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THE ROLE OF EPIGENETIC BIOMARKERS AND DNA METHYLATION ANALYSIS IN AGE ESTIMATION AND TISSUE IDENTIFICATION FOR MODERN FORENSIC INVESTIGATIONS

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ABSTRACT

DNA methylation has emerged as an important epigenetic biomarker for forensic investigations because it can provide biological information beyond conventional DNA profiling. This study examined the role of CpG methylation markers in forensic age estimation and tissue-identification interpretation using a secondary data-based computational design. A publicly available methylation dataset, GSE207605_GSE40279.csv.gz, containing 656 samples and 2,374 CpG markers with chronological ages ranging from 19 to 101 years, was analyzed. Data preprocessing confirmed complete methylation and age information, with no missing values. CpG markers were ranked according to their association with chronological age, and the strongest age-associated markers were used to develop a ridge regression-based age-estimation model. Model performance was evaluated using cross-validation and compared with a mean-age baseline model. The methylation-based model substantially outperformed the baseline, reducing mean absolute error from 11.92 years to 4.06 years and achieving an R^2 value of 0.862. These findings demonstrated that selected CpG methylation markers carried strong age-predictive information and supported their forensic value for donor age estimation. Direct tissue-identification modeling was not performed because tissue-origin labels were unavailable; however, tissue-specific methylation was discussed as a complementary forensic application. Overall, DNA methylation analysis showed strong potential for enhancing forensic biological inference through age estimation and tissue-origin interpretation.

KEYWORDS: DNA methylation, Epigenetic biomarkers, Forensic age estimation, CpG markers, Tissue identification

INTRODUCTION

As forensic investigations shifted away from traditional human ID needs, the need for more biological data increased. Standard DNA profiling was very useful at matching a biological trace to a known person, but was not very useful in the absence of a reference sample and/or where investigators were required to deduce the biological properties of the unknown donor. This constraint promoted the development of forensic DNA intelligence techniques, such as epigenetic analysis, that utilized molecular patterns that could provide clues to the age of the body, tissue of origin, and other biologically relevant patterns. DNA methylation emerged as a particularly relevant epigenetic mechanism, as methylation could be measured across various biological tissues, was relatively stable, and was informative (Maulani & Auerkari, 2020; Refn et al., 2023).

DNA methylation is the process of methylation, specifically by the addition of methyl groups to DNA, typically at cytosine-phosphate-guanine sites. These CpG methylation patterns were tissue-specific and even varied with chronological age. In forensic science, methylation was of great value in two particular aspects: ageing a donor and determining the tissue or body fluid source of a biological trace. Methylation markers were useful for biological age prediction with age-associated methylation markers and tissue-specific methylation markers for the

classification of body fluids such as blood, saliva, semen, and forensic biological materials (Ghai et al., 2020; Alghanim et al., 2020).

DNA methylation was identified as an important research focus for forensic aging, as methylation changes at a selected group of CpG sites correlated well with chronological age. These markers proved useful because they could be added to a statistical or machine-learning model for the creation of donor age estimates from biological samples. Systematic evidence suggested that there was a great potential for forensic application of methylation-based age estimation, especially if compact CpG panels were created for use in the laboratory (Maulani & Auerkari, 2020). Comparative studies also revealed that methylation detection technology, choice of markers, and statistical modelling approach might give different results in forensic age prediction, and that validation is needed before implementation in casework (Freire-Aradas et al., 2020).

There was significant attention given to methylation models based on blood, as the samples were widely available in both clinical contexts and in forensics. The analysis of blood DNA methylation even in living and deceased individuals showed that ageing could be utilized across various forensic scenarios, including post mortem (PM) scenarios (Dias et al., 2020). Other age prediction methods were also found to provide good chronological ages for small numbers of DNA methylation sites in combination with machine learning algorithms (Varshavsky et al., 2023). These advances helped to progress the use of CpG methylation biomarkers from a purely exploratory molecular characteristic to a practical forensic tool.

