk1C, GridION and PromethION, involves the passage of single-stranded DNA and RNA molecules through staphylococcal alpha-hemolysin (alphaHL) nanopores. A motor enzyme at the 5′ end of the nucleotide sequence and an applied ion current enables nucleotides to pass through the protein pore. Real-time measurements of electrical current changes during nucleotide passage allow for DNA sequence analysis and provide long read lengths (500 bp to 2.3 Mb) and high accuracy [54]. When $>$ 400 ng of high molecular weight gDNA is available, ONT allows for amplification-free protocols, which are ideal for WGS, WES, targeted sequencing, and the study of methylomes, variants, isoforms, haplotypes and metagenomes [55]. When DNA input is lower (1–100 ng or $<$ 1 ng), whole genome amplification (WGA) PCR-based or MDA-based is required, respectively, prior to sequencing.

Illumina is also offering long-reads sequencing on NovaSeq platforms, both using PCR-based or PCR-free protocols. DNA should be extracted from blood or saliva, the recommended input quantity is 50-ng DNA and the read length is 5–7 kb (Deeper insights into complex regions of the genome, accessed on 4 September 2023. Applications such as WGS, epigenomics, metagenomics and single-cell sequencing are the most suitable for leveraging Illumina's long-read capabilities, enabling the comprehensive analysis of complex genomic regions and diverse biological samples.

Third-generation sequencing approaches based on SBL are those launched by the BGI group and based on DNBSEQ instruments and single-tube long fragment read (stLFR) technology. It consists in the ligation of DNA fragments to adaptor sequences containing known DNA bases. This specific approach allows for long read lengths and it involves fragmenting DNA, ligating known adaptors and amplifying the fragments into DNA nanoballs. These nanoballs are sequenced using fluorescence-based detection to determine the DNA bases. DNBSEQ's advantages include long reads (> 10 kb), low error rates and cost-effectiveness. It is used in genomics research for WGS, transcriptomics and metagenomics, enabling a comprehensive analysis of complex genomes and diverse samples.

2.2 | Epigenomics and DNA methylomics

A particularly interesting omics discipline with great relevance in forensic applications is epigenomics. Epigenomics involves the high-throughput characterization of epigenetic modifications, including reversible changes to DNA and DNA-associated proteins, such as DNA methylation and histone modifications. The human genome is characterized by 0.6 billion cytosines and by 56 million CpG sites (e.g., those cytosines followed by a guanine), 80% of those are modified in mammalian genomes [56]. While the primary biological function of these modifications is to regulate gene expression, their use in forensics primarily revolves around establishing correlations between DNA methylation (5-methylcytosine, 5mC) and specific biological fluids or an individual's chronological age (see Section 3 for more details). With this rationale in mind, this sub-chapter will focus specifically on the high-throughput methodologies available for conducting 5mC DNA methylomics studies. Other approaches (e.g., targeted SNaPshot assays) are not the focus of this review paper and therefore will not be discussed in detail, despite their use in forensic contexts will be reported in the following section.

Bisulfite sequencing (BS) (also known as whole-genome bisulfite sequencing, WGBS) has emerged in the recent years as the preferred method for cost-effective and efficient exploration of the human methylome at the genome level with base-pair accuracy [57]. Briefly, bisulfite conversion consists in the treatment of DNA with sodium bisulfite under denaturing circumstances able to separate the double DNA strand and to make cytosines accessible to the bisulfite. The addition of bisulfite leads to the conversion of unmethylated cytosines into uracil bases, while preserving methylcytosines as unchanged, through a sequence of sulphonation, deamination and desulphonation reactions. Upon sequencing, unmethylated cytosines are converted to thymidines, whereas methylated cytosines resist deamination and are read as cytosines. This allows to map the methylation status of each cytosine in specific DNA regions, but requires high-quality DNA as degraded, fragmented or poor quality DNA can lead to incomplete or inaccurate conversions, overall affecting the reliability of the downstream methylation analysis. Other limiting factors consist in the amount of starting DNA, which should be minimum 5 μg for successful conversion, in the fact that the conversion procedure is highly harsh and degrading due to the extreme pHs and temperatures used in the process, in the high costs associated with the deep sequencing level required to overcome the under-representation of C- and G-containing dinucleotides in comparison with non-converted genomes [58] and in the difficulty in the alignment of the reads [59].

In order to overcome the limitations posed by the bisulfite conversion, a more gentle enzymatic conversion promoted by three enzymes, Ten-Eleven Translocation (TET) family dioxygenases, T4-phage beta-glucosyltransferase (T4-BGT) and apolipoprotein B mRNA editing enzyme catalytic subunit 3A (APOBEC3A), has been developed recently. First, TET2 transforms 5-methylcytosine (5-mC) into 5-hydroxymethylcytosine (5-hmC), followed by conversion to 5-formylcytosine (5-fC), and ultimately, to 5-carboxylcytosine (5-caC). T4-BGT then catalyses the glucosylation of both TET2-formed and genomic 5hmC to 5-(beta-glucosyloxymethyl)cytosine (5gmC). Finally, APOBEC3A catalyses the deamination of both methylated cytosines into thymines and unmethylated cytosines into uracils, enabling their discrimination. 5mC and 5hmC are sequenced as cytosines, whereas unmethylated cytosines are sequenced as thymines [58]. Based on this principle, New England Biolabs recently introduced an enzymatic methyl-seq (EM-seq) technique. In their approach, they employ TET2 to oxidise methylated cytosines and then apply APOBEC3A treatment to transform unmethylated cytosines into uracils. WGBS and EM-seq, collectively known as whole-genome methylation sequencing (WGMS), both convert 5-mC/C to C/T, making the analysis tools developed for WGBS equally applicable to EM-seq [60]. This approach requires Illumina library preparation by adaptor ligation before the conversion (in the same way required for bisulfite conversion), and provides increased CpG coverage in comparison with WGBS, reduced sequencing costs as it covers more genomic features by using the same number of reads and allows for methylation studies of samples with lower starting DNA input. In fact, EM-seq can provide reliable results even when applied to as little as 100 pg of DNA, offering methylation and CpG coverage comparable to DNA inputs ranging from 10 to 200 ng when using standard bisulfite conversion.

2.3 | Microbiomics

Microbiomics involves the study of the microbiome through metagenomic and/or metabarcoding techniques [61]. The term 'metagenomics' and its techniques were developed in 1998 to address the constraints of conventional microbiological methods, eliminating the

need for culturing individual organisms and enabling researchers to directly investigate varied microbial communities within their natural habitats [62]. Metagenomic approaches entail the examination of genetic material, primarily DNA, extracted from environmental samples containing a diverse array of microorganisms, including bacteria, archaea, viruses, and other microorganisms. Metagenomic analyses eliminate the need for a PCR amplification step, resulting in a high capacity for species identification and precise quantitative analyses. Importantly, this approach avoids biases introduced by the amplification step, enhancing the overall accuracy of results. Several platforms can be used for metagenomic studies, including Illumina, PacBio and ONT [63]. While short-read platforms are widespread for metabarcoding, long-read sequencing techniques like PacBio and ONT are becoming more popular for overcoming challenges associated with short reads allowing the process of assembling short reads into full genes. After sequencing, obtained sequences are assembled into contigs and aligned with reference genomes. For bacteria, widely used databases include SILVA, RDP, Greengenes and NCBI [64], while for fungi, UNITE and Warcup are popular choices [65]. The results from assembly or binning are utilized for forecasting taxonomic classification and functional pathways by referencing diverse databases. Computational tools have been specifically designed to support such analyses [66].

While metagenomics is aimed at analyzing the entire microbial population in a sample, metabarcoding (or metagenetics) refers to the targeted analysis of specific genetic markers or barcode regions, allowing for the identification of different taxa. The analysis of short fragments of specific genes (e.g., 16S rRNA for bacteria and archaea, 18S for most non-fungal eukaryotes and ITS for fungi) is based on amplicon analysis studies and differs from untargeted metagenomics approaches [67]. Amplicon metabarcoding sequences are typically obtained on Illumina platforms, and universal primers have been designed to target conserved areas within specific genes to discriminate distinct microbial taxa. For example, primers designed for the 16s rRNA gene target nine hypervariable regions, denoted as V1 through V9, and different combinations of these regions have been employed to investigate bacterial communities at the familial, genus and species levels [68]. However, less studied domains may lack such primers, making taxonomic attribution via metabarcoding complex. Universal primers used in metabarcoding may not cover all bacterial groups identified through metagenomic DNA sequencing [69]. Additionally, overestimations in bacterial abundance may occur due to varying numbers of 16S rRNA gene copies in bacterial genomes, which can range from 1 to 16. This leads to overestimation on the abundance of bacteria carrying a high number of 16s rRNA operons per cell [70]. Various techniques are available for quantifying and characterizing microbial diversity within metabarcoding datasets, including amplicon sequence variants (ASVs) and operational taxonomic units (OTUs). While OTUs have been widely used, ASVs are now preferred as they provide higher resolution by considering exact sequence variants instead of clusters of similar sequences. ASVs are based on sequencing a specific marker gene (e.g., 16S rRNA for bacteria/archaea or 18S rRNA for eukaryotes) using denoising algorithms like DADA2 [71] to identify unique sequence variants. This approach allows for clustering similar sequences together without applying a fixed similarity threshold, enabling to take into account sequencing errors, chimeras and other artifacts, and ultimately the generation of high-resolution variants that can be compared across different studies. Standard bioinformatic tools for processing metabarcoding data using ASVs include quality filtering to remove low-quality reads and correct sequencing errors, sequence dereplication to collapse identical sequences into a single representative sequence to reducing redundancy, denoising and chimera removal to eliminate artificial sequences created during PCR amplification, overall ensuring data accuracy.

2.4 | Transcriptomics

Transcriptomics represents the analysis of the complete set of transcripts in a cell, tissue, or organism, including their abundance and function in different conditions. Two high-throughput approaches have been developed to identify and quantify RNA transcripts, and they are either hybridization-based or sequencing-based.

Hybridization-based methods generally entail the incubation of fluorescently labelled cDNA with either custom-designed microarrays or commercially available high-density oligonucleotide microarrays. The dependence on pre-existing genome sequence information, the high levels of background noise due to cross-hybridization, the restricted dynamic range for detection due to both background noise and signal saturation and the challenging comparison of expression levels between distinct experiments led towards the development of improved methods for transcriptomics analyses [72]. Sequencing methods, instead, directly determine the cDNA sequence. Despite these approaches originally used Sanger sequencing (see Wang et al. [72] for a comprehensive review), with the introduction of NGS RNA-seq became the gold standard for transcriptomics analyses. With RNA-seq, a set of RNA molecules is transformed into a collection of cDNA fragments, with adaptors attached to either one or both ends of the fragments. Every individual molecule, whether subjected to amplification or not, is subsequently sequenced in a high-throughput fashion to generate short sequences from a single end (single-end sequencing) or both ends (paired-end sequencing). The read lengths typically range from 30 to 400 base pairs, depending on the specific DNA sequencing technology employed. After the sequencing process, the generated reads are then either mapped to a reference genome or reference transcripts, or assembled de novo. This leads to the creation of a comprehensive transcriptional map at a genome scale, which includes information about both the transcriptional structure and the expression levels associated with each gene. RNA-seq has the capability to resolve sequence variations, such as SNPs, within the transcribed regions, it has a higher dynamic range than microarrays, due to the lack of noise signal and upper limits for quantification and a higher quantitative accuracy and reproducibility. New applications of transcriptomics include single-cell transcriptomics [73] and spatial transcriptomics [74]. Their application in the forensic field has not been

exploited yet; therefore, this review will not focus on these specific topics.

2.5 | Proteomics

Proteomics refers to the study of the full set of proteins present in a cell, tissue or organism, and includes their identification, modification, quantification and localization [75]. Proteomic studies enable researchers to understand the complex interplay between genotype and phenotype, and to decipher how the genetic information is expressed in biological systems and influenced by extrinsic and intrinsic factors to result in a specific phenotype. Proteomics predominantly leverages high-throughput methodologies, with mass spectrometry approaches playing a pivotal role. Across each phase of the process, which encompasses sample preparation, fractionation, MS data acquisition, quantification and data analysis, numerous methods and tools have been developed and extensively reviewed [75, 76].

The two main techniques used in the field of mass spectrometry-based proteomics to analyze and characterize proteins are bottom-up (or shotgun proteomics) and top-down strategies. The main difference between the two is that in the first one, proteins are extracted and digested prior to their analysis, whereas in the second one, proteins are analyzed directly without enzymatic digestion. Depending on the technique chosen, different protocols for sample preparation are required, as well as different instrumentation and data processing strategies.

2.5.1 | Untargeted proteomics

In shotgun proteomics, peptides are separated, ionized and then transported into the mass spectrometer, where spectra of peptide fragment ions are recorded. Separation is aimed at decreasing the complexity of the sample and at increasing the accuracy and sensitivity of the experiment, and is generally achieved either via gel-based approaches (e.g., two-dimensional polyacrylamide gel electrophoresis, 2D PAGE) or via gel-free approaches (liquid chromatography, LC). For extensive reviews and comparisons on 2D PAGE and LC, see refs. [77, 78]. Following separation, ionization is a crucial step in the procedure that allows to convert polar, non-volatile and thermally unstable peptides into ionized analytes that can enter the gaseous phase in the MS while minimizing their degradation [75]. Ionization methods in proteomics are essentially matrix-assisted laser desorption ionization (MALDI) [79, 80] and electrospray ionization (ESI) [81, 82]. The distinguishing feature of MALDI is its ability to utilise a matrix mixed and deposited with the sample on the MALDI plate, which absorbs laser energy and subsequently transfers this energy to the acidified analytes. This swift, laser-induced heating then leads to the desorption of both the matrix and $[M + H]+$ ions of the analyte into the gaseous phase, predominantly generating single-charged positive ions.

In contrast, ESI operates by applying a high voltage (typically ranging from 2 to 6 kV) between the emitter located at the end of the separation pipeline and the entrance of the mass spectrometer. The physicochemical processes involved in ESI include the formation of a Taylor cone, which is followed by the generation and drying out of analyte-solvent droplets and the resulting creation of multiply charged ions [75]. Once ions are transported in the mass spectrometer, the mass analyzer filters and optionally fragments ions and separates them based on their mass-to-charge ratios, allowing for the assignment of peptide sequences, which in turn permits the inference of the corresponding proteins, their modifications and relative abundance. Ion trap (IT), Orbitrap and ion cyclotron resonance (ICR) mass analyzers employ ion separation methods that are dependent on the resonance frequency of ions m/z values and use a trapping system to select and fragment ions. Fragmentation can occur in separate higher-energy collisional dissociation (HCD) cells which allows for collision-induced dissociation (CID). An example is the hybrid configuration of Orbitrap with linear ion trap (LTQ-Orbitrap) where the HCD cell accumulates, isolates, and fragments peptide ions [83]. Alternatively, fragmentation can occur following electron capture dissociation (ECD) in instruments such as Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) [84]. In contrast, quadrupoles (Q) utilize m/z stability as their principle of separation and are considered ion-beam mass spectrometers, while time-of-flight (TOF) analysers rely on the flight time of ions for separation. Several mass analysers can also be combined in hybrid mass spectrometers to take advantage of the various properties that different analysers can offer and maximise dynamic range, speed, mass range, resolution, and sensitivity [75, 85–87]. The combination between ion sources and mass analysers is also variable. Usually, TOF mass analysers are interfaced with MALDI (MALDI-TOF instruments), whereas ion-beam and traps are usually associated with ESI ionizers. For extensive comparisons between the mass spectrometry instruments most used in proteomics, we recommend Yates et al. [75].

In many bottom-up applications, it is necessary to employ tandem data acquisition (MS/MS). Methods for data acquisition in bottom-up proteomics include untargeted (or discovery) approaches such as data-dependent acquisition (DDA) and data-independent acquisition (DIA), and targeted methods such as selected reaction monitoring (SRM, also known as multiple reaction monitoring or MRM) and parallel reaction monitoring (PRM).

In DDA, the mass spectrometer scans the peptide ions as they elute from the LC column and selects a specific number of the most intense peptide ions (often the 'top N' ions) from each scan for fragmentation. These selected ions are called precursor ions and are then fragmented to obtain the MS^2 spectra via the collision with a neutral gas (collision-activated or collision-induced dissociation, CAD or CID). Whilst the MS^1 spectra are used to infer protein abundance, MS^2 spectra are used to obtain sequence information required to identify the proteins present in the sample. Protein abundances are usually inferred via label-free quantification [88], as signal intensities in mass spectrometry data correlate with the quantities of ions responsible for generating those signals, subject to the dynamic range constraints of the instrument. Consequently, quantifying a peptide entails identifying all or a representative portion of the signals originating from its ions and subsequently integrating their signal intensities. Alternatively, relative quantification can be achieved via isotope labelling strategies,

such as iTRAQ [89] or SILAC [90]. Limits in both label and label-free quantitative approaches in shotgun proteomics stem from the inherent challenges associated with the partially random sampling approaches used to collect tandem mass spectra (MS/MS spectra) from peptides within complex proteome digests. Peptides with higher abundances are more frequently sampled, resulting in more accurate measurements. Conversely, peptides with lower abundances are sampled less frequently, leading to less precise quantifications [91].

With reference to protein identification, database searches allow then to match the experimental MS^n data against the anticipated, computationally generated fragmentation patterns of the analyzed peptides [92]. Advanced algorithms for post-processing search results, coupled with target-decoy search strategies, enable the statistical validation and assignment of meaningful confidence scores to peptide identifications [93].

