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Some tips on age estimation using DNA methylation in saliva samples as an index across the Japanese and Indonesian ethnicities

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ABSTRACT

Age estimation of unidentified bodies is of marked importance in forensic medicine. In previous studies, the analysis of DNA methylation in body fluids led to the identification of several age-related CpG sites in genes such as *EDARADD* and *FHL2*. However, limited information is available on whether interethnic differences may affect the age prediction results. In the present study, we examined the effect of ethnicity on the age prediction method based on methylation scores, which were determined via methylation-sensitive high-resolution melting. We found that there was a significant difference in methylation scores between Japanese and Indonesian participants of early 20 s group, and that the nationality coefficient was significant for age estimation when applying the existing method for the analysis of the methylation status of *EDARADD* and *FHL2*. This suggests that when using certain biochemical indicators as a predictor of age, the effects of ethnicity on DNA methylation should be considered to improve the accuracy of the estimation.

1. Introduction

Age estimation of unidentified remains is important for personal identification in crime scenes and forensic medicine in general. The age estimation methods used in the forensic field are roughly classified into morphological and biochemical. It has been reported that biochemical methods are more precise, since morphological methods are highly affected by error due to individual differences. Moreover, biological fluids, such as saliva and blood, which are commonly found in crime scenes, cannot be analysed with morphological techniques. In addition to biochemical age estimation methods, gene-based methods, such as those employing signal-joint T-cell receptors [1], telomere length [2,3], and somatic gene arrangement [4], are increasingly being used for the analysis of biological fluids and small specimens frequently found in disaster and crime scenes. However, while morphological similarities and differences among various ethnicities may be known, there is limited evidence of these among biochemical markers.

In recent years, the use of epigenetics in age estimation has been reported, where the amount of methylated cytosines in CpG sites has become known as a tissue-specific marker of age [5–11]. In fact, studies have reported age estimation of saliva samples based on DNA methylation of CpG sites located in *ELOVL2*, *EDARADD,* and *FHL2* [9,12,13]. Due to the cost and the complexity of DNA methylation measurement, there is an increasing expectation for using real-time PCR based techniques such as methylation-sensitive high-resolution melting (MS-HRM) [12,14] and methylation-specific PCR (MSP) [15]. However, limited information is available on whether these methods are accurate across different populations.

In our study, we took into consideration the nationality of the saliva sample donors (Japanese or Indonesian) and reported some results for age estimation via MS-HRM quantification of methylation in *ELOVL2*, *EDARADD,* and *FHL2*.

2. Materials and methods

All experimental protocols were approved by the epidemiological

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research ethics review committee of Hiroshima University (no. E-1073). We used donated saliva samples and opt-out residual specimens from other clinical tests. A written informed consent for donation was signed either by the donors themselves or the next of kin.

2.1. Sample collection, DNA extraction, and bisulfite conversion

Saliva samples from 79 healthy Japanese donors and 34 healthy Indonesian donors were collected in plastic tubes following paraffin wax chewing stimulation. Ethnicity information was collected through interviews. Donors were defined as "Japanese" if both donor and the donor's parents had Japanese nationality and had lived in Japan most of their life. Similarly, donors were defined as "Indonesian" if both donor and the donor's parents had Indonesian nationality and had lived in Indonesia most of their life. Age was calculated from the date (year and month) of birth. Age and sex information for the 113 samples is shown in Table 1. All samples were stored in a − 20 °C freezer within 12 h after collection for further studies. DNA was extracted from the saliva samples using QIA amp DNA Investigator Kit (Qiagen, Hilden, Germany) and treated with EpiTect Fast Bisulfite Conversion Kit (Qiagen) for bisulfite conversion according to the manufacturer's protocol.