Another important use of DNA methylation in forensics was the identification of tissue and body fluids. Crime samples were frequently complex, degraded, mixed, and/or limited in quantity, and identifying the biological source of a stain may be vital to the reconstruction of events. This was possible because tissue-specific methylation patterns mirrored the epigenetic regulation of various cell types, and DNA methylation markers were useful. Researchers have identified methylation markers in semen and saliva and shown their potential for forensic analysis (Ghai et al., 2020). Other studies employed pyrosequencing and qPCR/high-resolution melting methods to develop methylation markers for sperm, saliva, and blood, indicating that methylation-based assays could be used to assist laboratory body fluid identification (Alghanim et al., 2020).

If samples from a crime scene contained more than one type of body fluid, this information was of particular importance in a forensic investigation. Mixed body fluids were used in simulated crime scenarios to show the usefulness of applying methylation-based markers for distinguishing the biological components in complex samples (Gomaa et al., 2021). DNA methylation-based age prediction and body fluid typing were also studied together, building upon the usefulness of methylation analysis as a multi-purpose forensic tool that is not just used for age determination (Lee et al., 2022).

Despite these advances, there were still some issues to be addressed. Robustness of markers, population diversity, analytical platform, and sample type, and model validation, were key requirements in forensic inference based on DNA methylation. Age-prediction models created with one technology or population may not be as accurate in another (Freire-Aradas et al., 2020; Aliferi et al., 2022). Hair methylation studies found that biological materials needed to be validated before forensic application, depending on the biological materials used (Hao et al., 2021). Today, then, the focus in forensic science is on reproducing workflows, reporting uncertainty clearly, and reporting considerations carefully in the interpretation of methylation-derived predictions (Refn et al., 2023; Naue, 2023).

Objectives of the study

1. To evaluate the association between CpG DNA methylation biomarkers and chronological age using publicly available methylation data.
2. To develop and validate a DNA methylation-based age-estimation model for forensic biological inference.
3. To examine the forensic relevance of tissue-specific DNA methylation patterns for supporting tissue and body-fluid identification in modern investigations.

METHODOLOGY

Study Design

This study employed the secondary data-based computational design to investigate the use of epigenetic biomarkers and DNA methylation analysis in forensic age estimation and tissue identification. The analytical portion was on the age estimation using the CpG methylation data that was made available, and the tissue identification using a forensic interpretive approach due to the lack of tissue-origin labels for the data set.

Dataset Source and Selection

The study took the publicly available data GSE207605_GSE40279.csv.gz, which contained DNA methylation beta values of 2,374 CpG sites with 656 DNA methylation samples. The range of chronological age in the data was from 19 to 101 years. It was chosen because it contained methylation data at the CpG level, had no missing values, had a wide adult age range, and was appropriate for regression-based age-estimation analysis (Varshavsky et al., 2023).

Data Preprocessing
The dataset was imported and analyzed for the number of samples, the number of CG markers, age distribution, missing values, and range of methylation beta-values. Chronological age was separated as the outcome variable, and CpG methylation beta values as predictor variables. Assessment of missing values was carried out, and no imputation was needed as the data set did not have any missing values. The development of the models included the standardization of CpG predictors.

Feature Selection and Model Development
The markers were sorted by the correlation with chronological age based on CpG. CpG sites that had the strongest association with age were chosen to provide dimensionality reduction and better model interpretability. Methylation data sets will typically have a large number of correlated and high-dimensional predictors, where the primary age-estimation model was ridge regression. A baseline model using the mean-age prediction was also developed for comparison.

Model Validation and Statistical Analysis
For the methylation-based age-estimation model, cross-validation was used. The mean absolute error, root mean squared error, and coefficient of determination were measured to evaluate the model's performance. The methylation model was contrasted with the baseline model to see if there was a benefit in being able to predict chronological age using the methylation values. Descriptive statistics were used for summarising characteristics of the dataset, the distribution of methylation, and the methylation pattern related to age.

Tissue-Identification Framework
A forensic interpretive tissue identification was added as opposed to being a classification analysis itself. No labels of tissue-origin and body fluid labels were provided in the dataset, so the accuracy of tissue-identification was not calculated. The complementary forensic usage of tissue-specific DNA methylation was also discussed, where the DNA methylation profile of different tissues and body fluids may be different. With the framework provided, this study was able to link methylation-based age estimation with the broader role of epigenetic markers in forensics.

