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POINT OF VIEW

THE EPIGENETIC CLOCK CONCEPT AS AN EMERGING MOLECULAR TOOL FOR POST-MORTEM INTERVAL ESTIMATION IN FORENSIC INVESTIGATIONS

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ABSTRACT

Accurate estimation of the post-mortem interval (PMI) remains a significant challenge in forensic investigations, particularly when conventional methods, such as body temperature, rigor mortis, and entomological analysis, are confounded by environmental variables or late-stage decomposition. The epigenetic clock concept, which assesses DNA methylation patterns at specific cytosine–phosphate–guanine (CpG) sites, has recently emerged as a promising molecular tool for PMI estimation. Originally developed to measure biological age in living individuals, models such as Horvath's, GrimAge, and PhenoAge have shown potential in forensic contexts due to their tissue specificity and relative post-mortem stability across biological matrices, including blood, muscle, bone, and dental pulp. However, to date, the epigenetic clock concept has not been validated for PMI estimation, limiting direct forensic application. These methylation-based tools can be tested for more objective and reproducible PMI estimates, especially during early to intermediate post-mortem intervals, and can be further enhanced by integrating with protein degradation or microbiome-based analyses.

However, the forensic application of the epigenetic clock concept faces multiple challenges. Post-mortem DNA degradation, particularly in tropical climates, compromises methylation signal integrity and limits the reliability of time-sensitive analyses. Most existing models are trained on living datasets and lack post-mortem calibration across diverse populations and decomposition conditions. Additional barriers include the high cost of sequencing technologies, infrastructural limitations in low-resource settings, and the absence of universally accepted forensic validation protocols. Ethical concerns regarding genetic data privacy and the legal admissibility of molecular evidence must also be addressed. Future studies should prioritise the development of forensic-specific adaptations of the epigenetic clock concept tailored to regional and environmental contexts, establish standardised protocols for PMI estimation, and promote interdisciplinary collaboration among forensic pathologists, molecular biologists, and legal authorities. With rigorous validation and ethical oversight, the epigenetic clock concept may significantly enhance the accuracy and objectivity of medico-legal death investigations.

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INTRODUCTION

The post-mortem interval (PMI), defined as the time between death and body discovery, plays a main role in medico-legal death investigations, helping a wide spectrum of legal, investigative, and humanitarian functions. From identifying decedents in missing persons cases to validating witness timelines in homicides, PMI estimation remains a cornerstone of forensic casework[1]. Despite its importance, accurate PMI determination continues to pose significant challenges due to the complex interplay of intrinsic and extrinsic factors influencing the rate and pattern of decomposition[1]. Conventional techniques, such as evaluating rigor mortis, body temperature, entomological activity, or circumstantial evidence, are often limited by subjectivity, environmental variability, or late-stage decomposition[1,2].

In recent years, the forensic sciences have moved toward molecular and tissue-based methods to overcome these limitations. Techniques involving dental pulp histology[3], cadaveric microbiome profiling[4], and RNA decay analysis[5], have shown potential, particularly in early to intermediate PMIs. However, these approaches are often constrained by sample degradation, standardisation issues, and inter-individual variability. Among these emerging tools, the epigenetic clock, biomarkers based on DNA methylation (DNAm) at specific cytosine–phosphate–guanine (CpG) sites, have gained traction due to their correlation with biological age and apparent post-mortem stability[6].

Originally developed to estimate chronological age in living individuals, epigenetic clocks such as Horvath's, PhenoAge, and GrimAge are now being explored in forensic contexts for their potential to enhance PMI estimation. These models, rooted in molecular aging biology, may provide objective, reproducible, and time-sensitive insights into tissue degradation at the cellular level, offering a promising complement to conventional forensic pathology[7]. To date, epigenetic clocks have not been validated for PMI estimation[8,9]. As forensic medicine moves toward precision-based post-mortem diagnostics, understanding the applicability and limitations of the epigenetic clock concept in real-world death investigations becomes more important.