In DIA, all precursor ions identified in an MS^1 survey scan are subjected to fragmentation. During this process, fragment ions are collected within a set of wide isolation windows that cover the entire mass-to-charge ratio (*m/z*) range. In DIA-MS, the initial steps of sample processing and data acquisition closely resemble those of the single-shot DDA-MS approach. However, additional post-acquisition data processing steps are necessary to match the acquired MS data with spectral libraries. A spectral library serves as a repository containing various mass spectrometric and chromatographic parameters, such as precursor and fragment *m/z* values, fragment types, charges, and elution times (in case of in-house built libraries), for each individual peptide within the analyzed sample. Human spectral libraries are now available [94] and often eliminate the requirement to create customized libraries for every DIA-MS experiment, enhancing cross-laboratory consistency, reducing sample demands and optimizing the utilization of mass spectrometry instrument time [95]. Traditionally, these study-specific spectral libraries were generated through extensive proteomic characterization using the DDA-based approach on the same samples before conducting the DIA-MS analysis. However, it is important to note that the variability in DDA experiments and the subsequent spectral library generation across different laboratories can lead to discrepancies in the identification and quantification of proteins between studies. Furthermore, the extensive number of DDA-MS experiments required to construct study-specific spectral libraries can be costly and time consuming. Despite the current availability of human libraries for biomedical research, these are not always suitable for forensic experiments, where oftentimes samples are taphonomically degraded and are notably different from the 'fresh' samples used to generate such references libraries, therefore building internal custom libraries is still a requirement that brings in all the downsides previously discussed associated with the customization of libraries for DIA, and still limits the use of this technology in the forensic field. DIA offers some advantages in comparison with DDA, including high reproducibility across technical replicates as well as across different experiments, and significant reduction in missing values due to the cyclic fragmentation of all precursor ions [95]. The higher data processing demands and, more importantly, the need to generate study-specific libraries represents instead the major downside of DIA in comparison with DDA, particularly in situations like the forensic ones where the starting sample may be already extremely limited and therefore not suitable for conducting both DDA (to build the library) and DIA runs.

2.5.2 | Targeted proteomics

In contrast with untargeted approaches, targeted proteomics allows for accurate quantification of selected sets of peptides (and, therefore, proteins) of interest in a multiplex, highly sensitive, fast, and extremely reproducible way, therefore allowing also for the detection of low-abundance proteins or PTMs challenging to quantify using untargeted approaches [96–98]. SRM experiments typically involve triple quadrupole mass spectrometers, where the first quadrupole selects precursor ions, the second quadrupole induces their fragmentation and the third quadrupole isolates target-specific fragment ions for detection. Multiple fragment ions are usually monitored sequentially in this process. Unlike SRM, PRM experiments are conducted on mass spectrometry systems capable of capturing complete fragment spectra, including quadrupole-Orbitrap-type mass spectrometers, QqTOF, MALDI-TOF and MALDI-TOF/TOF systems [98]. In PRM, all fragment ions from a chosen precursor are simultaneously recorded. In both setups, an inclusion list is provided to the mass spectrometer, outlining precursor *m/z* ratio windows and retention time (RT) windows. In the case of SRM, the inclusion list also specifies the *m/z* values of the fragment ions to be monitored. A key aspect in targeted proteomics is the need of using libraries with fragmentation spectra that allow researchers to select the most ideal peptide candidates to subsequently identify proteins of interest, ensuring accurate identification and quantification of specific peptides or proteins, reducing interference and allowing the validation of the results, overall facilitating method development. Libraries can be empirically determined, and some are available online [99, 100] or generated via in silico predictions of the fragmentation patterns [101].

Targeted proteomics approaches allow to perform absolute quantification of proteins, in contrast with the label-free relative quantification approaches available for discovery studies. Quantification is usually performed with stable isotope-labelled internal standards or with reference peptides with known concentrations [98]. In both cases, the principle is that proteins (either non modified or post-translationally modified) can be assessed by quantifying the distinct peptides that make up their structure after undergoing proteolytic digestion. This application offers advantages in contrast with standard immunological quantitative assays (e.g. ELISAs) as it allows for multiplexing and it does not require specifically developed antibodies for its scope. Excellent reviews on the topic are Liebler and Zimmerman [102] and van Bentum and Selbach [98].

In general, bottom-up proteomics offers several advantages when compared with top-down approaches, including suitability to analyze complex samples, higher sensitivity in detecting low-abundance proteins, simplified data analysis, robustness and possibility to obtain relative and absolute quantitative measurements [103]. On the contrary, top-down proteomics offers advantages like the comprehensive

analysis of proteoforms (the specific molecular form of the protein resulting from combinations of genetic variation, alternative splicing, and post-translational modifications [104]), 100% sequence coverage and the consequent full characterization of PTMs [105]. A detailed review of top-down approaches in proteomics is out of the scope of this review, as their application in forensics is not exploited yet, but extensive reviews can be found in literature including those of Toby et al. [106], Catherman et al. [105] and Melby et al. [107].

2.6 | Metabolomics

Metabolomics is the comprehensive analysis of cellular and tissue metabolic products, a field that has seen significant development and implementation over the past two decades. It focuses on the characterization of small molecules within biological systems (≤1000 Da) [108, 109]. The interest in this field has grown due to the understanding that the components comprising the metabolome are the outcomes of variations in cellular regulatory processes, which can be influenced by genetic or environmental perturbations [110]. In addition to the commonly used profiling of polar functional metabolites, lipidomic analysis is gaining increasing popularity. Molecular lipids constitute a crucial component within cells, playing vital roles in energy retention, maintaining membrane integrity, and facilitating signaling processes [111]. Several key factors must be carefully considered when conducting metabolomic studies. Sampling, quenching, storage, and extraction play pivotal roles in ensuring consistent and comparable results within and across studies, making them essential in the context of forensic science and investigation, as previously discussed. The initial two steps in a metabolomics workflow are of paramount importance. They are designed to achieve systematic sampling, thus minimizing variation resulting from tissue heterogeneity. Additionally, prompt quenching is essential to arrest metabolism, ensuring that the extracts provide a dependable compound pool that accurately reflects the qualitative and quantitative composition of endogenous metabolites in the original, living tissue or at the time of the sampling [109, 112]. Indeed, creating a universal protocol that fits all situations can be challenging, especially considering the diverse materials, and working conditions involved. However, when it comes to quenching, the primary objectives are to prevent any disruption to the current levels of metabolites during the harvesting process and to effectively halt all enzyme and chemical activities. This is typically achieved by rapidly snap-freezing the samples in liquid nitrogen, followed by storage at −80°C [113, 114]. Besides the approach chosen, an efficient sample preparation protocol is characterized by four main attributes: (I) lack of selectivity, (II) simplicity, (III) reproducibility and (IV) consideration of any chemical or enzymatic reactions that could affect the metabolites after their extraction [115]. Another important aspect is the choice of replicates to be employed in a study. Biological replicates typically originate from distinct individuals of the same genotype. In contrast, technical replicates are generated by performing the complete analytical procedure either using the same initial bulk material or by repeatedly injecting the same extract. Technical replicates can be generated at various phases, including sampling, quenching, extraction, and analysis, allowing for independent replication of each stage in the process [109, 112, 116].

In the realm of metabolite identification, nuclear magnetic resonance (NMR) stands as the primary method for elucidating metabolite structures. NMR relies heavily on objective physical characteristics, resulting in a high level of reproducibility. This implies that factors such as instrument specifications, setup, sample type and data collection parameters, along with the spectroscopic data produced, should collectively furnish sufficient information for a definitive metabolite characterization. On the other hand, mass spectrometry (MS) coupled with gas chromatography (GC) and LC, offer broader coverage of metabolites within the system under investigation. However, a potential challenge for both NMR and MS lies in the ambiguity of compound identification. The combination of chemical information provided by NMR and MS in combination is highly efficient in metabolite identification [117]. In addition, ion mobility has provided significant advantages in structural biology by facilitating the differentiation of isomeric and isobaric ions and directly revealing their conformation [118].

2.6.1 | NMR

NMR is a spectroscopic technique based on the energetic transitions of nuclear spins in the presence of a robust magnetic field. Over the years, it has found extensive use in both clinical and non-clinical settings for metabolomics analysis. Since the acquisition of the first NMR spectrum in the 1940s, its primary application has been the identification and structural elucidation of organic compounds. This capability has been instrumental in exploring the behaviors of substantial biological molecules, such as proteins and nucleic acids [117]. Furthermore, the use of Stable Isotope Resolved Metabolomics (SIRM) has been employed in monitoring reactions across metabolic pathways by providing positional labelling information and, therefore, finding applications in various scientific fields, including medicine, nutrition, and toxicology. Recently, NMR has gained significant traction in the field of metabolomics for the analysis of complex mixtures. This trend is driven by several advantages offered by NMR compared to mass spectrometry (MS). First and foremost, NMR is known for its high inter-laboratory reproducibility and instrumental stability. Maintenance of the NMR instrument is remarkably straightforward, as the sample never encounters any components of the instrument. This not only ensures reliability but also minimizes the risk of sample carry-over between experiments [119]. In terms of metabolic coverage, approximately 60 metabolites can be detected in an untargeted ^{1}H NMR spectrum when employing a 600-MHz NMR spectrometer. This applies to samples such as human urine, and it requires minimal sample preparation [120]. NMR, and particularly high-resolution (HR) magic angle spinning (MAS)-NMR, provides another valuable capability – the analysis of intact tissues without the need for extraction. This approach allows for the direct examination of biological samples in their native state, providing insights into the metabolites' spatial distribution and interactions within the tissue. The final technical aspect to consider when using ^{1}H NMR is the solvent suppression

to obtain a flat baseline for better detection of lower abundant compounds and increased sensitivity [121]. Although more computationally intensive and time consuming, 2D NMR experiments offered better deconvolution of complex mixtures. As previously mentioned, the most common application of NMR is to determine metabolite structures by providing information about functional groups (revealing the position of chemical shifts), spatial or connecting protons (describing the multiplicity of signals and coupling patterns), as well as the count of equivalent protons (depicted through signal integrals) [117]. Despite the great potential of 1D ^{1}H NMR in elucidating compound structure, for the most complex molecules with complex multiplicities and second order effects, quantum-chemistry algorithms are commonly employed [122]. Identifying metabolites in metabolomics can pose difficulties due to the numerous overlapping signals. Reference libraries containing metabolites commonly present in matrices like urine, plasma and serum serve as valuable tools. To validate structures in metabolomic investigations, profiles are compared to standards in spectral databases. The combined use of NMR and MS analyses can provide both structure and exact masses, confirming compound identifications and eliminating the need for metabolites isolation [123]. In recent years, and of particular interest for forensic applications, is the use of quantitative NMR (qNMR) for the analysis of complex biological fluids and tissues. The quantification is based on the principle that the intensity of the signal is proportional to the number of nuclei of the compound and is optimized by controlling the longitudinal relaxation time. The complexity of this procedure lies in managing experimental conditions and accounting for factors such as pH, ionic strength, solubility, chemical interactions, and sample storage. The main advantage is the ability to perform quantification without the need for an internal standard or calibration curve, which is a requirement for MS platforms [123].

2.6.2 | MS-based approaches

Mass spectrometry-based metabolomics is a rapidly advancing technique widely employed in biomedical research, with a primary focus on mechanistic studies of pathological conditions and the discovery of biomarkers for diagnostic purposes. Similar to proteomics, the analytical platform for metabolomics consists of a source, a mass analyzer and a detector. The choice and combination of various components offer both advantages and disadvantages.

Regarding the source, the preferred choice is ESI, which is responsible for converting molecules into ions in the gas phase. ESI has the advantage of producing a large number of intact ions through charge exchange in solution, making it a 'soft ionization' method. In contrast, atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization ionization (APPI), which does not typically promote high source fragmentation, are less commonly used [124]. However, they complement ESI by their ability to profile non-polar compounds in dual-source instruments [125]. In LC-MS hardware, the mass analyzer is another essential component, and there are four main types to consider. Triple quadrupole (QQQ) and linear-ion trap (LIT) instruments are low-resolution tools. High-resolution instruments commonly applied in metabolomics include quadrupole-time of flight (Q-TOF), quadrupole-orbitrap (Q-Orbitrap), Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS). These instruments offer the capability to simultaneously aid in compound identification and quantification by resolving *m/z* values. Additionally, the use of CID instruments enables the acquisition of fragmentation patterns, which are valuable for structural analysis and improved compound identification by providing fragmentation patterns of precursor ions [125, 126].

MS metabolomics can be performed via direct infusion, by injecting the sample directly in the MS detector, or via use of separation techniques such as GC, capillary electrophoresis (CE) and LC [125]. The implementation of separation techniques requires deproteinization and extraction protocols to prevent the sample from interfering with the separation technique and, therefore, resulting in destructive and laborious protocols. However, this offers clear advantages in terms of compound separation and identification. Furthermore, besides the separation technique, MS-based methods offer higher sensitivity than NMR, larger selectivity, and greater metabolic coverage. In terms of chromatographic approaches, GC-MS is often considered the most cost-effective and user-friendly separation technique; however, the choice of separation strategy should be based on the properties of the compounds under investigation and the scope of the analysis. It also offers enhanced stability and reproducibility compared to some other methods. The underlying principle of GC-MS reduces its capability to exclusively examine substances that are volatile or readily volatilized and can undergo derivatization. The conventional derivatisation approach in GC-MS involves a two-step process involving oximation followed by silylation and plays a crucial role in the separation and identification of metabolites [127]. GC-MS also allows the detection of polar compounds as fatty acids, amino acids, amines, sterols and sugars. In terms of identifications, this happens via comparison with commercially available library (e.g., NIST and Fihen [128, 129]). CE-MS offers several advantages such as high-resolution and low sample consumption to detect polar metabolites. However, it shows low concentration sensitivity as well as narrow separation windows [130].

Even though none of the current methods is capable of comprehensively profile the entire metabolome, LC-MS is arguably the most common platform for metabolic phenotyping [131]. High- and ultra-performance liquid chromatography (HPLC/UPLC) offer a versatile tool to obtain efficient separations and improve MS data quality due and low background noise by reducing sample complexity. Semi-polar compounds (e.g., phenolic acids, fiavonoids, glycosylated steroids, alkaloids and other glycosylated species) are easily profiled using reverse-phase liquid chromatography (RPLC), normally using C18 columns. On the other hand, hydrophilic interaction liquid chromatography (HILIC) columns are ideal for profiling more polar compounds, including sugars, amino sugars, amino acids, vitamins, carboxylic acids, and nucleotides. Both GC-MS and HILIC-based approaches are highly compatible with all the MS platforms mentioned earlier. However, it is often necessary to combine different chromatographic techniques and

customize extraction protocols to achieve comprehensive coverage of the metabolome [112, 125].

Furthermore, as for proteomics, there are different MS data acquisition modes for metabolomics, namely full-scan, DDA and DIA. The typical approach for untargeted metabolomics begins with profiling in full-scan mode, which allows the assessment of mass-to-charge ratios (m/z) and relative abundances for all detected features. These features are automatically extracted during reprocessing, and the results are analyzed using both univariate and multivariate methods. Additionally, tandem mass spectrometry (MS/MS or MS^E) techniques are employed to further elucidate the structures and fragmentation patterns of detected metabolites. DDA combines full-scan MS with the analysis of MS/MS spectra, enabling both semiquantitative analysis and the collection of structural information for the profiled compounds. However, there is a risk associated with DDA: certain precursor ions may never be selected for fragmentation, potentially leading to the neglect of these compounds in the profiling process [132]. Moreover, MS/MS spectra tend to exhibit lower signal intensity when fragmentation is employed, which can compromise the quantitation of compounds and increase the risk of missing those present in low concentrations. In contrast, the DIA strategy is based on the fragmentation of all ions, making it more inclusive of compounds with lower intensity. In practice, multiple precursor ions are simultaneously fragmented. The two primary approaches for DIA are all-ion fragmentation and 'sequential window acquisition of all theoretical' fragment-ion spectra (SWATH), which uses predefined molecular mass values to create medium-sized pass windows for acquisition [133, 134]. Additionally, these profiling methods are typically designed to discover biomarkers in response to specific biological questions. They are often followed by targeted approaches for confirmation of identification and providing absolute quantification. One of the widely adopted techniques is MRM, commonly performed in triple quadrupole (QQQ) LC-MS instruments. MRM allows for the monitoring of specific precursor ions and product ions at a fast scan speed, with the capability to switch between polarities. However, it has the drawback of limited coverage. To overcome this limitation, PRM strategies have been developed for Orbitrap instruments. PRM enables the monitoring of groups of fragment ions and quantification in a high-resolution instrument [126].

To address complex biological questions, large-scale study designs are essential. These designs often involve measurements taken over time and may incorporate a multi-batch design. However, they also underscore a major limitation of MS-based metabolomics, which is the low replicability of results and the introduction of analytical or technical variation. This variation needs to be carefully accounted for and corrected during data pre-processing and processing to ensure the reliability of the results [135]. For this reason, in order to enhance reproducibility within and across studies, quality assurance (QA) and quality control (QC) approaches need to be considered for both untargeted approaches, that provide semi-quantitative data of as many metabolites as possible, targeted approaches, that give quantitative results via the monitoring of selected isotopically labelled internal standards or standards added to the analysis, and semi-targeted ones, that provide approximate quantification by analysis standards before the formal analysis of the sample under investigation [112, 136, 137]. QA encompasses standard operating procedures that ensure quality requirements are met during experimental procedures. In untargeted assays, where identification is putative and relies on reference libraries, QC measures can involve a mixture of authentic chemical standards distributed across the run and batches, similar to what is done for targeted and semi-targeted assays. However, this strategy may not fully capture the need for QC to reflect the combined metabolite profile of all biological specimens. To address this, a common approach is to use a small aliquot from each sample to create a pooled QC sample. This pooled QC is then divided and analyzed across the entire experiment, providing a more comprehensive assessment of the overall quality and consistency of the data [112, 137]. Another essential QA is the evaluation of artifacts created by the extraction protocol via the creation of extraction blanks. These are generated by performing the extraction without any samples and allow the evaluation of 'carryover' phenomena that can be excluded during data processing or run in series to assess system suitability [137].

3 | OMICS IN FORENSIC SCIENCES

This review aims to present readers with the cutting-edge advancements in the use of omics disciplines within the realm of forensics, concentrating specifically on their pivotal role in the investigation of deceased individuals, encompassing aspects such as determining cause of death, estimating PMIs, assessing age at death, and facilitating the identification of the cadaver (Figure 1).