RESULTS
Dataset Characteristics
The dataset analyzed was GSE207605_GSE40279.csv.gz, consisting of 656 samples with 2374 CpG methylation markers. All samples had chronological age and ranged from 19 to 101 years. The average age was 64.04 years with a median age of 65 years, suggesting a wide age range of adults. No missing values were found with the variable age and CpG methylation values. Table 1 presents an overview of the general characteristics of the DNA methylation data analysed.

Table 1. General characteristics of the analyzed methylation dataset

Characteristic	Result
Dataset name	GSE207605_GSE40279.csv.gz
Total samples	656
CpG methylation markers	2,374
Age range	19–101 years
Mean age	64.04 years
Median age	65 years
Standard deviation of age	14.74 years
Missing values	0
Main analytical use	DNA methylation-based age estimation

The dataset was thus appropriate for age estimation analysis using regression. The wide age range of adults served as a better foundation for model development than studies at more limited age ranges.

Age Distribution of Samples
The age distribution indicated that the majority of samples were aged 51 to 80. The most numerous were from the 61-70 years age group (167 samples), followed by the 51-60 years age group (140 samples) and the 71-80 years age group (138 samples). The youngest and oldest age groups were the age groups with fewer samples. The age group distribution of the study samples is presented in Table 2.

Table 2. Age-group distribution of the study samples

Age group	Number of samples	Percentage
19–30 years	14	2.13%

31–40 years	24	3.66%
41–50 years	83	12.65%
51–60 years	140	21.34%
61–70 years	167	25.46%
71–80 years	138	21.04%
81–90 years	80	12.20%
91–101 years	10	1.52%
Total	**656**	**100%**

The sample distribution showed it to be more likely to be a middle-aged and older population. This structure would have implications for the performance of adult age estimation modeling, but would also imply better performance with middle and older age groups than with younger adults. The age distribution of samples in the DNA methylation dataset is presented in Figure 1.

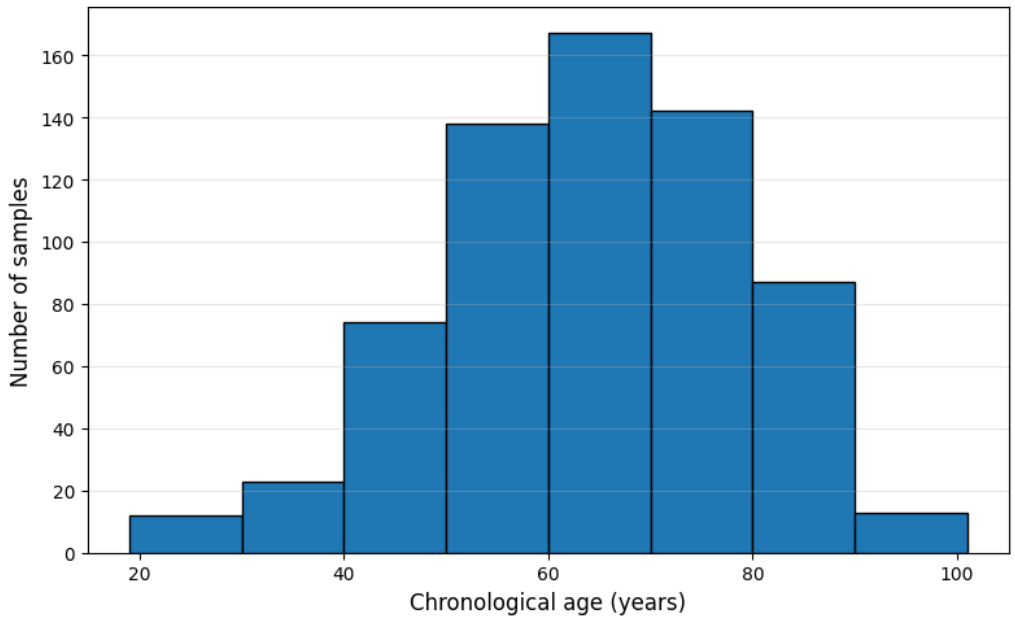

Figure 1. Age Distribution of Samples in the DNA Methylation Dataset

DNA Methylation Data Quality and CpG Marker Association

The methylation beta values were found between 0.00 and 1.00, as expected for DNA methylation beta-value data. The average beta level was 0.595, and the standard deviation was 0.323. These values showed significant differences in the methylation of CpG markers and samples.