2.2. High-resolution melting

For the PCR we used the following previously reported [12,14] pairs of primers: for *ELOVL2*, forward primer 5′-CGATTTGTAGGTTTAGT-3′ and reverse primer 5′-ACTACCAATCTAAACAA-3′ (91 bp, 10 CpG sites); for *EDARADD*, forward primer 5′-AGAAGGTTTGATTTTGGTTAGAT-3′ and reverse primer 5′-CCTCTCCCCATCTATTTAAT-3′ (139 bp, 4 CpG sites); for *FHL2*, forward primer 5′-TTTACCAAAACTCCTTTCTT-3′ and reverse primer 5′-GTGGGTAGATTTTTGTTATT-3′ (133 bp, 14 CpG sites). PCR amplification was carried out with a StepOne™ Plus (Thermo Fisher Scientific, Waltham, Massachusetts, USA) equipped with the HRM Software (version 3.1, Thermo Fisher Scientific). A total volume of 20 μL containing 1 × MeltDoctor HRM Master Mix (Thermo Fisher Scientific), 300 nM of each primer, and 40 ng of bisulfite-modified template was used. The anneal/extend temperatures of 50, 52, and 60 °C were used for *ELOVL2*, *EDARADD*, and *FHL2*, respectively. When HRM analysis was performed, the pre-melt and post-melt temperature regions were respectively set as follows: 68–69 °C and 82–83 °C for *ELOVL2*; 65–66 °C and 80–81 °C for *EDARADD*; and 68–69 °C and 82–83 °C for *FHL2*. The samples were analysed using HRM in duplicate. Other variables were set appropriately according to the manufacturer's protocols.

2.3. Calculating methylation scores

Fully methylated/unmethylated and bisulfite converted control DNA were purchased from Qiagen and mixed in appropriate ratios to make 0%, 1%, 10%, 25%, 50%, 65%, 80%, 90%, and 100% methylated control DNA. These were then used to prepare standard curves for each HRM measurement. The Df value of each control sample obtained by HRM was plotted, and standard curves for *ELOVL2* (non-linear), *EDARADD* (linear), and *FHL2* (non-linear) were developed in Excel (Microsoft Excel for Microsoft Office 365) to determine the value of the methylation score for the saliva samples.

Table 1
Age and sex information for samples used in this study.

	Japanese N = 79		Indonesian N = 34	
	Male	Female	Male	Female
Age < 10	4	4	0	0
Age 10–19	2	3	0	0
Age 20–29	9	9	12	15
Age 30–39	11	6	2	3
Age 40–49	3	10	0	0
Age 50–59	6	4	0	1
Age > 60	5	3	0	1
Total	40	39	14	20

2.4. Statistical analysis

Spearman's rank correlation coefficient (ρ) was used to measure the correlation between each of the methylation scores (*ELOVL2, EDARADD*, and *FHL2*) and age. According to the results of preliminary experiments from 48 Japanese samples (Supplementary Table 1), we did not find any significant correlation between chronological age and methylation score of *ELOVL2* in our experimental conditions (Supplementary Fig. 1). For this reason, we used the score of *EDARADD* and *FHL2* for the following multiple linear regression (MLR) analysis. An MLR analysis with reference to chronological age was done with the methylation scores of *EDARADD* and *FHL2*, sex (1 = male, 2 = female), and nationality (1 = Japanese, 2 = Indonesian) as the explanatory variables. Non-parametric Mann-Whitney's U test was used to compare the methylation scores between Japanese and Indonesian saliva samples. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) [16]. More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics. A p-value lower than 0.05 was considered statistically significant.

3. Results

In the "Age 23–25" group, there were significant differences in the methylation scores of *EDARADD* between Japanese and Indonesian saliva samples (Fig. 1a, d). In contrast, there were no significant differences in the methylation scores in the "Age 26–28" group (Fig. 1a, b). With respect to the methylation scores of *FHL2* among these groups, there were no significant differences (Fig. 1a, c, e). Considering methylation scores, sex, and nationality, we found that the *EDARADD* ($p = 0.000$), *FHL2* ($p = 0.000$), and nationality ($p = 0.005$) coefficients were significant for age estimation (Table 2). Contrastingly, the sex coefficient was not significant for the estimation of age (Table 2). Variance inflation factor (VIF) of the methylation scores of *EDARADD* and *FHL2* and sex and nationality coefficients, were 1.385, 1.372, 1.043, and 1.032, respectively. The regression models excluding sex from the explanatory variables are shown in Figs. 2 and 3. The mean absolute deviation (MAD) of a prediction model with nationality was 10.8 years (Fig. 2b), while that of a model without this factor was 11.1 years (Fig. 3b).