3.1 | Manner of death (MOD) and cause of death (COD)

One major challenge that forensic practitioners face during investigations is determining the cause of death, especially when death occurs unexpectedly or in unwitnessed circumstances [138]. Nowadays, biochemical and molecular high-throughput approaches allow the assessment of pathophysiological changes, drug abuse and intoxications, which play a central role in understanding the mechanisms that lead to death [138, 139]. This field is often referred to as forensic molecular pathology and involves the analysis of the tissue believed to have caused death (local molecular pathology) and the examination of the entire systemic pathophysiology to evaluate potential additional causes of death (systemic molecular pathology) [140]. The main analytical approaches have been previously introduced but important aspects to consider in implementing forensic molecular pathology are (I) existing pathology prior to insults, (II) changes in the process of death following insults, encompassing particular discoveries and non-specific alterations, and (III) modifications or artifacts occurring after death [140]. Manner

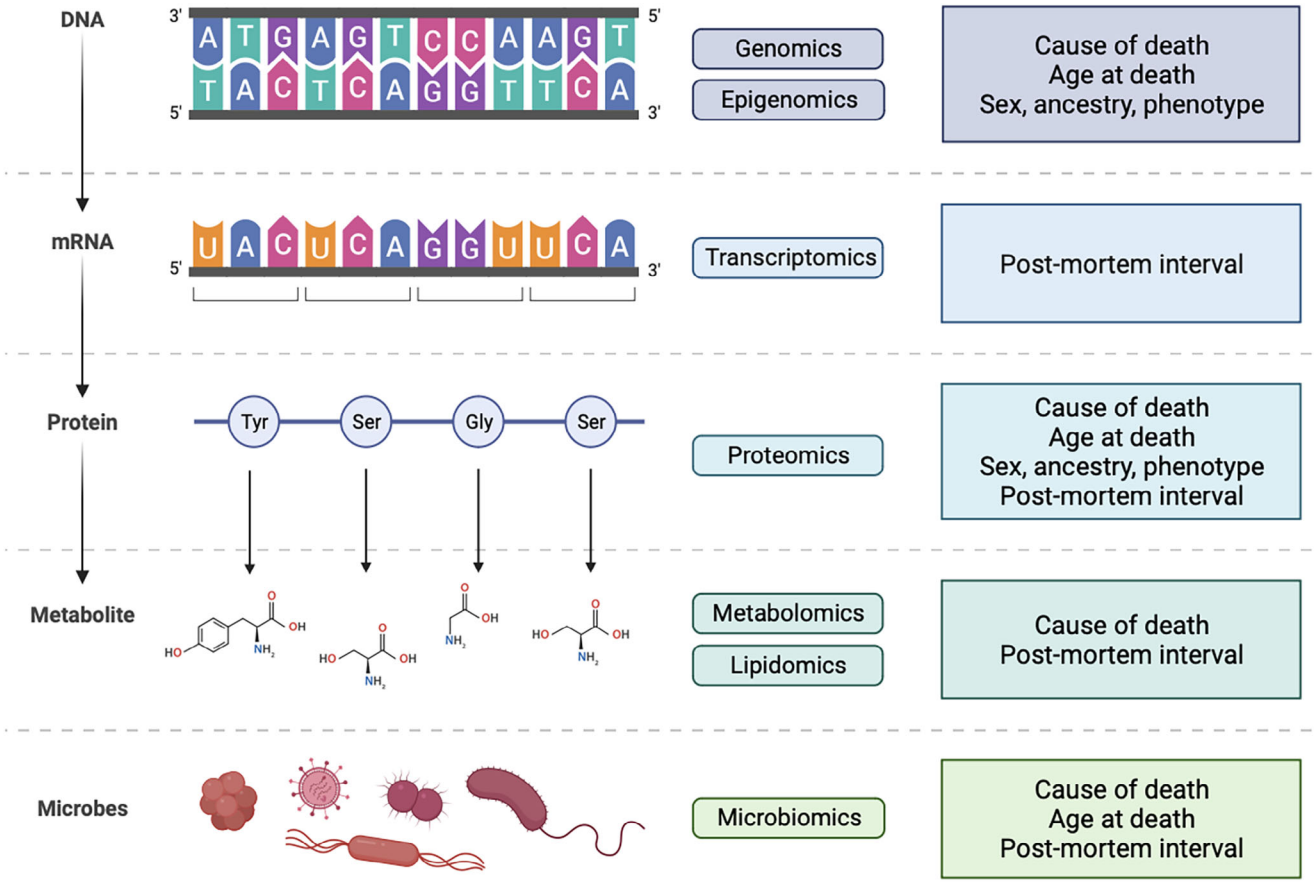

FIGURE 1 Schematic representation of the central dogma of molecular biology, the omic methodologies associated to each level and their application to the study of the cadaver in forensic science.

of death is defined as 'the underlying medical condition, disease or injury that begins a lethal chain of events resulting in death' while cause of death 'describes the way in which a death occurs, which may be homicide, suicide, accidental, natural or undetermined' (Maryland Department of Health, Office of Chief Medical Examiner, accessed on 4 October 2023).

3.1.1 | Search results

Using the Scopus database, the combined search terms were used: 'forens*', 'omic*', 'cause of death', 'forens* AND proteomic* AND cause of death', 'forens* AND metabolomic* AND cause of death', 'forens* AND metagenomic* AND cause of death', 'forens* AND metabarcod* AND cause of death', 'forens* AND metabarcod* AND cause of death', 'forens* AND omic* AND toxicology', 'toxicology AND omics AND human AND metabolomic*', 'forens* AND genomic* AND cause of death', 'forens* AND transcriptomic* AND cause of death', 'forens* AND wound AND age AND omic'*. Two hundred and forty-nine articles were retrieved, and 46 retained as considered appropriate for the topic of the current review. Eleven additional articles were also included manually for the pertinence to this specific thematic area.

3.1.2 | Genomics and epigenomics for COD and MOD

Genomics, primarily utilizing NGS, has found extensive application in cases involving sudden cardiac death (SCD). SCD is characterized by an individual in good health experiencing cardiac-related symptoms one hour prior to death, with underlying cardiac conditions being the cause. This technology plays a crucial role in understanding the genetic factors contributing to such cases [141]. A number of cardiac disorders have been associated with SCD and investigated via genome sequencing such as long QT syndrome (LQTS), short QT syndrome (SQTS), Brugada syndrome (BrS) or catecholaminergic polymorphic ventricular tachycardia (CPVT) and inherited cardiomyopathies [142]. A very comprehensive analysis performed by Sanchez et al. [143] involved 786 cases of individuals who did not die from violent causes. In 81.1% of these cases, the cause of death was determined during autopsy. For the remaining 283 cases, for which the cause was not assigned macroscopically, further investigation using Sanger sequencing revealed that 34.63% were attributed to coronary disease, and 12.72% were due to inherited cardiomyopathy. The accuracy of these findings was confirmed through histological analysis. The study also identified specific genetic variants, such as PKP2 and SCN5A, which have been previously reported by Kiehne and Kauferstein [144]. Additionally, compound

variants were identified as potential causes of rare cardiac conditions. This highlights the need for further research, ideally involving the analysis of family members to assess the potential inheritance of these conditions [143].

Another field of application is the study of hypertrophic cardiomyopathy (HCM), an autosomal dominant genetic disorder characterized by left ventricular hypertrophy. As a result, NGS represent a natural analytical platform for the *post-mortem* study of this condition. In a case study that involved heart tissue from 15 participants, it was observed that almost 50% of the cases (7/15 cases) did not exhibit any pathogenic mutations associated with HCM. This finding suggests that in these cases, the cause of HCM may not be attributed to genetic mutations commonly associated with the condition. However, in the remaining cohort, eight missense variants in five genes were identified (i.e., *MYBPC3*, *CAV3*, *PRKAG2*, *MYH6*, *MYH7*) with only two that were correlated with the conditions (i.e., MYBPC3p.Arg470Gln and MYBPC3p). Recent research findings have indicated that cases where there was a specific level of myocardial disarray, typically exceeding 5% within the heart, were often associated with pathogenic rare variants related to hypertrophic cardiomyopathy. Specifically, in the majority of such cases, which accounted for 88.9% of the nine cases studied, pathogenic rare variants were identified as a contributing factor to the condition [145]. A similar panel of genes was detected by Christiansen et al. [146] on a cohort of 97 deceased diagnosed with schizophrenia analyzed by MPS. Their analysis revealed that there was no overrepresentation of heart-related disease variants in deceased individuals with schizophrenia. However, the study did find that the collective genetic load of variants within the panel of genes under investigation was higher in individuals with schizophrenia.

An interesting case was reported involving a sudden unexpected death following a mild trauma during a physical altercation for an individual suffering from a psychotic disorder. Toxic causes of death were excluded following analysis with UPL-MS/MS. The study revealed the association of heterozygous variants in the *RYR2* gene, which are typically associated with CPVT. This association had not previously been linked to the cause of sudden death. The findings suggest that the confluence of the genetic variant and the traumatic trigger event might have been the underlying cause of the SCD [147].

Another marker for SCD is 8bp insertion/deletion polymorphism (rs150703258) within downstream of *NPC1* [148]. In a more recent study conducted on a cohort of 30 individuals at the Department of Medical and Surgical Sciences, University of Bologna, between 2018 and 2021, a comprehensive investigation was carried out. This study involved full autopsies, cardiovascular pathological examinations, and systematic toxicological analyses. The findings of this study confirmed the presence of several genetic variants that can be associated with SCD. These results contribute to our understanding of the genetic factors underlying SCD and provide valuable insights for forensic pathology and clinical practice [149]. The study, employing Illumina sequencing, identified 25 SNPs that may be indicative of a potential pathogenic association with SCD. A particular interest was raised by the missense variant rs2228314 in the *SREBF2* gene, associated with the metabolism of cholesterol, responsible for the insurgence of coronary atherosclerosis, a known cause of SCD. This hypothesis was also confirmed by the presence of *CACNA1C*, *KCND2* and *PRKAG2* variants all associated with lipid, cholesterol, arachidonic acid, and xenobiotics/drugs metabolisms [149]. Interestingly, the same study identified genetic variants mapping involved drug metabolism (i.e., *ABCB1*, *ABCB2*, *CYP2E1*, *CYP2C9 and CYP2J2*). These findings suggested the combined effects of drug abuse and arachidonic acid metabolism could be related to the incidence of SCD. This underscores the complex inter-play of genetic and environmental factors in contributing to SCD risk [149].

Another condition of interest is Marfan syndrome (MFS), which is an autosomal dominant genetic disorder. It has been associated with ~ 600 mutations in the *FBN1* gene, which encodes the extracellular connective protein fibrillin-1, in MFS patients. This genetic variation can result in a range of cardiovascular and skeletal abnormalities in affected individuals. In a case of study, Takahashi et al. [150] reported a case of a Japanese individual diagnosed with MFS who died due to aortic rupture. This case highlights the importance of considering genetic variants like the p.C1307Y substitution in the *FBN1* gene, which affects connective tissue integrity. Individuals carrying this variant should receive thorough medical attention to monitor for the potential development of aortic dissection and sudden death (also see Ref. [151]). However, it is essential to note that the pathophysiology of thoracic aortic dissection or rupture (TADR) cannot be solely attributed to the *FBN1* gene. A comprehensive study conducted with MPS and involving 1078 *post-mortem* cases of sudden death with cardiovascular genetics focus, including 34 cases of TADR, revealed that molecular testing of the TADR sub-panel of genes had a diagnostic yield of 23.5% in the TADR sub-group (n = 34). The genes with the highest number of pathogenic variants were *FBN1*, *TGFBR2*, *TGFBR1* and *MYLK* [152]. This finding underscores the significance of genetic variants in these specific genes in both *post-mortem* examination and clinical diagnosis of TADR.

Exome sequencing, which involves sequencing all the exons in a genome, has gained popularity in the investigation of SCD as well [153, 154]. Wang et al. [153] examined a case involving a 34-year-old male and identified a potential cause of SCD in the form of a heterozygous gene mutation, *KCNQ1* G643S. This genetic variant was considered a potential contributor to the occurrence of SCD. This underscores the utility of exome sequencing in uncovering genetic factors associated with SCD.

Another application of exome analysis is the investigation of sudden infant death syndrome (SIDS) via MPS. Neubauer et al. [155] extracted from alcohol-fixed and paraffin-embedded tissue blocks of 161 cases of SIDS, resulting in 155 successful sequencing, showing two potential causative variants in *SCN5A* (p.Arg1897Trp) and *RYR2* (c.2907-1G4C) in two cases, while further variants of interest were identified in *KCNE2*, *CAV3*, *RYR2* and *MYBPC3*. Altered ion channel functions were mostly identified as SIDS causes, while variants found in genes associated with cell adhesion proteins and or sarcomere proteins are associated with cardiomyopathies and might, therefore, contribute to the main cause of death related to SIDS [155]. In another recent case

study, exome sequencing was employed to investigate genes associated with congenital thrombophilia, including *SERPINC1*, *PROC*, *PROS1*, *F2*, *F5*, *PLG*, *MTHFR*, in a Japanese man in his 30s. The study revealed the presence of a c.416C > T p.A139V variant in the *PROS1* gene, which was considered potentially related to the cause of death, specifically pulmonary thromboembolism. In response to this finding, further biochemical testing was conducted on deceased family members due to the autosomal dominant inheritance pattern of the variant. This case underscores the significance of molecular autopsies in forensic investigations [156]. In the context of applying molecular autopsies, several crucial aspects related to variants of uncertain significance (VUS) need to be carefully evaluated. These aspects encompass economic considerations, ethical and legal constraints, the challenge of confidently interpreting results and the absence of standardized guidelines for the utilization of VUS findings. Addressing these issues is essential for ensuring the responsible and effective use of molecular autopsies in forensic investigations [157].

In contrast to the extensive body of literature on variant analysis, only a limited number of studies have explored the potential of analyzing methylation patterns within specific gene promoter regions. These patterns can undergo alterations in response to various acquired predispositions and causes of death. One study conducted on a small cohort of individuals (n = 19) demonstrated a significantly higher degree of p16 promoter methylation in individuals exposed to Pb^{2+} compared to the control group. This suggests that methylation analysis in specific gene promoter regions may offer valuable insights into the effects of environmental exposures and their relationship to causes of death [158]. This has clear implication in the analysis of industrial chemical poisoning that has direct relevance for forensic assessment of cause of death. Similarly, Nakatome et al. [159] evaluated the different degree of DNA methylation of nine circadian clock genes in relation with cause of death and methamphetamine intoxication in an autopsy sample (n = 32). Results revealed that *Per2*, *Per3*, *Cry1* and *Tim* showed differential methylation patterns in different individuals, and those individual exposed to methamphetamine displayed higher methylation in *Pyr1*. While no direct correlation with the cause of death has been established, methylation analysis holds the potential to aid in understanding the mechanisms leading to death in forensic settings, as suggested by a study conducted by Nakatome et al. in 2011 [159]. This potential is further confirmed by a recent and comprehensive study that analyzed 737 selected White males with known medical records. DNA samples extracted from blood were analysed using the Illumina Infinium HumanMethylation450 BeadChip. This study revealed that DNA methylation-based biomarkers of age acceleration have a strong relationship with common causes of death in the aging population, such as myocardial infarction, stroke and cancer [160]. These findings show the potential of employing DNA methylation for the estimation of the cause of death. However, they also highlight the need for methodological standardization and further testing to ensure the reliability and accuracy of such an approach. This represents a promising avenue for forensic investigations and understanding the underlying factors contributing to various causes of death.

3.1.3 | Microbiomics for COD and MOD

In recent decades, the *post-mortem* microbiome has gained significant attention and is now considered a promising tool to be routinely involved in forensic investigations. This is evident from the substantial number of reviews on this topic in the scientific literature [161–164]. Despite the main application of microbiomics in forensics has focused on the estimation of PMI by evaluating bacterial community succession over time, recently successful applications have included the potential use of microbiomics for the identification of COD and MOD [165]. A comprehensive cross-sectional study conducted by Pechal et al. [166] aimed to assess microbial communities in various anatomical sites, including the external auditory canal, eyes, nose, mouth, umbilicus, and rectum. The study focused on determining PMI, variations in anatomical sampling and the influence of the individual's health status before death. The results confirmed the existence of niche differentiation between different anatomical locations and indicated the potential for utilizing microbiome studies in forensic cases with short timescales, specifically in those with a PMI of less than 48 hours. This conclusion was supported by the observed stable turnover patterns in microbial communities over time. Additionally, the study indicated that microbial communities present before death continue to exist *post-mortem* and could potentially serve as indicators of an individual health status as well as for MOD. The study revealed interesting findings regarding microbial diversity in individuals who died from different causes. It was observed that individuals who died from heart disease had lower microbial diversity, while those associated with violent deaths exhibited a higher bacterial community diversity. These distinctions in microbial diversity could potentially serve as indicators in forensic investigations to help determine the cause of death. This suggests that microbiome analysis has the potential to provide insights into *antemortem* health (e.g., *Aemophilus* and *Fusobacterium* showed twice the abundance in healthy individuals, whereas *Rothia* exhibited only 9% of more abundant in heart disease) [166]. The same dataset was later reanalyzed with the specific purpose of evaluating M/COD from beta dispersion data [165]. Results were promising and were used to developed multinomial logistic regression models able to validate the medical examiner cause of death assessment, assigning a correct COD $\sim$ 62% of the times. However, the best results were obtained using binary logistic regressions (natural vs. accidental death; cardiovascular disease vs. drug-related death; disease vs. non-diseased state) for which cardiovascular disease versus drug-related deaths showed $\sim$ 79% of correct assignments. The study also included random forest (RF) classification and logistic regression performances using beta-dispersion, highlighting the importance of appropriate bioinformatics pipelines in forensic science [165]. To confirm the potential of microbiome to differential M/COD, skin microbiomes from eight cases were sequenced and showed differences at both phylum and genus levels among the different CODs. Despite the interesting results of this study, the authors also highlighted important aspects to be considered when using *post-mortem* microbiome, such as biases introduced by the original depositional environment, the time elapsed between retrieval and

sampling and the anatomic location selected for the samplings [167]. For this reason, future studies should thoroughly consider the technical aspects connected to the sampling strategies starting from the study design, to ensure the replicability of the results [163]. Another interesting application of microbiome analysis for understanding M/COD was presented by Javan et al. [168], who investigated the correlation between *post-mortem* microbial composition and drug abuse. Similarly to the previous studies, the sequencing of the 16S rRNA via NGS showed that it is possible to distinguish between cases of drug abuse and non-drug abuse.

3.1.4 | Metabolomics and proteomics for COD and MOD

Advanced high-throughput methods, other than nucleic acid based ones, have enabled the identification of metabolites associated with various human traits, including health, disease, toxicology, and the aging process. Between these methods, proteomics and metabolomics are becoming increasingly popular in the diagnosis and in the mechanistic explanation of pathological conditions and drugs addiction. Consequently, the field of forensic investigation for the determination of the manner and cause of death has rapidly adopted these microbial tools to aid in the analysis and provide valuable insights into the circumstances of death.