To find multiple CpG sites that showed a strong correlation with chronological age, correlation analysis was performed. The best age-associated CpG marker was cg16867657, which was positively correlated with age of 0.859. Both increased and decreased patterns of methylation with age were identified, suggesting that certain CpG sites became more methylated with age, and others less. Table 3 shows the top CpG methylation markers associated with chronological age.

Table 3. Top age-associated CpG methylation markers

Rank	CpG marker	Correlation with age	Direction of association
1	cg16867657	0.859	Positive
2	cg06639320	0.747	Positive
3	cg24724428	0.745	Positive
4	cg22454769	0.744	Positive
5	cg10501210	-0.726	Negative
6	cg24079702	0.708	Positive
7	cg07553761	0.700	Positive
8	cg21572722	0.687	Positive
9	cg19283806	-0.679	Negative
10	cg08234504	-0.669	Negative

The results revealed that CpG sites were having high age-related methylation signals in these data sets. Hypermethylation and hypomethylation patterns were found, underscoring multiple CpG markers as compared to a single marker for age estimation modelling. Figure 2 shows the top CpG methylation markers associated with chronological age.

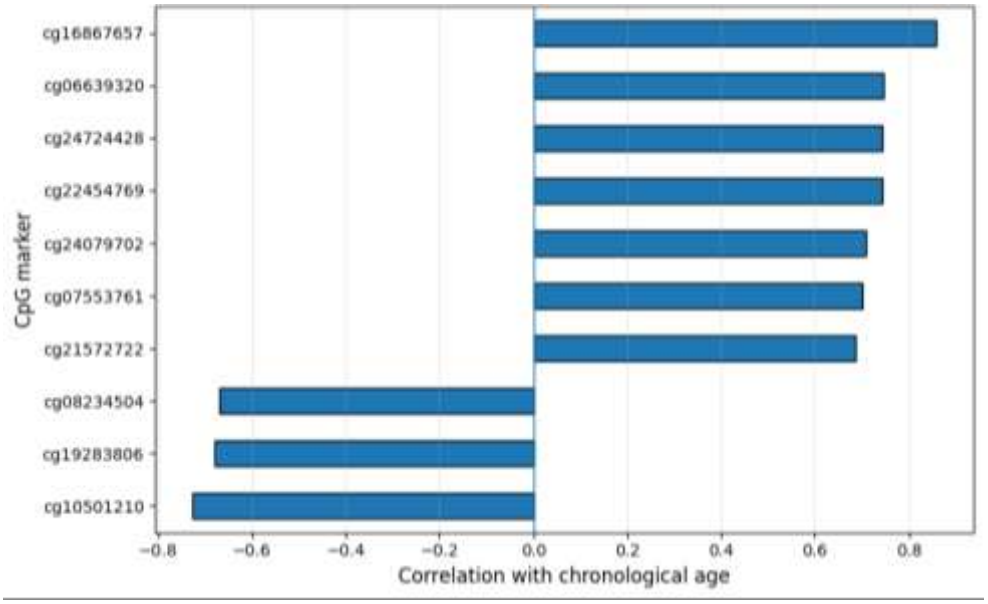

Figure 2. Top Age-Associated CpG Methylation Markers

Figure 3 illustrates the correlation pattern between chronological age and selected CpG methylation markers.