4. Discussion

In the present study, we uncovered a significant difference in age prediction among Japanese and Indonesian saliva samples while considering the MS-HRM *EDARADD* and *FHL2* methylation scores. Age prediction methods based on DNA methylation levels of saliva samples were previously reported [12,13]. However, to the best of our knowledge, no information is available on the possible influence of interethnic differences. For this reason, we carefully verified the nationalities of donors upon sample collection. In the present study, of the 113 samples used, we compared the methylation scores of groups "Age 23–25" and "Age 26–28", which have similar distributions between Japanese and Indonesians. Then, we found significant differences in the *EDARADD* methylation scores in the "Age 23–25" group between Japanese and Indonesian saliva samples. Although our prediction model did not present enough accuracy (Fig. 2: MAD = 10.8 years), our results suggest that, in some cases, we need to consider the population of origin in existing age predicting DNA methylation methods and adapt them in both forensic and clinical settings.

Disasters and crimes are a global occurrence and the victims are

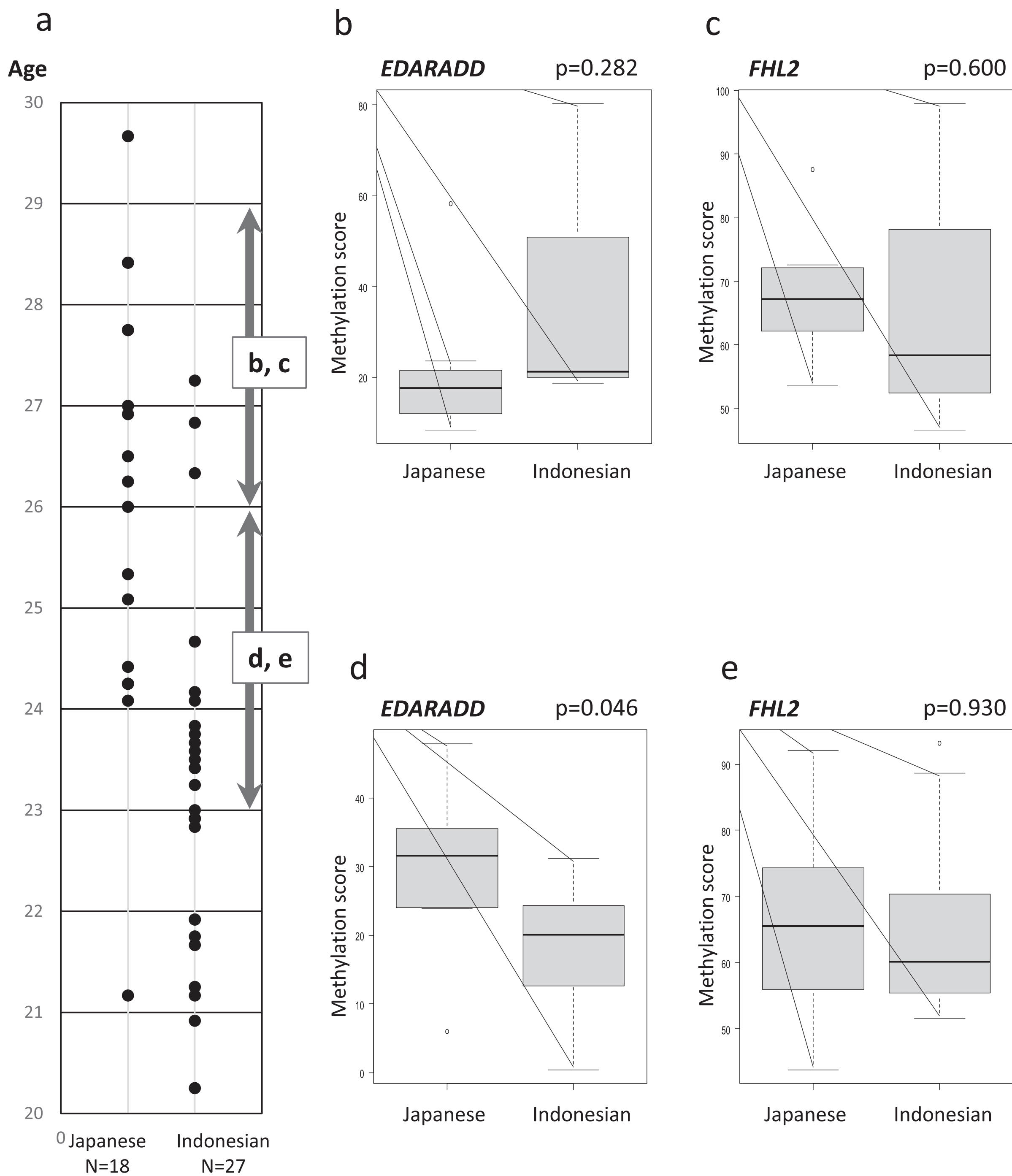
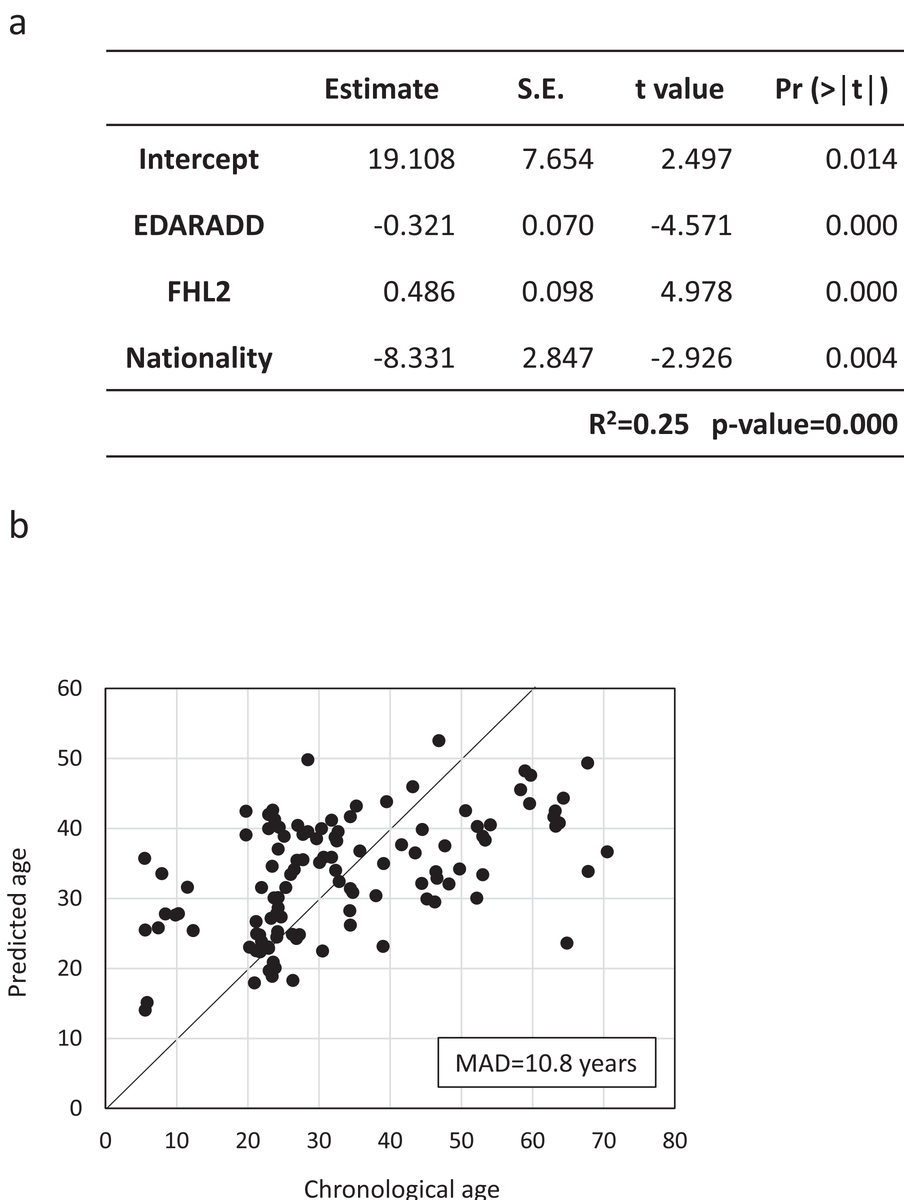