GC-MS and NMR metabolomics was performed by Wang et al. [169] on myocardial samples from rats to evaluate the possibility to detect myocardial ischemia (MI). The research findings indicate that when examining lethal ventricular tachyarrhythmia (LVTA) against control samples, PLS-DA yielded R^2Y and Q^2 values of 0.479 and 0.525 for GC-MS, and 0.626 and 0.981 for NMR. Additionally, for rats exhibiting severe atrioventricular block, the corresponding R^2Y and Q^2 values were 0.379 and 0.283 for GC-MS, and 0.691 and 0.994 for NMR. These results highlight the significant potential for effectively distinguishing between different causes of death. To confirm these findings, acute myocardial ischemia (AMI) was further investigated in an animal model including 30 rats belonging to three groups: control, sham, and AMI. The analysis of serum samples using UPLC-HRMS allowed for the classification of groups in a pairwise manner but also revealed nearly complete separation between all three groups when analyzed together, with $R^2Y = 0.987/Q^2 = 0.814$. The study also analyzed different machine learning models showing that multi-layer perceptron (MLP) provides the best accuracy (96.67%). The same study also tested a selection of seven metabolites from the MLP model (i.e., L-threonic acid, N-acetyl-L-cysteine, CMPF, glycocholic acid, L-tyrosine, cholic acid, and glycoursodeoxycholic acid) on serum samples from 17 cases (AMI, $n = 9$) showing 88.23% of accuracy [170]. A more complex rat model evaluated the possibility of characterize anaphylactic shock (AS, $n = 11$), mechanical asphyxia (MA, $n = 11$) and SCD ($n = 11$) using GC-HRMS on serum sample [171]. The study showed changes in glucose metabolism, the TCA cycle, glycolysis, lipid metabolism, creatinine catabolism, and purine metabolism according with the three CODs and pointed up the potential to classify them with a good degree of accuracy. However, the study highlighted biases that could be introduced by sample collection time, limited inter-individual variability in rats compared to humans, and low metabolic coverage offered by GC-MS in comparison with LC-MS [171]. Vitreous humor (VH), a target fluid for metabolomic analysis, was utilized in a targeted assay to compare cases of hypothermia fatalities ($n = 20$) to control cases ($n = 16$) by Rousseau et al. [172]. Orthogonal partial least-squares discriminant analysis (OPLS-DA) was applied to the profiles of 188 metabolites. This analysis captured 72% of the correlation between variables and 89% of the variance, with a predictive ability of $Q^2 = 0.51$, highlighting the effectiveness of the technique. Furthermore, good markers were identified in methionine sulfoxide, methionine, and acetylcarnitine [172]. Overall, these studies provide preliminary evidence that metabolomics could have real-world applications in understanding the cause of death in forensic science.

Similarly, the profiling of lipids, another class of less polar functional metabolites, can be employed to gain insights into the cause of death. Wu et al. [173] developed a rat model on serum samples to investigate LVTA linked to myocardial ion channel diseases using UPLC-MS. In their research, they analyzed serum samples and identified 749 lipids, of which 188 displayed the potential to discriminate the *post-mortem* condition. The advantage of this approach is also represented by the possibility to perform a comprehensive pathway analysis in order to explain the metabolic mechanism of the COD [173]. In a recent study, the integration of metabolomics and machine learning techniques was employed to assess the ability to differentiate between drowning cases and *post-mortem* submerging in a rat model. This investigation utilized various machine learning algorithms, including RF, partial least squares (PLS), support vector machine (SVM) and neural network (NN). The study findings demonstrated the exceptional performance of the RF algorithm, with an area under the curve (AUC) of 1 and an accuracy rate of 95% [174].

Only one study was found regarding the use of proteomics for the understanding of COD. The study aimed to identify potential biomarkers by conducting serum LC-MS/MS analysis on four cases of drowning and comparing them to a control group consisting of one man who died by hanging and one woman who died due to polytrauma. Through this analysis, ApoA1 and −1 antitrypsin were identified as biomarkers of particular interest. To validate these findings, immunonephelometry was performed using a BN ProSpec equipment from Siemens Healthcare Diagnostics (Marburg, Germany) on a larger cohort of drowning cases ($n = 16$) and compared to cases with other causes of death ($n = 9$, including four cardiovascular deaths, one hanging, two cases of polytrauma, one stabbing and one death from drug overdose) [175]. The use of advanced analytical techniques like LC-MS/MS, in combination with biomarker validation, offers promising insights into the distinctive biochemical profiles associated with drowning cases. By comparing these profiles to other causes of death, proteomics can contribute to more accurate and reliable forensic diagnoses, particularly in cases involving drowning.

Another approach that could have application in forensic settings is the analysis of the exosome (Exos). Exos are a specific type of extracellular vescicles involved in the regulations of most aspects of the cell life

cycle. In response to external stimuli, the plasma membrane undergoes its initial invagination. External active substances, including nucleic acids, proteins, lipids and metabolites, enter the cell along with surface proteins of the plasma membrane through the process of endocytosis, leading to the formation of endocytic vesicles forming endosomes (ESE). Early sorting endosomes initiate the process by endocytosing mitochondria, endoplasmic reticulum, Golgi bodies and nucleic acids, forming late-sorting endosomes (LSEs). Subsequently, under the influence of endosomal sorting complexes required for transport (ESCRT), LSEs undergo a secondary internalization, leading to the development of multiple inter-luminal vesicles (ILVs) of various sizes. Due to this mechanism, LSEs containing ILVs are termed multi-vesicular bodies (MVBs). Some MVBs are degraded by autophagosomes or lysosomes, while the majority fuse with the plasma membrane, releasing ILVs and eventually becoming exosomes [176]. Wang et al. [177] showed the possibility of applying it to the study of coronary disease, cardiomyopathy, myocarditis, and heart failure. The advantage of targeting these biomolecules is the possibility to analyze proteins, metabolites and miRNA. Exosomes, in fact, can be used to identify SCD without relying on cardiac tissue samples. They are easily accessible for forensic analysis, making them valuable tools in forensic medicine. Furthermore, due to the protective role of the lipid membrane of the exosome, the biomolecules contained in these structures could maintain longer stability *post-mortem* compared to circulating biomolecules, enhancing reliability of the COD assessment. One main limitation is the cost and the complexity of the Exos purification procedure [177].

A few studies have applied proteomics and/or metabolomics methods to investigate wound age for forensic purposes. Tarran et al. [178] performed experimental incisions on the skin of 18 adult rats from 5 minutes to 12 days before euthanasia, then excised the wounds and extracted proteins that were subjected to 2-DE and MALDI-TOF MS. They found differences in the level of hemoglobin, which was high and stable for wounds up to 3 hours, and dropped to control level by 12 hours. However, the authors stated that this analysis cannot be used on its own as hemoglobin is too abundant in tissue samples and proteins unique to specific time periods for healing may be more informative for forensic applications. Dammeier et al. [179] conducted a study to determine which projectile caused a lethal injury by penetrating bovine organs with 79 projectiles and identifying organ specific proteins on their surfaces. They performed tryptic 'on-surface' digestion and used an LTQ OrbitrapXL and SVMs, RFs, Gaussian naïve and multinomial naïve Bayes they obtained over 90% of classification accuracy for all models and > 99% accuracy for the multinomial naïve Bayes in discriminating the organs. This approach could also be applied to the analysis of other weapons to improve the forensic re-construction surrounding MOD. Instead, Cao et al. [180] focused on the application of metabolomics and tandem machine learning for the estimation of wound age inflicted on rats and sampled from 4 to 48 hours post-contusion. They found consistent changes in the metabolomics profile at specific time intervals and highlighted 43 endogenous compounds that could be used for wound ageing. Amongst four machine learning models tested, MLP was able to classify wound age with an accuracy of 92.6% for samples taken from 4 to 12 hours post-contusion.

3.1.5 | Toxicology methods for COD and MOD

The application of omics to forensic toxicology, the branch of toxicology that applies accepted and standardized analytical methods to cases and issues where drug effects may have administrative or medico-legal implications. In practice, forensic toxicology involves the analysis of ethanol, drugs of abuse (DOA), prescription drugs or poisons in various types of matrices (e.g., urine, blood) via pre-screen immunoassays for known and common substances or GC-MS, LC-MS(/MS) for confirmatory analysis or unknown compounds screening. In addition to identifying specific drug metabolites and monitoring their stability and changes over time, metabolomics can reveal endogenous biomarkers indicative of drug use or potential sample tampering, as well as the severity of intoxication. Furthermore, it provides insights into the mechanisms underlying drug actions, which is crucial for understanding their effects and addressing chronic toxicity, ultimately informing the development of suitable therapeutic interventions. In metabolomics-based toxicology, it is crucial to analyze all samples, including the target substance, from the same batch and under consistent conditions. This practice helps mitigate bias resulting from sampling, storage, or instrument variations, as emphasized earlier [139, 181]. To reinforce the significance of standardization in toxicology, the 'Human Toxome Project' was initiated. Its objective is to deduce, validate and share molecular pathways of toxicity (PoT) by employing endocrine disruption as a model. The project focused on examining the responses of MCF-7 human breast cancer cells, utilizing transcriptomics and metabolomics as initial approaches. More specifically, the project aimed to face several challenges including (I) Cell model and reference compound selection, (II) Cell model standardization and QA, (III) Omics and QA as well as bioinformatics integration of different outputs from platforms, (IV) mechanistic Human Toxome Knowledge-base and (V) real-word application. An example of the application of toxicology to the evaluation of COD was presented by Chighine et al. [183] who evaluated the possibility of identifying methadone intoxication in cases of perinatal asphyxia. In the study, urine samples from 10 newborns affected by perinatal asphyxia, 16 healthy control newborns and one affected by methadone intoxication were tested via ^{1}H NMR. Results of multivariate analysis showed that the metabolome profile of the intoxicated individual was similar to the perinatal asphyxia ones, and the separation with the control group was mostly driven by increased levels of lactate that could be associate with a witch to anaerobic glycolysis, caused by the switch to anaerobic metabolism. Finally, based on the metabolic profile of the asphyxia cohort, it was possible to distinguish between the individual that survived the asphyxia episode [183]. This shows the possibility not only to identify intoxication via metabolomics, but also to provide a mechanistic explanation of the metabolic changes induced by it. In another study, researchers investigated cases of hypoglycemia-related deaths, which included 19 instances of insulin intoxication, 19 diabetic cases and 38 instances of hanging, the latter serving as the control group. The analytical method employed was UPLC-qTOF mass spectrometry. Moreover, the study screened a total of 776 randomly chosen post-mortem cases. The results showed decreased levels in 12 acylcarnitines within the profiles of the 19 insulin intoxication samples,

demonstrating a notable distinction between the three groups. When the model was applied to the randomly selected cases, it correctly identified 46 instances as being associated with hypoglycemia-related deaths, five of which were subsequently confirmed through autopsy reports [184]. As previously mentioned, it is crucial to consider the time-dependent changes of non-endogenous compounds during the investigation. In a study involving 477 cases, femoral blood samples were collected at two different time points. LC-MS/MS and GC-HRMS were utilized for drug abuse analysis and the identification of specific DOA. The investigation demonstrated distinct behavioral variations based on the specific compound under examination. Specifically, concentrations exhibited a decrease for diazepam and nordiazepam, an increase for mirtazapine and citalopram, while morphine and codeine displayed relatively consistent behaviour. Furthermore, LC-MS/MS profiling proved that including methionine, phenylalanine and valine in the targeted analysis could be beneficial in *post-mortem* concentration changes of drug concentrations [185]. Bai et al. [186] recently investigated antipsychotic agents' fatal intoxications by building an animal model based on LC-MS metabolomics of blood samples. Ten mice were first administered chlorpromazine or olanzapine in known doses to simulate fatal-intoxication and compared to 10 control samples and metabolic profiles of the two groups showed clear differences when test with OPLS-DA with some pathways (i.e., beta oxidation of very long chain fatty acids, oxidation of branched chain fatty acids, TCA cycle, fatty acid biosynthesis, and arginine and proline metabolism) being disturbed by the intoxication. Additionally, L-acetylcarnitine, succinic acid, L-carnitine, and propionylcarnitine were confirmed via targeted and found to be good marker to evaluate potential antidepressant fatal intoxication [186]. Only one study was selected according to our search criteria that applied a multi-omics approach to toxicology [187]. The study aimed to identify biomarkers of cannabis use applying a combination of slow off-rate modified aptamers proteomics, semi-quantitative targeted LC-MS/MS metabolomics on plasma samples from eight discordant twin pairs and four concordant twin pairs. Furthermore, 11 cannabinoids and their metabolites in ethylenediaminetetraaceticacid plasma were quantified via LC-MS/MS. Results show that 13 proteins, three metabolites, and two lipids that were associated with THC-COOH. Besides, the mechanistic explanation of pathways influenced by cannabis administration, the authors were able to identify new biomarkers that could be used to identify cannabis use from plasma sample (e.g., Myc proto-oncogene protein) [187].

3.2 | *Post-mortem* interval

In the realm of forensic investigations, establishing an accurate timeline of events is crucial, particularly in criminal inquiries. The process of PMI estimation assumes a pivotal role in this context, representing a fundamental component of the investigative framework. It yields insights into the circumstances enveloping an individual's demise, while concurrently addressing critical questions pertaining to the cause of death. Furthermore, in cases characterised by missing persons, homicides or unidentified remains, precise PMI estimation facilitates the identification and subsequent legal action against those implicated in the loss of life.

PMI estimation has been originally addressed via classic approaches, such as the evaluation of early *post-mortem* physical changes (*algor* [188], *livor* and *rigor mortis* [189]), the analysis of biochemical parameters (such as potassium levels in the VH of the eye [190]), the morphological examination of the remains (to evaluate, for example, ocular changes [191] or the decomposition stage [192]) and the use of forensic entomology [193]. Despite their wide use in forensics, they suffer from limitations in terms of precision and reliability due to the effect that intrinsic and extrinsic variables can play on those estimates. Additionally, the experience of the operator conducting the analyses can affect the accuracy of the estimations. Lastly, the applicability of some of these approaches to specific *post-mortem* time windows poses some limits, that can eventually be partially overcome by combining multiple techniques together [194]. Therefore, the development of advanced methods for PMI estimation, such as those offered by the adoption of omics approaches, can significantly enhance the precision of PMI estimations and increase the reliability of forensic investigations, ultimately contributing to more accurate and accountable outcomes in the field of forensic science.

3.2.1 | Search results

Using the Scopus database, the combined search terms were used: 'forens* AND "metagenom* AND "PMI"', 'forens* AND metabarcod* AND "PMI"', 'forens* AND microbiom* AND *postmortem* interval', 'forens* AND microbiom* AND "PMI"', 'forens* AND transcriptom* AND *postmortem* interval', 'forens* AND transcriptom* AND "PMI"', 'forens* AND proteom* AND *postmortem* interval', 'forens* AND protein* AND *postmortem* interval', 'forens* AND proteom* AND "PMI"', 'forens* AND metabolom* AND "PMI"', 'lipid* AND *postmortem* interval", 'forens* AND lipidom* AND "PMI"', 'forens* AND omic* AND "PMI"'. Two hundred and seventy-seven articles were retrieved, and 75 retained appropriate for the topic of the current review. Twenty-nine additional articles were also included manually for the pertinence to this thematic area. Table 1 summarises all the studies that exploited omic technologies combined with machine learning algorithms for PMI estimation.

3.2.2 | Microbiomics for PMI estimation

The first studies exploiting the use of microbiomics for PMI estimation were those conducted by Hyde et al. [216], Pechal et al. [195] and Metcalf et al. [196]. Hyde et al. [216] conducted their study on two human cadavers at Southeast Texas Applied Forensic Science (STAFS) facility and sampled mouth and rectum at the onset of the bloat stage and at its end, and analyzed the 16S via pyrosequencing. They were able to demonstrate the aerobic to anaerobic microbial shift during decomposition and to identify novel bacterial species in comparison with culture-based studies. Pechal et al. [195], instead, placed three swine

TABLE 1 Research articles applying omics technologies and machine learning algorithms to estimate PMI, and the accuracy of the best model developed.