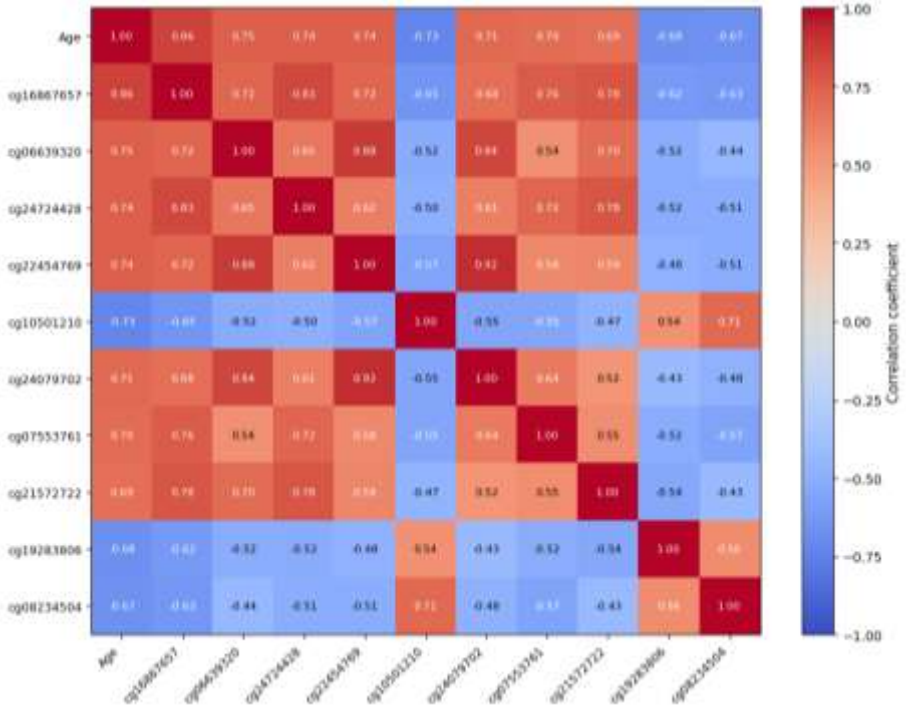

Figure 3. Correlation Heatmap of Chronological Age and Top CpG Methylation Markers

Age-Estimation Model Performance

The model using methylation data and ridge regression significantly outperformed the model based on the mean age. The baseline model showed a mean absolute error of 11.92 years in comparison to a mean absolute error of 4.06 years for the methylation-based model. The methylation model also performed well with an RMSE of 5.48 years and an R^2 of 0.862. The performance of the baseline and methylation-based age-estimation models is contrasted in Table 4.

Table 4. Performance of baseline and methylation-based age-estimation models

Model	MAE	RMSE	R^2
Mean-age baseline model	11.92 years	14.73 years	-0.001

Methylation-based ridge regression model	4.06 years	5.48 years	0.862

The results revealed that CpG methylation values were able to offer good predictive information for chronological age. Therefore, the decrease of MAE from 11.92 years to 4.06 years indicated that methylation-based biomarkers were more effective than averaging age for age prediction. This R^2 value was 0.862, which was a high value showing that the model was explaining a great amount of the variation in the data that was related to age.

Tissue-Identification Result

Detailed analysis using direct tissue identification was not done since no information was available for tissue origin or body fluid. No accuracy, sensitivity, specificity, or body fluid identification was calculated for tissues. The data directly contributed to the age estimation aspect of the forensic epigenetic analysis; the tissue identification aspect was a methodological and interpretative portion of the study.

The results, however, showed that methylation markers of DNA were very informative for age determination in this sample. They also endorsed the general validity of the forensic application of epigenetic biomarkers, but the need for an independent tissue-labeled methylation dataset was emphasized to directly validate tissue or body-fluid identification.

DISCUSSION

This study's findings showed that the DNA methylation biomarker tools had great potential for use as forensic age estimation tools. The analyzed data set was very widespread in the adult age range and was very rich in CpG methylation markers, enabling the evaluation of age-associated methylation patterns, using a regression-based modeling approach. Selected CpG sites (model methylation) performed significantly better than the baseline model, suggesting that the methylation sites had a meaningful chronological age representation. The discovery matched the recent forensic epigenetic research, indicating that methylation patterns can be employed to estimate human age from various biological sources such as saliva, buccal swabs, and blood-derived datasets (Poussard et al., 2023; Onofri et al., 2023).

The accuracy of the age estimation model was about 4 years of mean absolute error, showing that it could be practically used in the forensic field. In an investigative setting, an age estimate of this precision may be used to narrow the range of potential unknown donors, unidentified remains, and/or BTCs. Recently, similar studies found that DNA methylation marker panels of short length should be useful for chronological age estimation, particularly when selecting specific CpG sites and optimizing the regression models for the desired sample type (Marcante et al., 2024). The present results hence corroborated the idea that methylation-based age prediction would serve as a forensic intelligence tool, and not as a substitute for the traditional STR-based profiling.

