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TECHNICAL NOTE

Odontology

Molecular and morphological findings in a sample of oral surgery patients: What can we learn for multivariate concepts for age estimation?

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Abstract

It has already been proposed that a combined use of different molecular and morphological markers of aging in multivariate models may result in a greater accuracy of age estimation. However, such an approach can be complex and expensive, and not every combination may be useful. The significance and usefulness of combined analyses of D-aspartic acid in dentine, pentosidine in dentine, DNA methylation in buccal swabs at five genomic regions (*PDE4C*, *RPA2*, *ELOVL2*, *DDO*, and *EDARADD*), and third molar mineralization were tested by investigating a sample of 90 oral surgery patients. Machine learning models for age estimation were trained and evaluated, and the contribution of each parameter to multivariate models was tested by assessment of the predictor importance. For models based on D-aspartic acid, pentosidine, and the combination of both, mean absolute errors (MAEs) of 2.93, 3.41, and 2.68 years were calculated, respectively. The additional inclusion of the five DNAm markers did not improve the results. The sole DNAm-based model revealed a MAE of 4.14 years. In individuals under 28 years of age, the combination of the DNAm markers with the third molar mineralization stages reduced the MAE from 3.85 to 2.81 years. Our findings confirm that the combination of parameters in multivariate models may be very useful for age estimation. However, the inclusion of many parameters does not necessarily lead to better results. It is a task for future research to identify the best selection of parameters for the different requirements in forensic practice.

KEYWORDS
age estimation, D-aspartic acid, DNA methylation, multivariate models, pentosidine, tooth mineralization stages

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Highlights

- The combined use of different markers of aging in multivariate models may improve age estimation.
- Molecular markers (DNAm, D-aspartic acid, pentosidine) as well as morphological markers are promising candidates for such models.
- The most relevant features of multivariate models can be assessed by the predictor importance.
- Future research should identify the optimal combinations of markers for specific applications.

1 | INTRODUCTION

Methods for forensic age estimation are based on the evaluation of age-related alterations of the human organism. Classical approaches use morphological changes especially of teeth and the skeleton [1,2]. In the last decade, molecular approaches found their way into the methodological repertoire [3]. They are based on molecular modifications that accumulate with age and exhibit a close correlation with chronological age. Such alterations are the methylation of DNA (DNAm, [4–6]) as well as the accumulation of D-aspartic acid (D-Asp) and pentosidine (Pen) in permanent proteins [7–13].

Age-dependent DNAm patterns are the subject of intense research, and several models for the estimation of chronological age have been established (e.g., [14–20]). An important advantage of this approach is a broad (and in case of buccal swabs also noninvasive) applicability to diverse tissues [15]. Though the reported mean absolute deviations (MADs) for the currently best models are in the range of approximately 2–4 years [5,15–19,21], substantial errors in single cases have to be taken into account, especially in older ages [16,17,19].

Age estimation based on D-Asp in dentine is one of the most accurate methods for age estimation in adults, and the reported data reveal MADs of 0.72–3.4 [22–25]. This approach can also be applied to tissues more complex than dentine (e.g., epiglottis, bone, and intervertebral disks [26–28]), which, however, results in a significantly lower accuracy of age estimation [26,29,30].

Pen is an advanced glycation end product and accumulates with increasing age in long-living proteins [10–12]. The relationship between the Pen content of dentine and age is close [13], but significantly less close than the one between the D-Asp content of dentine and age.

Each of the approaches outlined above has its limitations, and most of these limitations are consequences of the complexity and variability of the aging process. Accordingly, one of the main problems of all methods of age estimation is an increasing scattering of data with increasing age. It has already been proposed that a combined use of different molecular and morphological markers may better address the complexity of aging and result in a higher accuracy of age estimation, especially in older ages [28,31].

However, molecular analyses for age estimation are demanding, the combined use of different approaches can be expensive, and not every combination may be useful. Thus, it would be important to know which combinations of markers actually offer significantly better results in defined case constellations of forensic practice.

So far, we do not know which combinations of D-Asp, Pen, DNAm, and morphological findings in multivariate models are useful. We approached this question by analyzing molecular and morphological parameters in parallel and investigated the significance of each parameter in multivariate models for age estimation. For ethical reasons, the investigation of a sample of oral surgery patients was the only possibility to get access to teeth (for the analysis of D-Asp and Pen), to buccal swabs (for the analysis of 5 DNAm markers), and as well to orthopantomograms (OPTGs, for the morphological staging of the tooth mineralization). The over-representation of younger ages in this group of patients could be accepted, since it was not our aim to develop new models for age estimation or to compare different methods, but to learn—in the sense of basic research—which of the analyzed parameters (D-Asp, Pen, 5 DNAm markers, tooth mineralization) may contribute to multivariate models of age estimation to which extent and which combinations of markers offer added value.

2 | MATERIALS AND METHODS

For 90 oral surgery patients, the following parameters were investigated:

- Stages of the tooth mineralization (third molars),
- DNA methylation at CG dinucleotides (CpGs) within the genes *PDE4C*, *RPA2*, *ELOVL2*, *DDO*, and *EDARADD*,
- D-aspartic acid content (D-Asp),
- Pentosidine content (Pen).

Based on these parameters, multivariate models for age estimation were developed and the impact of each parameter on the models was determined.

2.1 | Patients and material

90 patients (with known ages between 10.2 and 79.5 years) from the dental clinic of the University of Düsseldorf were included in the study after written consent. As far as possible, OPTGs, buccal swabs, and extracted teeth were collected from each patient. Table 1 gives an overview over the available material. The included extracted teeth were free

TABLE 1 Number of samples (all age-groups and individuals with ages under 28 years)

	All ages	Ages under 28 years
Teeth	66 (from 52 patients)	46 (from 37 patients)
Buccal swabs	88	71
Radiographs (OPTGs)	82	69

of caries and free of fillings, root canal treatments, and crowns. Patients with known diabetes mellitus were excluded from the study.