METHODS

To develop this 'Point of View' article, a focused literature exploration was conducted using databases including PubMed/MEDLINE, ScienceDirect, and Google Scholar. The search spanned publications from 2018 to 2025, employing keywords such as "post-mortem interval estimation", "DNA methylation", "epigenetic clocks", "forensic molecular tools", and "biological aging markers". Filters were applied to prioritise peer-reviewed articles, recent advancements, and studies directly related to forensic applications. Relevant data were extracted and organised using a structured format that included study objectives, techniques used, findings, and limitations. The collected literature was critically analysed by the authors, with emphasis on emerging molecular tools and their practical integration into forensic contexts. Final interpretations were developed through consensus among the researchers, aiming to provide an evidence-informed perspective on the evolving role of epigenetic clocks in PMI estimation.

THE EPIGENETIC CLOCK CONCEPT: MECHANISMS AND BIOLOGICAL BASIS

Epigenetic clock concepts are molecular tools that estimate biological age by analysing DNAm patterns at CpG sites[10]. These clocks emphasize stable chemical modifications to DNA that affect gene expression without altering the DNA sequence, giving a quantifiable measure of cellular aging[11–13]. The concept of epigenetic age acceleration (EAA), a divergence between biological and chronological age, has been associated with disease susceptibility and environmental influences[10].

Among the most recognized models, Horvath's clock uses 353 CpG sites across various tissues, while Hannum's clock, based on 71 CpG sites in blood, has been associated with aging-related diseases[12,13]. Second-generation models such as PhenoAge and GrimAge integrate clinical biomarkers and mortality predictors, enhancing accuracy in health span and survival prediction[14].

The DNAmTL clock, which estimates telomere length from DNAm patterns, provides additional mechanistic insights into aging[14].

These epigenetic clocks have key ageing mechanisms like epigenetic drift, the random loss of DNAm fidelity with age, leading to altered gene regulation[10]. Ageing is marked by global hypomethylation and localised hypermethylation, affecting transcription, DNA repair, and cellular homeostasis[13]. These modifications, along with chromatin remodelling and histone changes, contribute to age-related functional decline[12].

For forensic purposes, the post-mortem stability of DNAm is a key advantage. Methylation signs in tissues such as blood, brain, muscle, and teeth remain sufficiently intact for analysis shortly after death[15,16]. Blood is the most commonly used tissue in epigenetic clock studies due to its accessibility and predictive accuracy[10]. Age-predictive methylation markers have also been successfully validated in buccal cells, bone, and cortical tissue, making them applicable in diverse forensic contexts[9,15,].

Despite their promise, these models may be affected by confounders such as disease, lifestyle, or environmental exposures, underscoring the need for robust, forensic-specific calibration[9]. Nevertheless, the relative stability of DNAm post-mortem, combined with the increasing accuracy of these clocks, supports their integration into forensic workflows for both age-at-death and post-mortem interval estimation.

POST-MORTEM EPIGENETICS: MEDICAL AND FORENSIC APPLICATIONS

Although epigenetic clocks have become powerful tools for estimating age in living individuals, no published studies to date have directly applied this concept to PMI estimation. This limitation must be explicitly recognised, as the core of ageing-clock research has not yet been translated into validated forensic tools for PMI. Nevertheless, research on post-mortem epigenetics provides strong evidence that several epigenetic marks remain stable after death, supporting the biological plausibility of future molecular tools for PMI estimation.

A. Stability of DNA Methylation After Death

- In rat brain tissue, global levels of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) remain largely unchanged across PMIs up to 96 hours, indicating strong post-mortem stability of these cytosine modifications[17].
- Human, pig, and mouse brain studies have similarly demonstrated that 5mC, 5hmC, 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) show minimal degradation for at least 72 hours post-mortem[17].
- Immunohistochemical analyses further confirm that these cytosine modifications remain preserved for up to 4 to 5 days after death, although some other epigenetic marks, particularly histone acetylation, begin to decline with prolonged PMI[17].
- Quantitative data from the same study show no significant reduction in global 5mC levels for at least three days post-mortem, reinforcing the chemical stability of methylated cytosines[17].