Max PMI/ max PMSI	Host Species	Number of hosts	Sample type	Indoor/outdoor/ sub-merged	Location	Omics used	Instrument used	Best performing modelling method	Accuracy of the best model	Manuscript
5 days	Pig	3	Mouth, skin	Outdoor	Xenia, OH	16S	Pyrosequencing	RF	96% time variation explained	[195]
48 days	Mouse	40	Abdomen, skin body, skin head, soil	Indoor on soil	NA	16S, 18S, rRNA longer fragments	Illumina HiSeq, Pacific Biosciences RS	RF	MAE = 3.30 ± 2.52 days over 34 days PMI	[196]
48 h	Rat	36	Plasma	Indoor	Japan	Metabolomics	GC-MS	PLS	$R^2 = 0.98$	[197]
~500 ADD	Human	21	Ear, nose	Outdoor	ARF	16S	Illumina MiSeq	KNN	55 ADD	[198]
71 days for mouse, 82–143 days for human	Mouse and human	120 and 4	Abdomen, skin, soil	Indoor on soil and outdoor	STAFS	16S, 18S, ITS	Illumina HiSeq, Illumina MiSeq	RF	On human, RMSE 342.14 ADD over 2000 ADD PMI	[199]
48 h	Mouse	52	Muscle and serum	Indoor	Japan	Metabolomics	GC-MS	NLRA	Mean accuracy 0.27 ± 2.88 and 0.89 ± 2.31 h for muscle and serum	[200]
1739 min	Human	540	36 tissues	GTEx metadata	NA	mRNA	NA	LRM	$R^2 = 0.86$	[201]
72 h	Rat	84	Blood	Indoor	China	Metabolomics	GC-MS	oscPLS	51.82 and 55.96 h for male and female	[202]
1429 min	Sheep	36	AH	Indoor	Italy	Metabolomics	NMR	oCPLS2	SDEP = 59 min for PMI <500 min, 104 min for PMI from 500 to 1000 min and 118 min for PMI >1000 min	[203]
72 h	Rat	36	Blood	Indoor	China	Metabolomics	GC-MS	SVR	MSE = 10.33 h, $R^2 = 0.99$	[204]
15 days	Mouse	80	Brain, caecum, heart	Indoor	China	16S	IonS5XL	ANN	MAE =1.5 ± 0.8 h over 24 h PMI and 14.5 ± 4.4 h over 15 days PMI	[205]
22 days	Pig	5	Skin head, skin torso	Submerged pond	Michigan	16S	Illumina MiSeq	RF	MSE 3 days over 22 days PMSI	[165]
58.6 h	Human	31, 31 and 33	AH, VH, serum	Morgue	Russia	Metabolomics	NMR	MLR	RMAE = 0.45 ± 0.14 h	[206]
192 h	Human	63	Intestine	Morgue	China	16S	Illumina MiSeq	RF	MAE = 25.79 h, $R^2 = 0.91$	[46]

(Continues)

TABLE 1 (Continued)

Max PMI/ max PMSI	Host Species	Number of hosts	Sample type	Indoor/outdoor/ sub-merged	Location	Omics used	Instrument used	Best performing modelling method	Accuracy of the best model	Manuscript
9 months	Human	6	Rib	Outdoor	STAFS	16S, 18S	Illumina MiSeq, Illumina HiSeq	RF	MAE = 793.33 ADD (~ 34 days)	[207]
353 days	Pig bones	240	Rib and scapula	Submerged river	Virginia	16S	Illumina MiSeq	RF	RMSE ~ 28 days over 353 days PMSI, R2 0.94	[208]
579 days	Pig bones	190	Bone	Sub-merged lake	Virginia	16S	Illumina MiSeq	RF	RMSE ~ 37 days over 579 days PMSI, R2 0.96	[209]
38 h	Human	7	Blood	Morgue	Belgium	mRNA	Illumina NextSeq	GLM	RMSE 4.75 h over 38-h PMI	[210]
36 days	Mouse	65	Soil	Outdoor	China	16S	Illumina MiSeq	RF	MAE 1.27 days over 36 days PMI	[211]
14 days	Mouse	180	Gut	Sub-merged river	China	16S	Illumina NovaSeq	RF	MAE 0.818 ± 0.165 days over 14 days PMSI	[174]
30 days	Rat	140	Liver, lung, kidney, muscle	Indoor	China	Metabolomics	UPLC-Orbitrap	RF, LDA, SVM	MAE 0.42 days over 30 days, R2 0.93	[212]
30 days for rat, 28 days for human	Rat and human	84 and 9	Caecum	Indoor and morgue	China	16S	Illumina MiSeq	RF	On human, MAE 0.56 days over 7 days PMI and 4.51 days over 30 days PMI	[213]
86 h	Sheep	71	VH	Indoor	Italy	Metabolomics and potassium level	NMR	ptPLS2	RMSEP 5.7 h over <24-h PMI, 7.4 h for PMI between 24 and 48 h, and 8.4 h for PMI >48 h	[214]
170 h	Human	24	Pericardial fluids	Morgue	Italy	Metabolomics	NMR	oCPLS2	SDEP 33-34 h over 170-h PMI, 13–15 h over 100-h PMI	[215]

carcasses outdoor and sampled the oral and skin epinecrotic communities at selected time points up to 5 days PMI. Bacterial communities were analyzed via 16S amplicon pyrosequencing, and RF models developed were able to explain 96% of the time since placement of the carcasses using 10 selected taxa. Metcalf et al. [196] increased the sample size by using a smaller animal model (mouse, $n = 40$) indoor to estimate PMI starting from different body sites (abdominal, skin of body, skin of head) and sample types (soil underneath corpse and control soil without corpse) for a total of 223 samples collected. They sequenced both 16S and 18S amplicons using the Illumina HiSeq platform together with longer fragments of the rRNA genes on the Pacific Biosciences RS platform. With RF modelling, they obtained an error of approximately 3 days when using data from the skin of the head. The authors recommended the use of skin and soil sites instead of the abdominal cavity for increased accuracy, and found that the combined use of 16S and 18S dataset does not significantly improve the PMI estimations. Hyde et al. [217] used two donated cadavers at STAFS that were sampled in their mouth, on different skin sites and in the rectum on daily basis for 19 days. 16S metabarcoding was achieved with pyrosequencing and allowed the group to describe for the first time the successive bacterial changes associated with human decomposition. Due to the limited sample size, they were not able to develop regression models nor to make inferences about human decomposition in general. Guo et al. [218] instead sampled the oral and rectal microbiome of six rats placed outside up to 3 days *post-mortem*. They identified dominant taxa at several time points via 16S metabarcoding using the Illumina MiSeq platform but due to the limited sample size were not able to develop PMI predictive models. Johnson et al. [198] targeted instead the skin microbiota of ear canals and nose of 21 human cadavers located outdoor at the Anthropological Research Facility (ARF) at the University of Tennessee to evaluate the applicability of non-invasive samplings for PMI estimation. 16S metabarcoding was conducted on the Illumina MiSeq platform and several regression techniques were tested to identify the best predictors for PMI. They found out that the combination of the two anatomical sites increases the accuracy of the estimation, that results in a mean absolute error (MAE) of 55 accumulated degree days (ADD) (approximately two days of decomposition in the warm months in Tennessee) when considering a PMI of 500 ADDs. In order to evaluate the principles governing the decomposition process on different soil substrates, Metcalf et al. [199] placed mouse carcasses indoor on three soil types and sampled their skin, abdominal cavity and gravesoil for a maximum PMI of 71 days, and conducted in parallel human decomposition studies on four donated bodies at STAFS by sampling their skin and associated gravesoil over a maximum period of 143 days. Similarly to their previous study [196], they did full metabarcoding analyses on archaeal, bacterial, microbial eukaryotic and fungal communities. Their RF regression model resulted in an error of 2–3 days over the first 2 weeks of decomposition. Soil types, seasons and host species did not affect the accuracy of the estimations. They also showed that soil is a crucial source of decomposer microbial communities, which are found at very low abundance at the beginning of the experiment and that increase with the progression of the decomposition. Javan et al. [219] conducted a study on human cadavers from criminal investigations ($n = 27$) with PMI ranging from 3.5 to 240 hours. Specifically, they sampled internal organs, mouth and blood and performed 16S metabarcoding. They were able to show that sex, internal organ chosen for the analysis and taxonomic depth (e.g., genus vs. species level) all play a role in explaining the variance of the PMI predictive models, and that temperature, on the contrary, did not play a key role in contributing to the differences in the community structure. DeBruyn and Hauther [220] sampled the caecum of four human individuals placed at ARF and performed metabarcoding on the 16S via Illumina MiSeq. They identified a common decay community among the different cadavers characterised by an increased richness and a decreased diversity with increasing PMIs. Pechal et al. [166] conducted the largest study so far on 188 human cadavers which were sampled during routine death investigations. Specifically, they sampled ears, eyes, nose, mouth, rectum, and umbilicus and conducted metabarcoding of the 16S gene with either Illumina HiSeq and MiSeq instruments. The PMI range was from <24 hours to more than 73 hours *post-mortem*, and was determined either on the basis of taphonomic alterations or on corroborated eye witness reports. Their study was aimed at exploring the ecology and the microbial successions in different anatomical districts postmortem, and not at developing a model for PMI estimation. Their findings suggest that the *post-mortem* microbiome reflects the *antemortem* one for at least 24–48 hours *post-mortem*, similarly to what found by Iancu et al. [167]. In their study, they sampled eight bodies at the morgue by swabbing their face and hands upon their arrival and after 12 hours. Sequencing of the 16S with Illumina MiSeq revealed minor variations in the microbial populations at the two time points considered, and identified correlations between the skin microbiome and cause of death, in line with what observed by Pechal et al. [166]. Fu et al. [221] focused exclusively on the fungal successions associated with PMI by using juvenile pigs as human proxies both indoor (for up to 56 days) and outdoor (for up to 14 days). Both cadaveric and soil fungal communities were sampled at selected time points and the ITS1 region was targeted for subsequent metabarcoding analyses with Illumina MiSeq. They identified fungal species correlated with accumulated degree hours (ADH) that were proposed as indicators for PMI estimation. Moreover, due to the similarity in terms of fungal communities observed between the carcasses and the gravesoil, they proposed the use of fungal analyses to ascertain the location from which a cadaver has been moved. Liu et al. [205] sampled several internal organs (brain, heart and caecum) of 80 mice left to decompose indoor for a period of 15 days. 16S gene was sequenced via amplicon sequencing with IonS5XL and several machine learning algorithms including RF, SVM and artificial neural network were used to estimate PMI. The best result was obtained when using data from the caecum and artificial neural network, with MAE = 1.5 hours for up to 24 hours decomposition and MAE = 14.5 hours for up to 15 days decomposition. Despite the great accuracy obtained, it has to be noted that controlled indoor decomposition conditions are far from the reality in forensic scenarios, therefore results of these models should be interpreted carefully before their use in real life forensic contexts. Hu et al. [222] conducted a large study on human cadavers, by sampling the gut microflora from the large intestine (vermiform appendix and

transverse colon) of 63 human cadavers from morgues with PMI ranging between 5 and 192 hours, conducted metabarcoding analyses on the 16S gene on Illumina MiSeq platform and applied RF modelling to develop PMI prediction models. Results showed that the microbiome of the appendix may be a good indicator for PMI, with MAE = 25 h for a maximum PMI of 192 hours. Li et al. [213] have recently compared rat and human thanatomicrobiomes by sampling caecum feces at selected time points (up to 30 days) from 84 rats and from nine humans at the time of forensic autopsies (PMI ranging from 3 to 28 days). 16S gene amplicons were sequenced via Illumina MiSeq. They divided the samples in two PMI windows (0–7 and 9–30 days) based on MLP modelling and established then regression models using RF in each group. Overall, the model on rats for 0–7 days had a MAE of 0.58 days and the one for 9–30 days had a MAE of 3.16 days. Subsequently, they selected shared taxa between the caecum of rats and human and predicted the human PMI based on the rat model previously developed. The MAE for the PMI group of 0–7 days was 0.56 days, and the MAE for the PMI group of 9–30 days was 4.51 days overall supporting the potential translation of PMI models based on animal proxies to the estimation of PMI in humans.

In contrast with the significant amount of literature available for PMI estimation using microbiomics from soft tissues, much less is known regarding the microbial profile of bones *post-mortem*. Damann et al. [223] analyzed bone samples from 12 human individuals and three soil samples, and found the presence of gut bacteria in the partially skeletonised remains, and a profile more similar to soil bacteria in dry remains. Similarly, Deel et al. [207] investigated the microbiome of ribs from six individuals at selected time points *post-mortem* and in different seasons, and successfully identified a core bone decomposer microbiome characterized by environmental taxa present in the surroundings of the bones (e.g., skin and soil). They managed to develop a RF model that predicted the PMI with an accuracy of 34 days over 1–9 months PMI timeframe, providing investigators with a new tool for the estimation of PMI from skeletal remains.

Other studies focused on the analysis of the soil associated with the cadaver/carcass (gravesoil) to estimate PMI also in circumstances when the body has been moved from its original location. The first studies focused on the characterization of the communities associated with decomposition and on their shifts over time. Carter et al. [224] explored the microbial succession in soils underneath pig carcasses in summer and winter by analyzing the 16s rRNA gene with Illumina HiSeq 2000, and demonstrated that soil microbial successions associated with the phenomenon of decomposition are predictable and reproducible, and that the seasonality plays a key role in the soil microbial communities and should therefore be taken into account when exploring the use of soil microbiome analyses for PMI estimation. Cobaugh et al. [225] collected soil from beneath four human cadavers at ARF throughout decomposition (up to 198 days) and analyzed the 16S gene with MiSeq. They identified distinct communities and predominant taxa in association with specific decomposition stages, human-associated bacteria present in the soil up to 198 days PMI, and potential taxa for PMI estimation from soil samples. Similarly, Finley et al. [226] evaluated the microbial communities associated with human cadavers ($n = 18$) either exposed on the soil surface or buried at the Forensic Anthropology Research Facility at Texas State University (FARF), that decomposed for up to 303 days. The analysis of the 16S via Illumina MiSeq revealed the presence of distinct communities when comparing buried versus surface gravesoil, with the latter being more stable throughout decomposition. Metcalf et al. [196, 199] in their studies targeted the 16S, 18S and ITS regions to identify the gravesoil communities associated with both human and mice decomposition and showed that the microbial communities in the decomposing tissues become similar to those in the gravesoil and that soil microbes play a key role as decomposers. Olakanye et al. [227] evaluated the sub-surface decomposition of three stillborn piglets and the parallel process of litter decomposition by using Illumina MiSeq to sequence the 16S gene. They identified some taxa that could be used as *post-mortem* microbial clocks in such subsurface conditions when a decomposing body is present. Adserias-Garriga et al. [228] conducted a study at ARF using three donated cadavers and investigating the bacterial successions in the gravesoil via 16S sequencing. They also identified the migration of bacterial communities from the cadaver to the soil, and proposed the use of the growth curve of *Firmicutes* from human remains to estimate PMI placed in outdoor conditions similar to those experienced by cadavers in Tennessee in summer months. Procopio et al. [229, 230] investigated both the bacterial (16S) and the fungal communities (ITS1-ITS2) associated with the gravesoil of four pig burials for up to 6 months in England and identified specific microbial shifts in association with increasing PMIs, as well as the presence of mammalian-derived microbial species in the soil, similarly to what reported by Cobaugh et al. [225], for up to 6 months post deposition. Recently, Cui et al. [211] developed for the first time a RF model to identify biomarker taxa from gravesoil for PMI estimation. The model was developed on $n = 65$ mouse carcasses buried in a forest and predicted PMI with a MAE = 1.27 days for up to 36 days decomposition. Despite the promising results obtained, similar studies on human cadavers and in other conditions (e.g. exposed vs. buried, different seasons/climates) are yet to be performed.

In the context of this review paper, it is also vital to bridge the gap between studies focusing on PMIs in terrestrial conditions and those conducted in aquatic environments (*post-mortem* submersion interval, PMSI). Benbow et al. [231] presented the first metabarcoding study on the evaluation of the *post-mortem* skin microbiome (epinecrotic biofilm) of swine remains sub-merged in a temperate head-water stream during two seasons via pyrosequencing the 16S gene. They showed an increased bacterial richness over increasing PMSIs and significant variations between the bacterial community structures found at the same PMSI but in different seasons. Lang et al. [232] performed a similar study but targeted the bacterial automated ribosomal intergenic spacer instead of the standard 16S gene and compared the swine epinecrotic biofilm against the epilithic biofilm present on ceramic tiles exposed to two different water streams. They showed the presence of changing communities on both biofilms analyzed, and identified distinctive shifts associated with the first weeks of PMSI with a potential for their use in PMSI estimations. They recommended, however, to pay attention to the influence of environmental factors (water type, environment, seasonality) as these are likely to affect the community shifts

and have, therefore, to be kept into account prior to conducting such estimations. More recently, Kaszubinski et al. [233] simulated a cold case by dressing in human clothing five pig carcasses and by placing them at 1m depth into a pond. Skin and mouth swabs were collected regularly up to 22 days and the 16S gene amplicons were analyzed via MiSeq sequencing. They highlighted common community shifts along the PMSIs across the five pig replicates, supporting the potential for microbiome analyses to estimate PMSI when adequate samples are collected at the time of the discovery of the cadaver, and developed a RF model with a mean squared error of ± 3 days over the PMSI investigated. Cartozzo et al. [208, 209] investigated the possibility to perform PMSI estimation starting from the bone microbiome of swine bones sub-merged in a freshwater river for 353 days ($n = 240$, ribs and scapulae) and in a freshwater lake for 579 days ($n = 190$, ribs and scapulae), respectively, via 16S analysis with MiSeq. Bone types affected the results, but an increase of the bacterial diversity was observed consistently with increasing PMSIs for all bone types. They also developed an estimation model on ribs and obtained a root-mean-squared-error (RMSE) of 28 days for the river environment and on scapulae with an RMSE of 37 days in the lake environment. Finally, Zhang et al. [174] focused on the gut microbial successions of mice sub-merged in freshwater (16S gene) and either died of drowning or CO_2 asphixia. They noticed that the cause of death did not cause variations in the *post-mortem* microbial successions and developed a regression model for PMSI estimation with a MAE = 0.81 days for a maximum PMSI of 14 days. The results obtained, despite the limited number of study available, clearly support the use of *post-mortem* microbial successions for PMI estimation also in water environments.

Something that has to be considered when conducting microbiomics studies for PMI estimation is the storage condition of the body *post-mortem*, and in particular, the effect of freezing and thawing on the *post-mortem* microbiome. The vast majority of the works on *post-mortem* microbiomics rely indeed on the analysis of human or animal bodies that were preserved frozen until the start of the experiments. Pechal et al. [234] showed a shift in the *post-mortem* microbial communities (structure and abundance) during the thawing process of two pediatric cases, whereas Ogbanga et al. [235] showed that storing bodies frozen preserves the composition and abundance of their pre-freezing microbiome despite reporting some statistically non-significant changes to the *post-mortem* microbiome post freezing and thawing. These results, despite limited to a few studies, highlight the needs for a better understanding of the effects that storage conditions can have on the bodies, to ensure a correct interpretation of the findings for the identification of potential biomarkers for PMI estimation and human profiling.

While acknowledging the relevance of microbiome analysis in estimating PMIs, it is worth noting that some studies have delved into utilizing microbiome data from larvae and insects for PMI estimation [236–238]. However, it is important to emphasize that these specific investigations, while noteworthy, fall beyond the scope of this review and would not be explored in detail here. Finally, it is worth mentioning that several methodological papers have addressed the crucial aspects of extracting and preserving microbial samples for metabarcoding analyses, including kit and instrumental comparisons [239, 240] and sample storage evaluations [241], overall providing valuable insights into optimizing the techniques used in this domain.

3.2.3 | Transcriptomics for PMI estimation

In the pursuit of refining PMI estimation techniques, thanatotranscriptomics emerges as a powerful tool with distinct advantages. *Post-mortem* mRNA profiling can, in fact, offer high accuracy estimations of relatively short PMIs (from hours to days), and is, therefore, able to complement the standard approaches used in forensic pathology for PMI prediction. Despite the vast number of studies conducted historically on RNA degradation of specific genes and their association with PMI estimation (and for which numerous review papers are available [242, 243]), this section will focus on the systematic analysis of transcripts (therefore, following a transcriptomics approach) and will not include the targeted works just mentioned.