Fig. 1. Differences in the methylation scores between Japanese and Indonesian saliva samples. Mann–Whitney's *U* test. (a) Age distribution of participants in their 20 s; (b) *EDARADD* of Age 26–28; (c) *FHL2* of Age 26–28; (d) *EDARADD* of Age 23–25; (e) *FHL2* of Age 23–25.

Table 2

Estimated regression coefficients. A multiple linear regression (MLR) analysis; ***$p < 0.001$. The result of the MLR analysis of chronological age considering the methylation scores of *EDARADD* and *FHL2*, sex, and nationality as the explanatory variables (79 "Japanese" and 34 "Indonesian" saliva samples).

	Regression coefficient (estimate)	95% CI lower	95% CI upper	S.E.	P-value	
Intercept	21.180	4.739	37.620	8.294	0.012	*
EDARADD	−0.329	−0.471	−0.188	0.072	0.000	***
FHL2	0.494	0.299	0.690	0.099	0.000	***
Sex	−1.740	−6.980	3.500	2.644	0.512	
Nationality	−8.174	−13.852	−2.497	2.864	0.005	**
				$R^2 = 0.246$	**p-value = 0.000**	



a

	Estimate	S.E.	t value	Pr (>|t|)
Intercept	19.108	7.654	2.497	0.014
EDARADD	-0.321	0.070	-4.571	0.000
FHL2	0.486	0.098	4.978	0.000
Nationality	-8.331	2.847	-2.926	0.004
			R^2=0.25	**p-value=0.000**

Fig. 2. Validation of the predicted age through multiple linear regression analysis considering the methylation scores of *EDARADD* and *FHL2* and information on nationality (79 “Japanese” and 34 “Indonesian” saliva samples). (a) Coefficients in this study. (b) Relation between predicted and chronological ages.

diverse. In Japan, the number of foreign residents is increasing every year. At the end of 2019, the number of foreign residents was ~ 2.9 million, mostly from Asian countries [17]. In our study, identifying the donors’ nationality purely based on appearance would have been challenging. In the current scenario, our population consists of a mixture of people from multiple regions of different countries, therefore, we need to confirm if it is reasonable to include biochemical indicators for age estimation.