B. Histone Modifications and Chromatin Structure Post-Mortem

- According to the same study mentioned above, histone methylation marks (such as H3K4me3 and H3K27me3) were found to remain stable for 48–72 hours post-mortem in animal brain tissue[17].
- By contrast, histone acetylation marks (H3K9ac, H3K27ac, H4K5ac, etc.) decline more rapidly: some of these marks start to decrease within 24 hours after death[17-20].
- Chromatin immunoprecipitation experiments further support that nucleosome-bound DNA remains associated with histones in the human brain for as long as 30 hours post-mortem and that differences in histone methylation at specific gene loci can still be recovered[21].
- A forensic genetics-oriented review also argues that nucleosomes may provide structural resilience after death, protecting DNA and allowing certain

histone post-translational modifications (PTMs) to persist even in degraded samples[19].

C. *Forensic Implications and Research Gap*

These data collectively demonstrate that epigenetic features can resist decay at least during early-to-intermediate PMIs, which means it is biologically plausible to develop post-mortem molecular markers. However, the lack of any study calibrating established epigenetic ageing clocks to PMI remains a critical gap. No research has, for example, measured how clock-CpG sites change in a time-dependent manner after death. Therefore, while the epigenetic marks themselves are preserved, the clock algorithms have not been tested in post-mortem settings, and their forensic utility remains speculative[8,22,23].

Therefore, current evidence highlights the need for dedicated post-mortem epigenetic research to determine whether specific methylation signatures or adapted clock models could eventually contribute to reliable PMI estimation.

MOLECULAR ADVANTAGES AND CLINICAL CHALLENGES IN PMI ESTIMATION

A. *Molecular Advantages*

- **Oxidative Dynamics as a Forensic Signal**
  Post-mortem biochemical processes can actively reshape cytosine modifications via oxidative stress, which may themselves serve as forensic markers. In rat cerebellum, increasing post-mortem intervals (up to 540 minutes) were correlated with elevated levels of reactive oxygen species (ROS), accompanied by a significant decrease in 5mC and a concurrent increase in 5hmC[24]. This suggests that ROS-driven oxidation may drive conversion of 5mC to 5hmC, providing a mechanistic basis for developing “oxidative epigenetic clocks” that reflect time since death.

- **Stability of Epigenetic Marks Despite Post-Mortem Delay**
  Although some methylation changes may occur, other epigenetic modifications remain robust for hours to days after death. A well-controlled study on pig and mouse brain tissue, and even human neocortex, found that cytosine modifications (5mC, 5hmC, and further oxidized forms) remained stable for at least 72 hours, even under varying post-mortem conditions[17]. This resilience supports the feasibility of recovering meaningful methylation data in real forensic settings where sampling may be delayed.

- **Resilience of DNA Methylation in Forensic Samples**
  Environmental insults, such as heat, ultraviolet (UV) exposure, humidity, and pH shifts, pose a major threat to forensic DNA. However, empirical research demonstrates that methylation-specific markers can survive in dried stains despite these challenges. In blood, saliva, and menstrual-blood stains exposed to UV, high temperature, variable pH, or salt, bisulfite-PCR assays successfully detected methylation at tissue-specific CpG sites[25]. This suggests that methylation-based forensic assays may remain robust even in degraded or environmentally exposed evidence.

- **Long-Term Storage Stability**
  For forensic applications, it is important that methylation data remain reliable during storage. A longitudinal human study showed that global DNA methylation and hydroxymethylation were stable for up to 18 months when stored at –20 °C and –80 °C, even through multiple freeze–thaw cycles[26]. This provides confidence that DNA samples from forensic contexts, when stored appropriately, can preserve epigenetic information over extended periods.

B. Forensic-Translational Challenges

- **Environmental Confounders and Sample Variability**
  While some epigenetic marks are resilient, real-world environmental factors still threaten data integrity. Elevated temperature, high humidity, and substrate type (porous vs. non-porous) significantly influence DNA degradation and may bias methylation measurements[27]. For example, relative humidity may accelerate microbial growth and strand breakage, complicating the interpretation of methylation signals in crime-scene samples[28].

- **Temperature and Humidity Effects on Specific CpGs**
  Experimental work on blood samples has shown that extreme conditions alter methylation at certain loci: under 0°C or 55°C and relative humidities of 12% and 58%, the methylation level of the *TRIM59* gene exhibited a modest but statistically significant regression over time[28]. Such sensitivity implies that epigenetic PMI models must account for environmental history to avoid systematic error[28].