Experiments conducted on animal models revealed increased levels of mRNA transcripts in liver and brain associated with stress, apoptosis, inflammation, and other molecular functions from 1 hour from death for up to 96 hours *post-mortem*, potentially associated with the response of the cells still alive after death fighting for survival or with the fast decay of specific gene repressors leading to an increased transcription of such genes [244]. Similar findings were also found by Javan et al. [245], which showed the up-regulation of pro-apoptotic genes and the down-regulation of anti-apoptotic genes *post-mortem* in human livers with increasing PMIs, and the stability of mRNA molecules in decomposing tissues for up to 48 hours *post-mortem*. Tolbert et al. [246], instead, identified an initial up-regulation of antiapoptotic genes followed by the up-regulation of apoptotic genes in human prostate tissues *post-mortem*. Halawa et al. [247] found no significant increase in inflammation-related gene transcripts in animal brains for up to 6 hours *post-mortem* at room temperature, but identified increased inflammation signals following *post-mortem* heat stress. Therefore, temperature at which bodies are found should be carefully recorded and taken into account when conducting transcriptomics studies on human cadavers for PMI estimation. Additionally, these results suggest that the *post-mortem* response is organ- and species-dependent, so specific studies should be conducted to identify suitable mRNA markers, human- and organ-specific. More recently, a work conducted longitudinally on human blood from seven individuals at known PMIs (up to 38 hours) identified two groups of up- and down-regulated genes associated with increasing time elapsed since death [210]. In general, they observed the activation of DNA damage repair genes and the suppression of apoptotic pathways and developed a PMI prediction model with an accuracy of 4.75 hours for PMIs up to 38 hours *post-mortem*. Another study conducted on data published on the Genotype-Tissue Expression (GTEx) database, focused on human blood samples ante mortem and *post-mortem* at different PMIs and showed the up-regulation of genes associated with changes in DNA synthesis, deactivation of the immune response, increase of cell necrosis, inactivation of carbohydrate metabolism, synthesis of lipids and ion transport and

finally with blood coagulation and response to stress, particularly between 7 and 14 hours *post-mortem* [201]. They also investigated the transcriptomics signature in other tissues and their correlation with PMI and developed a model with a prediction accuracy of 9.45 min when using multiple tissues. By exploring the same GTEx database, Zhu et al. [248] confirmed that the mRNA signature associated with PMI for up to 27 hours is tissue-specific, gene-specific and genotype-dependent, and that the majority of transcripts decreases with increasing PMIs as a result of mRNA degradation, but that other transcripts associated with specific biological functions taking place *post-mortem* increase, as highlighted also above. Despite not proposing specific models for PMI estimation, the authors proposed the use of up-regulated mRNA markers to predict long PMIs, and down-regulated genes for the prediction of shorter PMIs.

3.2.4 | Proteomics for PMI estimation

Proteomics, traditionally renowned for its pivotal role in various scientific disciplines, has recently found applications in fields such as bioarcheology and paleontology. Notably, researchers have harnessed proteomic techniques for tasks ranging from species identification to the validation of ancient molecules within ancient specimens. Building on these successes, investigators have shifted their focus towards shorter timescales, sparking a new era in forensic science [249]. This section explores the use of proteomics in the estimation of PMI, by spanning from skeletal remains to soft tissues.

The use of proteomics as a way to address the PMI of skeletal remains is very recent, with the first publications on this matter being those of Procopio et al. [250, 251] that developed an optmized protocol for proteomic analyses of skeletal remains for forensic applications, and that explored the inter- and intra-skeletal variability of different skeletal elements and the proteomics differences associated with a specific age-at-death (AAD) to evaluate to which extent proteomics could have been used to estimate PMI in forensic contexts. Following their findings, they were then able to investigate the use of bone proteomics for PMI estimation, by conducting experiments with four piglets that were buried and allowed to decompose for up to 6 months [252]. At selected time points, bones were collected and proteomic analyses were conducted with LC-MS/MS to identify proteome variations (reduction in proteome complexity, increase of PTMs) associated with increasing PMIs. Specifically, they found a significant reduction in blood/plasma and muscle proteins and a significant increase of biglycan deamidation in association with prolonged PMIs, setting the bases for the application of bone proteomics to forensic contexts. In a similar way, Prieto-Bonete et al. [253] evaluated the proteome of $n = 40$ human skeletal remains from cemeteries at different PMIs (5–20 years) and identified 32 proteins that allowed them to discriminate between $<$ 12 years versus $>$ 12 years PMI groups.

Bone proteomics was also conducted on sub-merged animal remains in the attempt to evaluate the applicability of this methodology to estimate PMSI. Mizukami et al. [254] showed that there are specific proteins which abundance and/or PTMs levels correlate with the PMSI, therefore supporting the use of proteomics also in such contexts. The same group applied then bone proteomics to human skeletal remains, either from an experimental taphonomy experiment ($n = 4$) conducted at FARF [255], and from bones collected from Italian cemeteries ($n = 14$) [256]. They found new potential biomarkers for PMI and AAD estimation, but the number of the samples was not large enough to apply modelling algorithms for the development of regression models. It also has to be noted that future proteomic studies on human bones sampled from curated skeletal collections should be carefully conducted, as Gent et al. [257] applied proteomics to experimentally macerated animal bones and made readers aware of an alarming alteration in the bone proteome complexity and in the PTMs in any treated specimen.

In addition to bones, other biological sources have been examined through proteomics for shorter PMI estimation. It is important to specify that this review is not aimed at exploring the targeted studies conducted to evaluate the degradation pattern of specific proteins *post-mortem* by using non-omics approaches, as other reviews can be accessed to deepen this topic. A few publications have focused on muscle tissue and on the systematic characterization of the protein markers associated with PMI with a proteomic approach. Li et al. [258] sampled muscle tissues from four rats at selected time points (up to 144 hours *post-mortem*) and applied MALDI-TOF imaging to identify degrading proteins in situ. They developed then a genetic algorithm (GA) prediction model based on the results obtained, which used five peptide peaks able to separate the samples based on their PMI and obtained an accuracy of 83.72% in the cross-validation for the attribution of the samples to the right PMI time-window (0, 48, 96 and 144 hours). They used the same approach also to identify potential PMI markers from human and rat liver samples for up to 6 days PMI, obtaining an accuracy of 92.16% in the cross validation when considering the attribution of the sample to the same time-windows previously reported [259]. Choi et al. [260] conducted proteomic analyses on rat and mouse skeletal muscles for up to 120-h post-mortem, and identified specific proteins which showed a consistent degradation over the course of time, in both species analyzed. Brockbals et al. [261] sampled skeletal muscles from nine decomposing human cadavers for up to 3 months. They proposed the use of peptide ratios for the estimation of PMI and managed to split the samples in sub-groups ($<$ 200 ADDs, $<$ 655 ADD and $<$ 1535) successfully. Battistini et el. [262] experimentally degraded pig muscle samples by exposing them at 21°C and 6°C for increasing time points (up to 120 h). Then they applied mass spectrometry proteomics and identified a few protein targets for potential PMI assessment.

Other works conducted by Nolan et al. have used, instead, the decomposition fluid and have applied LC-MS/MS proteomics for the determination of markers associated with PMI. In their first work on four pig analogues, they identified a range of peptides consistently found in the fluids and mainly originated from hemoglobin [263]. Subsequently they expanded the study to 16 pigs and tested the decomposition fluids via proteomics in both summer and winter months [264]. They identified peptides, again derived mainly from hemoglobin and from other ubiquitous enzymes, that consistently

increased earlier during the PMI and then decreased markedly in both seasons when they were expressed in ADDs. Despite the differences noticed for other peptides depending on the season under investigation, the authors emphasized overall the potential that peptide analysis has for PMI estimation [265].

3.2.5 | Metabolomics for PMI estimation

In the realm of PMI estimation, the emerging field of metabolomics has taken a central role. This section explores the use of metabolomics and its pivotal role in determining the biochemical changes that occur in the *post-mortem* state. By examining the dynamic metabolic profiles of cadavers, metabolomics offers a fresh and promising perspective on enhancing the accuracy of PMI determination.

Within this field, two primary approaches have prominently emerged: NMR and MS. Originally, NMR studies have involved the metabolic profiling of animal tissues or fluids *post-mortem*, in particular of blood, muscles and ocular fluids and tissues. Hirakawa et al. analyzed $n = 72$ rat muscles and identified correlations between the metabolic profiles, cause of death and PMI [266]. By conducting categorical and multivariate analyses, they developed a method for PMI estimation able to discriminate PMIs < 15 min, 1–4 hours or > 8 hours with an overall accuracy of 84 and 96% with k nearest neighbors (k-NN) and SVMs, respectively. Rosa et al. [267] monitored the modifications of goats VH for up to 24 hours *post-mortem* at selected time points and multivariate regression analyses showed time-related metabolic compositional changes including the increase of lactate, hypoxanthine, free amino acids, creatine and myo-inositol and the decrease of glucose and 3-hydroxybutyrate. More recently, Locci et al. [203] proposed the use of NMR to estimate PMI from ovine AH samples collected for up to 1429-min *post-mortem*. By using multivariate statistics, they obtained a prediction error of 59 min for PMI < 500 min, 104 min for PMI from 1500 to 1000 min and 118 min for PMI < 1000 min. They also compared results of the NMR analysis of AH against the potassium concentration levels, a well-known marker for PMI, and demonstrated that the AH metabolomic profile presents a greater predictive power than potassium, recommending, therefore, its use in forensic caseworks [268]. Similar results were obtained when comparing VH and potassium levels in a ovine model; in fact, VH predicts PMI better than potassium concentrations for up to 48 hours *post-mortem*, whereas for longer PMIs (up to 86 hours), the combination of VH metabolites and potassium levels offers a better PMI estimation when compared with the single approaches [214]. Zelentsova et al. [206] compared the human serum, AH and VH profiles ($n = 33$ for serum and $n = 31$ for AH ad VH) *post-mortem* in order to identify a model for PMI estimation. As per their previous observations in animal samples [269], AH and VH seem to be better candidates for PMI estimation than serum. By developing a multivariate linear regression (MLR) model for PMI estimation from AH and VH, they obtained a RMAE = 0.45 hours (max PMI = 58.6 hours) for both fluids, and a slightly higher error for serum samples when using a univariate model. Recently, another body fluid has been investigated for PMI estimation via metabolomics. Chighine et al. [215] applied for the first time NMR on $n = 24$ human pericardial fluid samples for PMI estimations ranging from 16 to 170 hours, and by adopting a multivariate model obtained a prediction error of 33 h over the full PMI investigated, and of 14 hours when narrowing the time window to 100 hours, showing the potential of the use of this fluid (normally collected in real forensic scenarios) for successful PMI estimations.

In parallel with NMR studies, GC and LC approaches have also been extensively used to address PMI from a metabolomics point of view. Kang et al. [270] used UPLC/Q-TOF MS on liver of rats decomposing for up to 48-h PMI and applied PCA, PLS-DA and oPLS-DA to identify the metabolites associated with PMI. Sato et al. [197] used GC-MS/MS to identify metabolites associated with the same PMIs in rats' plasma. With PLS regression model based on the obtained variable importance in the projection (VIP) scores, they successfully predicted mean PMIs of 0.19±0.81 for 0 hours PMI, 4.04±2.81 for 3 hours PMI, 7.22±1.50 for 6 hours PMI, 10.51±1.02 for 12- hours PMI, 20.70±5.99 for 24 hours PMI and 48.50±5.73 for 48 hours PMI. Similarly to Sato et al., also Donaldson and Lamont [271] performed GC-MS on plasma from four rats over increasing PMIs and identified 26 metabolites' abundances being correlated with the time elapsed since death. Rats blood was also analyzed by Wu et al. [202], for a PMI up to 72 hours. They used GC-MS and analyzed the data via oscPLS regression model, obtaining estimates of 49.88 ± 6.26 and 56.91 ± 5.59 h over 60 hours PMI for male and female rats, respectively. Blood and muscles from decomposing mice ($n = 52$) were investigated also by Kaszynski et al. [200] for up to 48 hours PMI. They conducted GC-MS and generated PMI estimation models using a non-linear regression analysis algorithm. Muscle samples gave a maximum absolute error of 5 hours for up to 24 hours PMI and of 2 hours for the sample with 48 hours PMI, whereas serum samples gave a maximum absolute error of 0.9 hours for up to 12 hours PMI, 4 hours for samples with 24 hours PMI and of 1 hours for the sample with 48 hours PMI. Rat muscles decomposed for a longer time frame (up to 168 hours) were investigated in Du et al. [272] via LC-MS. The authors identified 59 metabolites via PLS-DA being correlated with PMI especially after 48 hours *post-mortem*, and proposed their use as biomarkers for PMI estimation. Tan et al. [273] studied instead the *post-mortem* changes in the rat retinal metabolome for up to 48 hours by using UHPLC-MS/MS. Despite not aiming at addressing PMI within their study, they provided the baseline for subsequent studies on ocular tissues for PMI estimation via metabolomics. More recently, Pesko et al. [274] performed a comparison between rat ($n = 8$) and human ($n = 6$) muscle tissues for PMI estimations up to 19 days using LC-MS. They identified similar markers of those found in the other studies previously mentioned, but did not develop estimation models based on the obtained data. Lu et al. [212] conducted a multi-tissue analysis of *post-mortem* metabolomics profiles on a large number of samples ($n = 140$ rats) for a PMI of up to 30 days. Specifically, they sampled liver, muscle, lung, and kidney and conducted multivariate analysis on each organ, as well as multi-organ models, for the establishment for the best model for PMI estimation. They obtained an accuracy of 93% and an AUROC of 0.96 with the multi-organ model, recommending the use of multi-organ models for advancements in the estimation of PMI.

There have also been studies focusing on specific causes of death and the associated PMI estimation model based on metabolomics. An example is the one done by Dai et al. [204] where the blood of rats killed by DDVP poisoning was sampled and analyzed at selected PMIs up to 72 hours. Support vector regression (SVR) models were created by using the metabolites found associated with PMI and obtained estimations with a minimum mean squared error (MSE) of 10.33 hours. Another one is the work of Zhang et al. [174] which assessed PMSI by profiling the metabolome of drowned rats within 24 h of decomposition with LC-MS/MS. They identified selected candidates for PMSI estimation with RF modelling and obtained an MAE = 1 hour. They also used the same approach to identify the cause of death, and more details can be found in Section 3.1.

A few studies combined the two methodological approaches, namely NMR and MS. Zelentsova et al. [269] proposed a study on rabbit blood serum, aqueous (AH) and VH for PMIs up to 31 hours *post-mortem* and showed that metabolomic changes in VH and AH take place slower than those in serum, recommending, therefore, these ocular fluids for the identification of PMI markers over serum samples. In a very similar work, Snytnikova et al. compared human AH and blood serum [275] and cornea and AH metabolic changes *post-mortem* [276] via a combined NMR and ESI-MS approach. They identified specific metabolomic changes in the fluids and tissues examined, in association with the disruption of the biochemical cycles post-mortem. Also in these cases, however, the focus was not the identification of PMI markers but the characterization of the events taking place *post-mortem* in specific fluids and tissues.

A separate mention should be made to the works that have used lipidomics from tissues for PMI estimation. Wood and Shirley [277] have used LC-MS/MS for the investigation of the lipidomic profile in skeletal muscles and their association with PMI. They sampled human muscles from donated cadavers at ARF, which were decomposing for up to 24 days. The results showed a decline in specific lipids over the *post-mortem* period investigated; however, the authors did not propose a PMI estimation model due to the limited sample size available.

Also in this case, similarly to the application of proteomics to skeletal remains, particular attention should be put on the treatment of the remains prior to the application of metabolomic and lipidomic analyses. A study conducted by Bonicelli et al. [278] showed how detrimental the pre-processing of skeletal remains is on the subsequent analysis of the bone metabolomic profile, therefore macerated or treated samples should be avoided when conducting such studies.

3.3 | Age at death

AAD estimation is another main challenge of forensic science. For individuals that have not yet reached complete maturation, developmental traits are the ideal target for estimating chronological age. When these traits are no longer useful for assessment, physiological degeneration represent a valid alternative and can focus on both molecular and biochemical characteristics of the tissue [17]. Among these, the main omics techniques applied are genome-wide methylation, transcriptomics, microbiomics and, more recently, proteomics (Table 2).

3.3.1 | Search results

Using the Scopus database, the combined search terms were used: 'age AND forens* AND genomic* AND human', 'age AND forens* AND methylation AND human', 'age AND forens* AND transcript* AND human', 'age AND forens* AND proteomic* AND human', 'age AND forens* AND metabolomic* AND human', 'age AND forens* AND microbiome* AND human'. Three hundred and sixty-seven articles were retrieved and 24 retained as considered appropriate for the topic of the current review. Nine additional articles were also included manually for the pertinence to this specific thematic area.

3.3.2 | DNA methylation for AAD estimation

DNA methylation is by far the most investigated area for AAD estimation. This targets several fluids and tissues to accommodate different forensic scenarios and decomposition stages. In this section, only studies employing WGS were considered. However, most studies present in literature are based on targeted analysis of age-related CpG (AR-CpGs) to evaluate tissue and population-specific variation as well as the effect of certain pathological conditions on the degree of DNA methylation and, as a result, of the age estimate. The interest in this approach stems from its potential applications in various fields, including disease prevention, treatment, forensics, and the enhancement of overall quality of life. For this reason, the sequencing of 450,000 CpG markers of whole blood from 656 human participants, aged between 19 and 101, provides a comprehensive model to elucidate the complexity of human ageing and its potential application in age estimation for forensic purposes [280]. The study identified 70,387 methylation markers associated with age, and through the application of the elastic net algorithm, a model was developed that included 71 methylation markers. This model achieved an impressive accuracy of 96% with an error of 3.9 years in age estimation. The accuracy remained high at 91% with an error of 4.9 years when validated in an independent test set. Notably, all selected markers were linked to age-related biological processes, including Alzheimer's disease, cancer, tissue degradation, DNA damage, and oxidative stress. Additionally, it was observed that, in this cohort, male individuals appeared to age 4% faster than their female counterparts. When the model was tested using methylation profiles from other tissues, which were obtained from the Cancer Genome Atlas [296], it demonstrated a correlation of $R = 0.72$ with a tissue-consistent offset. This finding suggests that methylation patterns are not tissue-specific and can provide reliable age estimates across different tissue types. Furthermore, the authors emphasized that methylomes in young individuals exhibit specific similarities that gradually decrease as changes accumulate with age. This observation aids in understanding epigenetic drift, as evidenced by the evaluation

TABLE 2 Research articles applying omics technologies and machine learning algorithms to estimate AAD, and the accuracy of the best model developed.