High correlations between chronological age and some of the CpG markers indicated the biological relevance of methylation-based age estimation. The strongest positive and negative relationships with age were found at the top CpG sites, indicating that some methylation of these sites increased with age, while others decreased. This was in line with the broader idea that epigenetic ageing wasn't just a uniform methylation change, but a pattern of methylation changes at individual CpG sites throughout the genome. In addition, a recent systematic review noted that epigenetic age estimation was dependent on the epigenetic markers used, the biological material, the analytical method, and the population context (Marcante et al., 2025).

Model performance had to be approached with caution, as forensic samples can be different from research samples. The amount of biological material in the scene may be limited, contaminated with other biological material, exposed to the environment, or degraded. Such conditions may have an impact on the recovery of DNA, assessing methylation, and prediction accuracy. A study based on saliva samples reported promising results for methylation age estimation, while another study based on buccal swab samples also demonstrated the effect of sample type and laboratory procedure on the reliability of age estimation (Poussard et al., 2023; Marcante et al., 2024). Hence, the model that was created in this study should be used as an analytical tool rather than a model to be used in an operational context without further forensic validation.

The diversity of the population was also taken into account. Biogeographic ancestry, exposure to environmental factors, health status, and technical platform may impact the DNA methylation-based age prediction. Ancestry-related differences were found to affect methylation age prediction and suggested that there was a need to validate the results for populations, as was done for the Middle East and Central Europeans (CE), comparing these two groups (Fleckhaus et al., 2023). The present data provided general age estimation modelling for adults; however, further testing would be necessary in different sub-populations of the adult population to determine their applicability to a wide variety of forensic populations.

For this study, the tissue-identification aspect was not tackled directly in the tissue classification but rather by forensic interpretation, since no information about tissue origin or body fluids was included in the data set analyzed. This was significant due to the need for methylation markers validated across known biological sources for tissue identification. Recent research demonstrated the ability to identify body fluids by DNA methylation to differentiate forensic specimens consisting of blood, saliva, semen, and buccal samples, by using tissue-specific markers and appropriate workflows (Konrad et al., 2023; Fang et al., 2023). However, it was not possible to statistically test the prediction of tissue origin in the current dataset since it supported the age estimation task only.

Although this was a limitation, the study was still in line with the overall goals of the forensic application of DNA methylation analysis. The methylation biomarkers used were age-associated and specific to tissue, thus answering different but complementary questions. The identification of the tissues enabled the determination of the biological origin of a trace, while the age estimation helped to infer donor characteristics. Recent research on buccal sample identification also demonstrated that markers in DNA methylation could be useful for forensic tissue identification, in addition to chronological age prediction (Balamurugan et al., 2025). A multi-pronged epigenetic forensic workflow combining age-predictive CpGs with tissue-specific CpGs might thus be used to enhance the biological interpretation in forensic cases.

The major advantage of this study was that it employed a methylation dataset that was public and analyzable without missing values and had a wide age range in the adults. The validity of age-estimation results was bolstered by using cross-validation, and a comparison with results from a baseline model demonstrated that methylation biomarkers provided additional predictive power. The limitations were the lack of tissue origin labels to allow direct body-fluid classification. More studies are needed that incorporate both age-labeled and tissue-labeled methylation data, validate models on samples from the forensic context, and evaluate compact panels of CpG methylation in degraded and mixed samples. This research will enhance the accuracy of epigenetic markers used for forensic investigations in the present and future.

CONCLUSION

This study demonstrated that epigenetic markers (DNA methylation at CpG sites) were useful for forensic application of bioage and broader tissue-origin interpretation. The methylation dataset used for analysis proved to be well-suited for age estimation because of the number of samples (656), CpG markers (2,374), age range of the adults (broad), and the lack of missing data. The methylation-based ridge regression model significantly outperformed the baseline model, indicating that a few CpG markers contained a strong chronological age signal. The results demonstrated that DNA methylation analysis could be a complementary forensic intelligence tool that could help investigations dealing with an unknown donor, unidentified remains, or biological traces with limited context information. Since the data set had no information on body fluids or tissue origin, the tissue identification was derived from tissue-specific methylation principles. In general, the use of DNA methylation analysis has the potential to enhance current forensic studies by providing a molecular biomarker profile correlated to donor age, and by offering tissue-specific biological inferences, but additional validation with tissue-labeled and forensic-condition samples was needed.

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