- **Interpretive Complexity from Oxidative Changes**
  The conversion of 5mC to 5hmC (and potentially further oxidised forms) post-mortem raises a significant interpretive challenge: How much of the observed signal reflects pre-mortem biology versus post-mortem chemical change? Without proper calibration, one risks mistaking an oxidative artefact for a reliable clock tick. This problem must be addressed through time-resolved calibration studies in controlled settings[24,29].

- **Validation and Standard Operating Procedures (SOPs)**
  To translate epigenetic markers into forensic practice, rigorous validation is essential. There is currently a lack of forensic-specific SOPs that define:

  1. Sampling protocols (how soon after death, what tissue, how preserved),
  2. Conversion methods (bisulfite vs enzymatic),
  3. Quality-control thresholds (DNA integrity, methylation signal consistency), and
  4. Inter-laboratory reproducibility[30–32].

  Without these, method variability could undermine legal admissibility.

- **Ethical and Legal Implications**
  The use of post-mortem methylation data in forensic contexts raises ethical and legal issues. For instance, how should the uncertainty inherent in methylation-based PMI estimates be reported in a court of law? Moreover, because methylation patterns may reflect ante-mortem exposures, there are concerns about privacy for the deceased and their relatives. A framework must be developed, in consultation with ethicists and legal professionals, to guide the responsible use, interpretation, and reporting of such data[23,33].

C. Future Directions

Based on the current evidence and interpretive considerations discussed in this 'Point of View' article, we recommend several key avenues for advancing the forensic application of epigenetic ageing clocks for post-mortem interval (PMI) estimation:

- Conduct longitudinal studies in cadaveric or animal models, sampling tissues at multiple PMIs to track changes in ROS levels and cytosine modifications under controlled environmental conditions.
- Utilise kinetic methylation data to train predictive "molecular clock" models that correlate oxidised epigenetic marks with PMI rather than chronological age, incorporating environmental and tissue-specific covariates.
- Investigate and validate minimally damaging DNA treatment protocols (e.g., enzymatic conversion) in forensic-type tissues to maximise DNA yield while preserving methylation information.

- Establish comprehensive SOPs encompassing sample collection, storage, DNA extraction, conversion, methylation quantification, and quality control to improve reproducibility and legal admissibility.
- Collaborate with forensic, legal, and bioethics experts to define guidelines on data privacy, consent (post-mortem), uncertainty reporting, and courtroom admissibility for methylation-based forensic evidence.

CONCLUSIONS

The epigenetic clock concept, based on DNA methylation, offers a promising molecular approach for estimating PMI. Their capacity to reflect biochemical changes at the cellular level, particularly when combined with kinetic models of oxidative methylation, provides greater precision and objectivity than many traditional PMI methods.

Nevertheless, realising their full forensic potential requires substantial research and validation effort. Time-resolved studies to map post-mortem methylation kinetics, together with the development of forensic-specific predictive models, are essential. Similarly, method optimisation (example: conversion protocols for degraded DNA) and standardised workflows must be established to ensure reproducibility, reliability, and legal admissibility.

Finally, the deployment of methylation-based PMI tools must be accompanied by robust ethical and legal frameworks. Given that methylation signatures may reflect ante-mortem exposures, issues of data privacy, consent, and uncertainty reporting cannot be ignored. Interdisciplinary collaboration among forensic scientists, molecular biologists, ethicists, and legal professionals will be critical to translate these molecular clocks into practically useful and ethically sound instruments in medico-legal death investigations.

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CONFLICTS OF INTEREST

The authors declared no conflicts of interest.

ETHICAL ISSUES

None.

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None.

AUTHOR CONTRIBUTIONS

MSNA: Conception and design of the work; the acquisition, analysis, and interpretation of data for the work; drafting the work; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. **HMAMH:** Conception of the work; revising the draft critically for important intellectual content; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. **PAOKP:** Interpretation of data for the work; drafting the work and revising it critically for important intellectual content; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. **DMDDD:** Design of the work; interpretation of data for the work; revising the draft critically for important intellectual content; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. **TDDTT:** Conception and design of the work; revising the draft critically for important intellectual content; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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