AAD range	Host Species	Sample type	Omics used	Instrument used	Best performing modelling method	Accuracy of the best model	Reference
10–101	Human ($n = 49$)	Whole blood	Methylomics	Illumina Infinium HumanMethylation BeadChip	RKHS	$R^2 = 0.98$, RMSE = 2.98	[279]
19–101	Human ($n = 656$)	Whole blood	Methylomics	Illumina Infinium HumanMethylation BeadChip	glmnet	$R^2 = 0.863$, RMSE = 7.22	[280]
20–80	Human ($n = 4409$)	Blood	Methylomics	Illumina Infinium HumanMethylation BeadChip	SVR	MAE = 2.8	[281]
17–77	Human ($n = 21$)	Teeth	Methylomics	MALDI-TOF	MLR	MAE = 2.45	[282]
2–90	Human ($n = 1156$)	Whole blood	Methylomics	Illumina Infinium HumanMethylation BeadChip	GRNN	$R^2 = 0.96$, MAE = 3.3	[283]
37–43	Human	Whole blood ($n = 394$), buffy coat ($n = 852$)	Methylomics	Illumina Infinium HumanMethylation BeadChip	RFR	MAD 3.21	[284]
20–59	Humam ($n = 41$)	Multi-tissue (whole blood, saliva, semen, menstrual blood and vaginal secretions)	Methylomics	Illumina Infinium HumanMethylation BeadChip	Elastic net	r = 0.73, MAE = 7.8	[285]
6–15	Human ($n = 48$)	Blood	Methylomics	Illumina Infinium HumanMethylation BeadChip	MLR	$R^2 = 0.941$, MAE = 0.33	[286]
31–112	Human ($n = 12$)	Bone	Methylomics	Illumina Infinium HumanMethylation BeadChip		r = 0.964, MAD = 6.4	[287]
19–101	Human ($n = 991$)	Whole blood	Methylomics	Illumina Infinium HumanMethylation BeadChip	MLR	$R^2 = 0.891$, MAD = 3.76	[288]
2–87	Human ($n = 180$)	Whole blood, mesenchimal stromal, breast, brain	Methylomics	Illumina Infinium HumanMethylation BeadChip	RFR	MAD 3.44	[289]
0–88	Humam ($n = 756$)	Whole blood	Methylomics	Illumina Infinium HumanMethylation BeadChip	Elastic net	MAE = 2.6	[290]
20–69	Human ($n = 22$)	Blood	miRNA	Illumina Hiseq 2500	AdaBoost	MAE = 5.52	[291]
	Human ($n = 8959$)	Faecal ($n = 4434$), saliva ($n = 2550$), skin ($n = 1975$)	6S-V4 rRNA		RFR	$R^2 = 73.91$, MAE = 3.8	[292]
18–56/1–87	Human ($n = 63/n = 308$)	Peripheralvenous blood	Methylomics	Illumina Infinium HumanMethylation BeadChip	MLR	$R^2 > 0.97$, MAE 2.53	[293]
19–73	Human ($n = 100$)	Whole blood	circRNA	Illumina Hiseq 4000	RFR	S.rho. = 069, MAE = 8.767	[294]
17–104	Human ($n = 171$)	Blood	circRNA	Illumina Hiseq 4000	RFR	r = 0.96. MAE = 0.68	[295]

of the entropy of a CpG marker. The increasing entropy indicates that the methylation state of the marker becomes less predictable with age, with methylation fraction tending to approach 50%. In this cohort, approximately 70% of the markers tended toward a methylation fraction of 50%. These findings regarding epigenetic drift were corroborated by a similar study, which showed that 97% of age-associated methylation is affected by drift. Lastly, the study explored the relationship between methylomes and transcriptomes of 488 participants and found that expression patterns linked to aging were correlated with nearby methylation markers associated with age [280].

Before focusing on the application of forensic age estimation using DNA methylation, it is important to consider employing high-dimensional variable selection methods due to the substantial number of CpG sites typically sequenced using NGS. In a study conducted by Xu et al. [281], 49 female blood samples spanning ages from 20 to 80 years were subjected to analysis using Sequenom Massarray technology. PCR products were designed to cover a total of 95 CpG sites, and from these, 11 were selected to construct age prediction models. Various methods, including MLR, Multivariate Nonlinear Regression, Back Propagation Neural Network and SVM, were compared in this analysis. Among these methods, the SVR model emerged as the most reliable, showing the smallest mean absolute deviation (MAD) from the actual chronological age (2.8 years) and an average accuracy of 4.7 years, utilizing only six out of the 11 selected CpG loci. Moreover, SVR demonstrated lower cross-validated error when compared to the linear regression model. This innovative approach provides precise age estimation, proving to be a valuable tool in forensic practice for individual age assessment and monitoring the aging process in related applications. A more recent and comprehensive study by Vidaki et al. [283] compared MLR with generalized regression neural network model (GRNN) on a blood sample from 1156 donors, aged 2–90 years. The GRNN model achieved an impressive R^2 = 0.96 and MAE of 3.3 years for the training set and 4.4 years for a blind test set (n = 231) with only 16 CpGs. This represents a significant improvement compared to MLR, that showed instead a MAE of 4.6 years (R^2 = 0.92). The authors advise caution when interpreting these results and recommend to consider pathological condition when developing these models, as they could have a great influence in the DNA methylation pattern and its correlation with age. Furthermore, when the model is applied to saliva samples, a decrease in performance is observed, underscoring the need for fluid-specific studies [283]. Another study tested the assessment of a model performance based on 13 AR-CpGs selected in silico using a test dataset of sequenced blood and demonstrated similar outcomes, with a MAD of 3.16 years and a RMSE of 3.93 years. A simplified version of the model, focusing solely on the top four markers (*ELOVL2*, *F5*, *KLF14* and *TRIM59*), yielded a RMSE of 4.19 years and a MAD of 3.24 years for the test dataset. During cross-validation on the training set, the reduced model exhibited a RMSE of 4.63 years and a MAD of 3.64 years [284]. Lau and Fung [288] investigated the effectiveness of four variable selection methods: forward selection, LASSO, elastic net and SCAD. These methods were applied to multiple linear regression (MLR), RF regression, SVMs using a polynomial function, neural networks with one hidden layer and neural networks with two hidden layers. The evaluation was carried out using MAD and RMSE on a dataset of 991 whole blood samples. Their analysis revealed that the best model, constructed using forward selection with 16 selected markers, demonstrated outstanding performance in predicting age, achieving a MAD of 3.76 years and a RMSE of 5.01 years. In another study that involved the sequencing of 180 blood samples and examined the performance of MLR, SVMs and RF regression, it was found that SVM (MAD = 3.44) outperformed MLR (MAD = 3.46) and RF (MAD = 3.56) [289].

HumanMethylation450 BeadChip collected profiles from the NCBI repository, aimed to establish a multi-tissue model for estimating age in forensic applications. The results identified 10 age-related CpG markers (AR-CpGs) from a dataset of 41 samples, including whole blood, saliva, semen, menstrual blood,and vaginal secretions. When tested on a validation set of 24 samples from four different tissues, the model produced a MAD of 5.6 years for buccal swabs, 6.9 years for vaginal fluid and 7.8 years for blood, demonstrating its potential in age estimation across various tissues [285]. Using Illumina MethylationEPIC (EPIC) array, 756 DNA methylation profiles were acquired from whole blood samples with the purpose of isolating blood-specific AR-CpGs [290]. According to Spearman's coefficient, 19 CpGs were positively correlated and 33 negatively correlated with age. The most striking outcome of the EPIC BeadChip analysis is the identification of new genes specific for blood analysis, namely *LHFPL4*, *SLC12A8*, *EGFEM1P*, *GPR158*, *TAL1*, *KIAA1755*, *LOC730668*, *DUSP16* and *FAM65C*, with 16 sites hypomethylated and five hypermethylated. Further, applying elastic net regression feature selection on 816,127 CpGs, 425 CpG markers were selected for their capability to provide a very accurate age estimation with a MAD of 0.68 years in the training set and of 2.6 years in a test set of 277 individuals. To decrease the variable count, a selection criterion was applied, choosing markers with an R^2 value greater than 0.5 at a false discovery rate (FDR) below 0.05. This process resulted in the selection of 10 markers based on the regression of age against CpG methylation levels. Subsequently, forward selection was used to further reduce these markers to six, ultimately producing a model that exhibited a MAD of 4.6 years in the test set. It is worth noting that this model primarily included markers specific to blood samples [290]. Another example of the importance of testing other variables that could influence methylation was provided by a study from Xiao et al. [293], who developed a pool of AR-CpGs for male (chr6:11044864ELOVL2, chr1:207997068C1orf132, cg19283806CCDC102B, cg17740900 and chr10:73740306CHST3) and female (hr6:11044867ELOVL2,chr1:207997060C1orf132, chr2:106015757FHL2, cg26947034, chr16: 67184108B3GNT9 and chr20:44658203SLC12A5) individuals in Han population.

Another critical focus of genomic analysis in forensic science is bone, which becomes the primary source of human remains for medium to long-term cases. Dealing with fully skeletonized remains poses significant challenges due to the DNA's poor preservation in such samples. The standard procedure for extracting DNA from bone powder generally involves several stages. First, the bone powder is incubated in a lysis buffer, which breaks down tissue and cell structures chemically. Next, the sample is incubated in a concentrated salt binding buffer to facilitate DNA binding to silica, usually prepared in columns. Subsequently, the DNA is washed with an ethanol-based solution to reduce the possibility of inhibitor contamination and then eluted in a low-concentration salt buffer [297]. The first successful methylation study that included also bone samples was carried out by Horvath and colleagues [298]. Together with skeletal tissues, other 30 anatomic sites and tissues were considered. Results showed that bone and bone marrow had older DNA methylation ages compared to other tissues. Reppe et al. [299] tested 84 biopsy bone material to evaluate methylation

patterns. These studies identified 63 CpGs with higher methylation levels in osteoporotic women compared to postmenopausal healthy individuals. It is worth noting that these studies often use ideally preserved materials that may not be readily available in forensic settings. Shi et al. [286] conducted a study that focused on analyzing AR-CpG (age-related CpG sites) in a sample of 124 Chinese children, aged 2–15, using the Illumina HumanMethylation450 BeadChip. They examined both skeletal age (SA) and dental age (DA). Combining the results from SA and DA, the model achieved an accuracy of 88.6% with an error of 0.47 years for male and 94.1% accuracy with a 0.33 years error for female. In another study, DNA methylation in 32 bone samples was analyzed, and this data was supplemented with published data from 133 additional bone donors, revealing 108 AR-CpG sites. Estimations of AAD were found to be very accurate with an error of 7.1 years when applied to a sample aged 49–112 years. Furthermore, it was observed that previously developed prediction formulas performed with considerable accuracy on the new sample [287]. The complexity of working with this material is highlighted by Lee et al. [300], who attempted the sequencing of skeletal remains from 28 male and four female aged from 31 to 96 years using the HumanMethylationEPIC BeadChip (Illumina). Out of the entire sample, only six yielded enough DNA to meet the minimum requirement for Illumina's BeadChip array and, after sequencing, only 12 displayed high-quality and were employed in the estimation. The estimation demonstrated an R value >0.95, with errors falling within the range of 5–7 years. Interestingly, *TMEM51*, *TRIM59*, *ELOVL2* and *EPHA6* exhibited moderate-to-weak relationships, indicating the need for further research to establish a comprehensive method for forensic science [300].

A very different approach instead consists in the use of MALDI-TOF mass spectrometry to analyze DNA methylation levels. Despite targeting *ELOVL2*, *FHL2* and *PENK* genes from 21 modern teeth (aged 17–77 years) [282], this approach uses a high throughput technology in contrast with targeted genomics studies and, for this reason, was included in this review paper. The study found that if DNA is obtained from dental pulp, it can be detected within approximately 2.25 years of error (with SD of 0.52). Similarly, if DNA is extracted from cementum, it can be identified within around 2.45 years (with SD of 0.53). However, when DNA is recovered solely from dentin and ADD could be detected within roughly 7.07 years (with SD of 0.57 years) [282].

3.3.3 | Transcriptomics for AAD estimation

Another category of biomolecules with the potential for estimating AAD is RNA. MicroRNAs (miRNAs) are a type of small non-coding RNA known for their resistance to degradation and their tissue-specific expression. For this reason, they are considered a useful target for forensic examinations. Fang et al. [291], utilizing the HiSeq 2500 platform to sequence miRNAs from blood samples of 220 individuals aged 20–69 years, successfully identified 485 miRNAs. Among them, miR-451a, miR-486-5p, let-7i-5p, let-7f-5p and let-7g-5p were found to be the most abundant in blood. They applied several models, including SVM, decision tree, k-nearest neighbor (kNN), stochastic gradient descent (SGD), RF, GLR and AdaBoost. The best-performing model, using the AdaBoost algorithm, achieved a MAE of 5.52 and 7.46 years for males and females, respectively. Interestingly, among the markers correlating with age, functional analysis unveiled associations with DNA replication, cell apoptosis and the regulation of glycerol channel activity [291]. Circular RNAs (circRNAs) were investigated in the blood of thirteen Chinese unrelated healthy subjects aged 20–62, leading to the identification of 40,000 circRNAs. Subsequently, 28 circRNA markers were chosen for validation in 30 unrelated healthy subjects using real-time quantitative polymerase chain reaction (RT-qPCR). Finally, 100 blood samples from individuals aged 19–73 were employed to assess the age estimation capabilities of the five AR-circRNAs. The RF model provided a MAE of 9.126 years, and notably, there was a noticeable increase in the error for the female subset of the sample compared to the male subset [294]. In another study, miRNAs were combined with circRNAs [177]. The sample consisted of four young participants (20–29 years) and four elderly participants (50–62 years) from circRNA-seq. Additionally, 171 samples of miRNA microarray data from public datasets were used, along with an additional validation set comprising 40 subjects (19–73 years), which were tested using RT-qPCR to assess non-coding RNAs (ncRNAs). A total of 27 circRNAs displayed a significant correlation with age. Among these, 13 were selected for RT-qPCR validation. Additionally, 18 age-related miRNAs were chosen for validation. In terms of the machine learning algorithms assessed, RF demonstrated the best performance for estimating age-associated disease (AAD) in both the training set (MAE = 3.68 years, R^2 0.96) and the testing set (MAE = 6.840 years, R^2 0.77) [177].

3.3.4 | Microbiomics for AAD estimation

If we consider ageing as a perturbation of homeostasis, it is clear that this is reflected in one's microbiome. This has attracted attention for understanding investigating age-related trends. However, the microbiome is highly variable, influenced by both personal and external environmental factors [301] and therefore very few attempts are present in literature for the estimation of AAD in forensic sciences. A large study investigated gut microbiome from fecal samples (United States n = 2.588, United Kingdom n = 936, Colombia n = 437 and China n = 4.963). The V4 hypervariable region of the 16S rRNA gene was sequenced with Illumina MiSeq platform [302]. Despite there was not an attempt to estimate age for forensic applications, they found that microbial richness increased in Colombian, U.S. and U.K. populations for individual aged 20–45 years. Additionally, the findings showed that women, especially younger adults, exhibited a greater degree of alpha diversity compared to their middle-aged counterparts. Moreover, women in the United States and the United Kingdom demonstrated a notably higher predicted microbiota age than men, whereas distinctions were only discernible among middle-aged Chinese adults [302]. Results of these studies were expanded by Huang et al. [292] that compared oral, gut and skin (hand and forehead) microbiomes to predict age in adults. Modelling was performed using RF, and

the best results were for the models developed on skin microbiome that could estimate age with a SD of 3.8±0.45 years and of 4.5±0.14 years for the oral microbiome, while the error was 11.5±0.12 years for the gut microbiome. Interestingly, the relationship prediction seems to plateau after 60 years with the exception of gut microbiome [292]. Two more studies based on an Italian sample composed of oral and skin microbiomes on 50 subjects showed a positive relationship between the abundance of *Spirochaetota* and *Synergistota* and the age of the enrolled participants [235, 303].

3.3.5 | Proteomics for AAD estimation

Proteomics is, so far, the MS-based profiling approach that has produced the best results, although preliminary, for the estimation of AAD. Procopio et al. [252] used an animal model to investigate *post-mortem* decay in porcine bones. Besides proving the efficacy of proteomics in assessing PMI, the study also highlights the negative relationship between fetuin-A and age. A further animal model based on rats aged between 1 week and 1.5 years confirmed a drop in fetuin-A levels. The study further reports a positive trend of Chromogranin-A with age. In contrast serum, albumin increased until sexual maturity, peaking earlier in females (3–4 weeks) than male (6–8 weeks). Biglycan and prothrombin were positively correlated with age while negative trend were seen for apolipoprotein A-1, vimentin, osteopontin and matrilin-1 [304]. Even fewer studies have used human samples and, interestingly, the same proteins previously highlighted for animal bones were confirmed, including a study conducted on archeological human remains [305] and a study conducted on experimentally buried individuals [255]. In addition to variation of protein abundances associated with age, a study found a strong positive correlation between deamidation of LUM-FNALQYLR ($R = 0.68$) with age, suggesting that PTMs on specific proteins might consistently accumulate or decrease with age as a result of physiological ageing [256]. Despite these few studies highlighted the potential of proteomics for AAD estimation, larger sample sizes should be employed in the future in order to account for inter-individual variability, which is higher in human than animal organisms. Furthermore, it seems that certain protein biomarkers are stable after death, making them ideal target for taphonomically degraded remains while other quickly degrade after death, allowing the applicability of forensic proteomic studies for both PMI and AAD estimation [230, 256].

3.4 | Personal identification of the cadaver

In forensic sciences and anthropology, the identification of human remains is particularly challenging when bodies have undergone extensive decomposition or have become skeletonised. These situations demand innovative sets of techniques and methodologies due to the absence of traditional identifying features, such as intact facial structures or readily accessible fingerprints. Amongst the available omics approaches, genomics and proteomics play the most crucial role when dealing with the identification of the cadaver.

3.4.1 | Search results

Using the Scopus database, the combined search terms were used: 'forens* AND identification AND genom* AND skelet*', 'forens* AND identification AND proteom*'. One hundred and ninety-one articles were retrieved and 37 retained as considered appropriate for the topic of the current review. Eight additional articles were also included manually for the pertinence to this specific thematic area.