In the present study, all saliva samples were collected following paraffin wax chewing stimulation. It is known that saliva contains oral epithelial cells, lymphocytes, and neutrophils [18]. Several studies have reported age-correlated methylation sites of *EDARADD* in buccal epithelial cells [19] and of *FHL2* in blood cells [14,19]. Therefore, we can reasonably say that in the present study there is a correlation between chronological age and each methylation score detected in the saliva sample. However, in our preliminary examination with 48 Japanese samples, methylation of *ELOVL2*, the well-known age-correlated factor in saliva [12,19], did not correlate with the chronological age significantly. Even though we used the same primer sequences that were used in a previous study that estimated age using saliva samples [12], some of our processes, such as the real-time PCR machine and chemical regents, were different. Thus, we adjusted the protocol for PCR and MS-HRM analysis to suit our conditions. However, the methylation scores of *EDARADD* and *FHL2* in 79 Japanese samples were obtained from appropriate real-time PCR reactions, but we failed to obtain appropriate reactions for *ELOVL2* in some samples (n = 31). In addition, although we used chewing stimulation (a gum test method) to collect the saliva samples in this study, there was no information about the saliva collection method (stimulated or non-stimulated) in the previous study. Salivary DNA has cellular heterogeneity and there is a possibility that the proportion of cells differs among collection methods. These factors can explain the low correlation observed in the present study compared with previous ones.

Our study, however, presents some limitations. First, the sample size was not large and focused on Japanese and Indonesian participants alone. In addition, Indonesian participants were aged between 20 and 50 years, with a particular bias towards the 20 s. This was due to the difficulty in collecting Indonesian saliva samples with reliable interview

a

	Estimate	S.E.	t value	Pr (>|t|)
Intercept	6.075	6.434	0.944	0.347
EDARADD	-0.307	0.072	-4.243	0.000
FHL2	0.511	0.100	5.090	0.000
				R^2=0.198 p-value=0.000

b

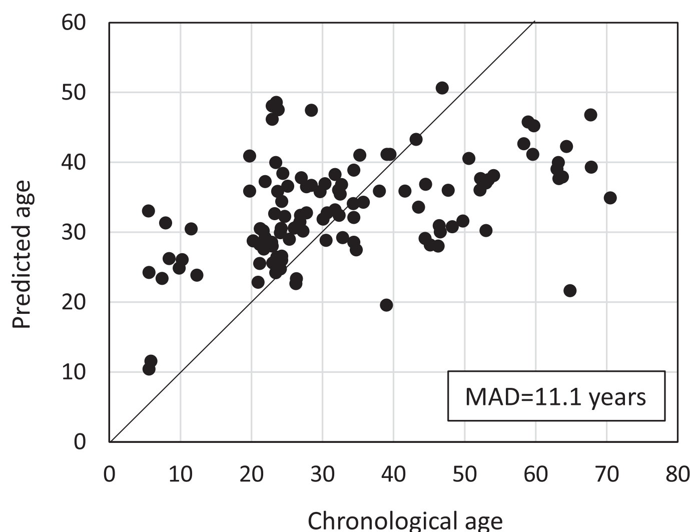


Fig. 3. Validation of the predicted age through multiple linear regression analysis considering the methylation scores of *EDARADD* and *FHL2* only (79 "Japanese" and 34 "Indonesian" saliva samples). (a) Coefficients in this study. (b) Relation between predicted and chronological ages.

results from young and elderly donors in Japan. To develop the accurate age estimation model, we need more samples. Second, we could not include *ELOVL2* in the main analysis. Adding *ELOVL2* to the analysis would probably improve the accuracy of the prediction formula. Third, the saliva samples were frozen immediately after collection. In our preliminary results, we observed that methylation was subject to variation in a 4 °C refrigerator (Supplementary Fig. 2). As mentioned in a previous study [14], storage conditions of samples must be maintained during examination to measure saliva DNA methylation. Further investigations are needed to define the effects of prolonged storage and preservation methods on saliva samples.

In conclusion, we believe that DNA phenotyping has considerable potential in age prediction and resolving forensic issues. However, there was a significant difference in methylation scores between Japanese and Indonesian participants of early 20 s group. Additionally, considering methylation scores, sex, and nationality, we found that the nationality coefficient was significant for age estimation when applying the existing method for the analysis of the methylation status of *EDARADD* and *FHL2*. Therefore, when focusing on the development of improved age prediction models using certain biological indicators, ethnic information should be considered.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.legalmed.2022.102042.

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