3.4.2 | Proteomics for personal identification

Amongst the various evidentiary samples, hair is one of those where oftentimes nuclear DNA analyses fail. In such contexts, GVPs were proven to work for the correct imputation of SNPs alleles [306] and many works in the last years managed to increase the discrimination power by applying targeted peptide assays [307]. Recently, researchers managed to successfully identify GVPs from shed hair lacking root nuclear root DNA [308], from hair shafts of decreasing lengths up to 0.12 cm [309] and from anagen head hairs shorter than 1 mm [310]. Interestingly, they demonstrated that the body site of origin does not affect the correct genetic identification of hair shafts [311, 312] nor the hair color [313]. Hair GVP could be used to potentially distinguish gender and ethnicity [314, 315] and to discriminate monozygotic twins [173]. However, for the remits of this review paper, which is focused on the identification of the cadaver, it should be highlighted that only a few works so far explored the applicability of such analyses to damaged hairs or to hair from highly decomposed cadavers, and this topic requires, therefore, additional investigation in order to clarify to which extent this approach on human hair can be viable. Chu et al. [316] explored the effect of an explosive blast on the hair proteome and on the subsequent GVP identification. They successfully conducted proteome profiling on hair damaged by the explosion, supporting the potential use of GVPs in hair for personal identification in situations involving explosive blasts and laying promising foundations for the use of hair GVPs in challenging forensic scenarios. Something less clear is, instead, to which extent decomposition can affect the hair proteome, and the consequent possibility to apply GVP analyses on hair shafts from cadavers in various decomposition stages. Despite it is frequent to find scalp hair on highly decomposed individuals, hair undergoes changes associated with decomposition shortly after death and develop a darkened band around the root end [317]. Donfack et al. [318] has applied proteomics to hair segments either containing or not containing this darkened band and showed that banded hair are severely affected by protein degradation, particularly in the banded section. Therefore, additional work should be conducted to evaluate to which extent hair GVPs can be applied for the identification of decomposed human cadavers. Similarly, also fingermarks could

be a reservoir of GVPs able to assist in human identification in conjunction with dactyloscopic information, particularly considering how frequently touch DNA analyses fail. Recently, a protocol has been developed to attempt to extract GVPs from human skin cells, and this should enable the future application of proteomic genotyping also to these samples [319].

Several methodological papers have been published aimed at efficiently extracting proteins from hair shafts [320–322], at improving data analysis and search algorithms for the discovery of GVPs [321], at allowing the co-extraction of proteins and mtDNA from a range of hair types to provide additional probative value to hair samples without sacrificing the mtDNA analyses [323] and at improving the mass spectrometry outcomes by exploring the use of alternative LC-MS/MS platforms and by testing PRM and MRM as a way to validate the existing methods for GVPs inferred genotypes [324]. Interestingly, the age of the donor, nor the storage conditions of the samples or the experience of the operators conducting the analysis are able to affect the GVP results, despite the yield can be different and batch effects can exist [325]. These results support the application of GVPs to forensic caseworks, where the storage conditions, length of storage and operators, are not always consistent within the same laboratory or across laboratories.

Based on the same principles, GVPs can also be used on bones for human identification in association with mtDNA analyses when the genomic DNA content is not adequate (quantity/quality) to obtain a successful identification (e.g. small bone fragments with low bone mineral density, bones processed *post-mortem* with physical/chemical treatments able to alter the DNA content [326]). Results showed that proteomics can be a valid method for human identification of compromised skeletal remains [307, 327]. Another application of proteomics to mineralized tissues is the analysis of sexually dimorphic amelogenin protein fragments in human enamel to estimate the sex of the individual when DNA analysis fail. This application is particularly viable in archaeological contexts where frequently DNA is too degraded to offer an exhaustive answer regarding the sex of the remains. Stewart et al. [328] proposed for the first time the use of nano LC-MS/MS to identify the peptide regions of amelogenin, a protein expressed from X and Y chromosomes in a dimorphic way and present in dental enamel, the hardest tissue in vertebrates. They were able to extract enamel proteins (including the two non-amelogenin proteins present in dental enamel ameloblastin and enamelin) in a non-destructive way by depositing a droplet of acid on the tooth surface and identified Y-chromosome-specific amelogenin peptides demonstrating the identification of gender dimorphic peptides for forensic and archaeological applications. In a subsequent work, they also showed that this method works for both adult and juvenile samples in a minimally disruptive way, overcoming the limitations encountered when using morphological assessments for sex identification of juvenile skeletons [329]. Gowland et al. [330] demonstrated also that sufficient peptide signal can be recovered also from developing perinatal teeth not fully mineralized and originated from archaeological sites. Similarly, Parker et al. [331] showed that amelogenin proteins, AMELX_HUMAN and AMELY_HUMAN can be obtained from ancient enamel samples (including deciduous teeth) and analyzed via LC-MS/MS from open-air archaeological contexts from least 7300 years ago. They proposed a new statistical framework to maximize the confident attribution of samples to either male or female sex by developing also a probability curve to calculate the probability of female sex in function of the AMELX_HUMAN signal. Proteomic analyses on ancient enamel seem to offer better sex estimation than DNA-based sex estimates, as the genomic signal decreases in older burials whereas the proteomic one remains stable. In the work of Buonasera et al. [332], proteomics sex estimation was possible in 100% of the samples for 55 individuals dated between 2440 and 100 B.P., whereas genomic attribution was possible only in 91% of the cases and osteological ones in 51% of the cases. It is, therefore, reasonable to think that the application of such methods for highly compromised skeletal samples may also become the gold-standard in forensic contexts for sex attribution. Lugli et al. [333] used the enamel proteomics principle to determine the sex of the so-called 'Lovers of Modena', 1600 years old, and confidently classified them as both males, shining a new light on the understanding of the funerary practices in Late Antique Italy. Gasparini et al. [334] performed enamel sex estimation of skeletal remains from two VII century CE necropolises and identified the sex of skeletons buried in non-gendered graves. Similarly, Mays et al. [335] identified the sex of highly degraded skeletal remains dated 1st century BCE overcoming the limitation posed by high-throughput DNA sequencing, that failed as no aDNA survived in such remains. Olszewski et al. [336] recently conducted proteomic analyses on human remains from a Dutch East India Company burial ground in South Africa and managed confirm the biological sex of the remains from these poorly preserved samples.

In order to maximize the success rate of the enamel peptide approach for sex identification, Wasinger et al. [337] developed a MRM method to target the amelogenin isoforms for sex determination and applied it to Iron Age individuals (2000–1000 years B.P.), and Froment et al. [338] used PRM MS approach to detect sex-specific amelogenin peptides in 5000 years old human teeth. Lately, Casas-Ferreira [339] proposed two fast methods for the assignation of biological sex to prehistoric human remains with run times of one and three minutes per analysis, which open the possibility to perform AMEL analyses to a greater number of laboratories due to the reduced cost of the instrumentation required for such analyses.

Recently, some concerns have been raised on the sex attribution based on Y amelogenin-specific peptides in association with a condition, known as Y amelogenin allele deletion, that affects 1% of the global population but 10% of Indian phenotypically normal biological males. By using either a PCR-based amelogenin sex test, or the proteomic method previously described, individuals with Y amelogenin deletion may be falsely attributed to female individuals, with impact on forensic investigations and archaeological research [340].

3.4.3 | Genomics for personal identification

MPS methods have recently gained a significant interest in forensics, due to their enhanced sensitivity in comparison with standard

STR analyses, that offers increased potential for personal identification also when DNA reference samples are not available. By exploting the recent advances in MPS, several panels have been introduced on the market to achieve human identification, ancestry determination and phenotype prediction for forensic purposes. Amongst the most used ones, it should be mentioned the Illumina ForenSeq™ DNA Signature Prep Kit, the first kit approved for upload to the National DNA Index System (NDIS) for casework that runs on the MiSeq FGx™ Forensic Genomics System [341]. It combines over 200 markers in a unified workflow, including 27 autosomal STRs, seven X-chromosomal, 24 Y-chromosomal haplotype markers and 94 identificative SNPs, plus 56 ancestry and 22 phenotypic informative SNPs. It can analyze up to 96 DNA samples in <2 hours, eliminating the necessity for conducting multiple STR tests. Other panels include the HIrisPlex-S System for the simultaneous prediction of eye, hair and skin colour from trace DNA, but this one is based on two SNaPshot-based multiplex assays targeting specific SNPs instead of on the MPS technology [342]. The ForenSeq™ DNA Signature Prep Kit has been already tested on bone powder extracts from a 140 years old skeleton from South Dakota, USA, and obtained results from 5/26 Y-STRs, 34/34 Y SNPs, 166/166 ancestry-informative SNPs, 24/24 phenotype-informative SNPs, 102/102 human identity SNPs, 27/29 autosomal STRs (plus amelogenin) and 4/8 X-STRs [343]. The kit allowed the researchers to establish the ancestry of the individual as European and to discover that the individual was a male with light red hair and brown eyes. Similarly, but using the HIrisPlex system, Chaitanya et al. analysed the SNPs profiles for 49 samples originated from bones or teeth from the World War II and obtained and eye and hair color predictions from all the skeletal samples analysed [342]. Equally good results were obtained by Draus-Barini et al. [344], which successfully obtained eye and hair color from 22 out of 23 old bone samples approximately 800 years old using the HIrisPlex system. Also when samples are partially degraded and give only partial STR profiles with standard CE analyses, MPS can provide higher allele recovery and valuable investigative information, as showed in the study by Almohammed et al. [345] where 30 challenging bone samples (e.g. aged bones that partially failed STR profiling) were tested with the ForenSeq™ DNA Signature Prep Kit and gave full STR profiles and indications on ancestry and phenotype prediction for the majority of the samples analyzed. Elkins et al. [346] used the same kit and obtained biogeographical ancestry prediction from 15 historic human samples from 1600 to 1700s (tooth, temporal bone, femur, and tibia) and phenotype predictions of eye, hair and skin color from four samples (tooth, temporal bone, femur and fibula). Ambers et al. [347] applied MPS on two skeletons recovered from a historical shipwreck (*La Belle*) discovered in 1995 off the coast of Texas that sunk at the bottom of the bay in 1686. The ForenSeq™ DNA Signature Prep Kit allowed them to obtain partial Y-STRs (18/24 and 5/24 for the two skeletons), almost complete ancestry informative and phenotype informative SNPs (56/56 and 49/56 aiSNPs and 22/22 and 18/22 piSNPs), 15/27 and 22/27 autosomal STRs, 94/94 and 66/94 identity informative SNPs and 4/7 and 1/7 X-STRs. Kukla-Bartoszek et al. [348] performed eye, hair and skin color predictions on 63 bone samples with a PMI up to 80 years using the HIrisPlex-S panel and the Ion Torrent technology. 55.6% of the samples gave a full DNA profile for eye, hair and skin colour prediction, 7.9% failed and 36.5% gave a partial profile where only eye and/or hair colour was predicted but not the skin one. Interestingly, full profiles were obtained from samples quantified as little as 49pg of template DNA and the samples that failed for HIrisPlex-S failed also for standard STR analyses. Aged bone remains from the World War II and from the 3rd to the 18th centuries AD were also tested with the HIrisPlex panel by Zupanič Pajnic et al. [349], which obtained hair and eye colour prediction from 27.3% of ancient skeletons and from 50% of the skeletons from the World War II. When conducting forensic phenotyping from old skeletal remains, it is important to consider the variability that different samples may provide due to the differences in DNA yield existing in such samples. As a result, Inkret et al. [350] recommend to conduct multiple samplings (three bones per skeleton) to overcome such limitations and to obtain a better quality consensus profile. To target the same markers included in the commercial panels, recently in-house developed panels have been created, as shown by Melchionda et al. [351] and by Sguazzi et al. [241]; however, they still have to be tested on skeletal remains.

3.5 | Integration of omics approaches

Integration of multiple omic datasets is a new and developing field for which methodologies are yet to be fully established. However, the combination of omics technologies for data acquisition, boosted by the recent advances in NGS platforms and spectrometric techniques, and the possibility to computationally combine molecules from different functional levels, has enabled to gain a more comprehensive holistic view of system biology. Forensic science is no exception, and the application of an integrative acquisition and processing strategy could assist in explaining important features such as the cause of death, *post-mortem* molecular degradation trends and, potentially, AAD for the entire system. This is in contrast with more traditional approaches that aim to reduce the size of a biological model to prove a specific hypothesis. However, data-driven integrative approaches serve as a complementary tool for system-level investigations that should be further enhanced and validated through traditional hypothesis (HP) testing [352, 353]. The more traditional statistical approach commonly employed in biology is univariate analysis, where one HP is tested for one variable independently in a controlled experiment. This includes, among others, *t*-tests, *F*-tests or non-parametric equivalent tests that assess the expression/abundance of a specific molecule across the population. HP testing is evaluated using *p-values*. Bivariate analysis, instead, accounts for the relationship between two variables to infer on causation mechanisms. Finally, multivariate analysis considers a large number of variables (e.g. genes, transcripts, proteins and metabolites) simultaneously to obtain the holistic view of a specific biological model. It is important to emphasize that these three approaches are not mutually exclusive but highly complementary. Multivariate analysis serves as the initial step in identifying candidate molecules for use in more tailored experimental designs, which may involve uni- and

bi-variate analyses. This complementary role enables the elucidation of causally driven hypotheses, which would be challenging to achieve with the sole application of data-driven analysis [352, 353].

Despite its significant potential, omics integration is accompanied by certain technical limitations. First, omics analysis often involves acquiring a larger number of biomolecules (variables) than the sample size, which can lead to computational challenges. Overfitting, where a statistical model captures noise in the data, may result in the description of molecular networks that lack biological significance. This issue is closely connected to collinearity, which arises from spurious correlations in high-dimensional datasets, potentially highlighting relationships between compounds that are irrelevant for addressing a specific biological question. Besides these problems related to statistical models, acquiring omics data from various platforms results in the obtainment of significantly different data matrices in terms of format, dimensionality and, scale, among others, that requires to be accounted for. This is known as heterogeneity, and is currently one of the main challenges when it comes to create balanced models across different omics. As a result, normalization and filtering become essential steps in the integration workflows [352–354]. In terms of practical application, Athienity and Spyrun [354] provided the following list of potential approaches joint dimensionality reduction (jDR), correlation and covariance-based jDR (COR), factor analysis (FA), probabilistic/Bayesian models (PR), similarity (Kernel) based (KB), network-based integration (NB), regression-based (RB) and deep learning (DL). While a detailed discussion of these approaches goes beyond the scope of this review, it is crucial to keep in mind that there is no one-size-fits-all model, and the selection of a computational tool should always be data-driven. This choice should take into account all the limitations mentioned earlier when discussing results and providing interpretations.

While many studies have utilized a single omics methodology to explore forensic inquiries or have combined one omics approach with other non-omics techniques [355–359], there remains a notable scarcity of research that delves into the use of multiple omics approaches and in their integration to approach the same questions from diverse perspectives. Burcham et al. [360] used a murine model that included various organs to investigate the bacterial dynamics during decomposition. Their multi-omics approach included metagenomics and metatranscriptomics, that were both conducted on an Illumina HiSeq platform. Specifically, they identified transcripts associated with metabolic pathways being highly expressed during the migration and colonization of Clostridium bacteria in the host's organs, and an increase of gene transcripts associated with stress response and dormancy later on during the progression of the decomposition, following the bacterial succession patterns. Mok et al. [361] conducted both proteomics and metabolomics on maggots on porcine corpses for PMI estimation, identified a total of 573 metabolites and more than 800 pig-derived proteins and proposed the use of specific metabolites as biomarkers for PMI based on their quantitative patterns. However, both studies did not integrate the two omics but instead focused on analyzing each separately to understand their individual contributions to the *post-mortem* decay.

Bonicelli et al. [362] proposed the combination of proteomics, metabolomics and lipidomics on human bones to investigate the PMI, defining this approach 'Forens-OMICS'. In first place, they investigated the three omics blocks independently using univariate and multivariate analyses. Subsequently, they applied the Data Integration Analysis for Biomarker discovery using Latent variable approaches for Omics studies (DIABLO) method to identify the best markers able to discriminate the individuals based on their PMI. Despite the study did not result in the development of an integrated multi-omics model for PMI, due to the small sample size, it set the bases for the future application of the Forens-OMICS method to greater sample sizes for PMI and AAD estimation. Salignon et al. [363] recently explored the combined use of proteomics and the analysis of small RNAs for age prediction in blood plasma samples (n = 103 human subjects). The proteomic analyses were performed in an untargeted way to first identify potential biomarkers among a pool of 612 profiled proteins. Targeted MRM approach was then performed on 31 proteins that showed consistent trends with age. Between the small RNAs, they identified 288 miRNAs and 229 transfer RNAs (tRNAs), of which the top 10 miRNAs, tRNAs and fragments of tRNAs (tRFs) correlated with AAD were retained for the analyses. Results showed a good performance for all the classes of molecules, with $R^2 = 0.59 \pm 0.02$ for the proteins and $R^2 = 0.42 \pm 0.03$ for the small RNAs. Finally, the combination of the two classes of compounds showed a clear increase in performance ($R^2 = 0.70 \pm 0.01$).

4 | CONCLUSIONS

In conclusion, the landscape of forensic science is undergoing a transformative shift with the integration of omics disciplines and multi-omics approaches. While their use in judicial caseworks still requires efforts to meet admissibility requirements, the potential of these approaches in generating intelligence data for forensic investigations is already evident. The state-of-the-art platforms for omics analyses, their applications in forensic sciences, particularly in determining the cause of death and identifying the deceased, as well as the growing body of research in the field of multi-omics, all point to a promising future for modern forensic research. As these approaches continue to evolve and meet the stringent standards necessary for courtroom admissibility, they hold the potential to re-shape the very foundations of forensic science, enhancing its effectiveness and reliability in solving complex criminal cases. The fusion of advanced molecular technologies and forensic expertise represents a pivotal step forward in our relentless pursuit of truth and justice.

AUTHOR CONTRIBUTIONS

N.P. and A.B. equally contributed to the work.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Not applicable.

ORCID

Noemi Procopio https://orcid.org/0000-0002-7461-7586

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How to cite this article: Procopio, N., & Bonicelli, A. (2024). From flesh to bones: Multi-omics approaches in forensic science. *Proteomics*, e2200335. https://doi.org/10.1002/pmic.202200335

AUTHOR BIOGRAPHIES

Noemi Procopio, PhD., is a Senior Research Fellow in Forensic Taphonomy at the University of Central Lancashire, and a UKRI Future Leaders Fellow. Her Forens-OMICS team's research involves the application of proteomics, metabolomics and DNA methylomics strategies to forensic science, particularly to skeletal remains, for *post-mortem* interval and age-at-death estimations. Additionally, as part of her research, she uses metabarcoding and NGS platforms for microbial and human DNA analyses, for human identification and PMI estimation.

Andrea Bonicelli, PhD., is a Research Associate at the University of Central Lancashire investigating *post-mortem* biomolecules degradation for the Future Leader Fellowship-funded Forens-OMICS. He is responsible for application of GC-/LC-MS metabolomics, LC-MS proteomics and microbiome analysis and for the integration of omics outputs.