2.2 | Staging of tooth mineralization

As morphological parameter to be included into the multivariate models, we chose the third molar mineralization (categorized by the Demirjian stages A–H [32]), since the mineralization of the third molars covers a broad age range that is of particular importance for age estimation in living persons (e.g., criminal responsibility, age of majority). Due to the proof-of-concept design of our study, we did not include the mineralization of other teeth. The stages were not used for a classical morphological age estimation, but as such (A-H) directly introduced into the multivariate models.

In this context, staging of tooth mineralization appeared only useful as long as tooth maturation is not yet completed. According to the data of Olze et al. [33], the development of third molars in the German population should be completed no later than the age of approximately 28.5 years (23.1 ± 1.8 years, considering a threefold standard deviation). Therefore, we staged the third molar mineralization only in a subsample of 69 individuals younger than 28 years (10.2–27.5 years).

The degree of tooth mineralization was categorized into eight stages (A–H), as described by Demirjian et al. [32].

The classification was carried out independently by two experienced forensic scientists. In case of deviating results, the x-ray image was reviewed and the stages were categorized after reaching consensus.

2.3 | D-aspartic acid content in dentine

Dentine samples from 66 teeth from patients with ages between 12.7 and 79.5 years were analyzed according to Ritz-Timme [34], in brief:

Crown and apical third of the root were cut off with a water-cooled diamond drill, and cement and pulp tissue were removed. The remaining root was washed, and the samples were lyophilized and crushed in a hydraulic press. The resulting powder was stored by −20°C until further analysis. 10 mg of dentine powder was hydrolyzed for 6 h with 1 ml 6 N HCl at 100°C. After drying, the samples were derivatized as described. The D- and L-aspartic acid contents were finally analyzed by gas chromatography (GC-2014 Shimadzu; flame ionization detector), using a chiral capillary column (Chirasil-L-Val, Chrompack, Frankfurt).

2.4 | Pentosidine content in dentine

Analyses were performed in dentine from 66 teeth from patients with ages between 12.7 and 79.5 years according to Odetti et al. [35] and modified by Greis et al. [13], in brief: 50 mg tooth powder was hydrolyzed with 1 ml 6 N HCl at 110°C for 18 h. After drying, 1 ml 0.01 M heptafluorobutyric acid (HFBA) was added. The solution was filtered through syringe filters (Ø 25 mm, 0.45 µm pore diameter) and dried again. The samples were dissolved in 350 µl pyridoxine-HFBA and analyzed by high-performance liquid chromatography (HPLC) as described.

2.5 | DNA methylation

The DNAm markers in *ELOVL2*, *DDO*, *RPA2*, *PDE4C*, and *EDARADD* (Table 2) were analyzed in 88 buccal swabs from patients with ages between 10.2 and 79.5 years.

DNA was extracted using the NucleoSpin® Tissue Kit from Macherey-Nagel. The extraction was performed according to the standard protocol for human tissue with 3-h lysis at 56°C in a shaking thermal block (ThermoMixer® C, Eppendorf). DNA was eluted in 100 µl BE buffer. DNA extracts were stored at −20°C. Quantitation was performed using the Quantiplex® Pro Kit (Qiagen) and the Applied Biosystems™ 7500 Real-Time PCR System following the manufacturer's instructions with default settings. Bisulfite conversion was performed using the EZ DNA Methylation-Gold™ Kit (Zymo Research) following the manufacturer's instructions. If possible, the recommended amount of 200–500 ng DNA was applied. Bisulfite-converted DNA was amplified using the PyroMark PCR Kit (Qiagen) with the respective primers under the manufacturer's

TABLE 2 Analyzed CpGs with CpG number, CpG ID, position, and reference

Gene ID	CpG Number	CpG ID	Position	Reference
ELOVL2	6	cg16867657	chr.6:11044877	Naue et al. [15]
RPA2	3	cg25410668	chr.1:28241577	Naue et al. [15]
DDO	1	cg02872426	chr.6:110736772	Naue et al. [15]
EDARADD	2	cg09809672	chr1:236557683	Baekert et al. [16]
PDE4C	1	cg17861230	chr19:18233127	Weidner et al. [17]

conditions for bisulfite-converted DNA: 95°C, 15 min; 45× cycles (94°C, 30 s; 56°C, 30 s; 72°C, 30 s); 72°C, 10 min; 4°C, hold. Primer sequences were taken from the original papers (Table 2). For the subsequent pyrosequencing analysis, 10–20 µl of biotinylated PCR product was immobilized to 1 µl Streptavidin Sepharose™ HP beads (GE Healthcare). The sequencing primers were designed as previously described [15–17]. Pyrosequencing was performed using the PyroMark Q24 Advanced CpG Reagents Kit (Qiagen) and the PyroMark Q24 Advanced System (Qiagen).

2.6 | Statistical evaluation

Uni- and multivariate models for age estimation were developed, and the impact of each parameter (tooth mineralization stages, D-Asp, Pen, and DNAm markers) for the multivariate models was tested. Univariate models were only developed for the molecular methods.

Prediction of individual age was performed using random forests, that is, an ensemble of decision trees. We repeatedly drew subsamples of subjects from the training set and estimated the optimal decision tree on this reduced number of subjects. These were then combined into a final prediction allowing to obtain better performance than obtainable from any of the constituent predictors by itself. In practice, we repeatedly sampled 85% of the training cases (with replacement).

For each run, we trained a decision tree using the CART algorithm to identify the best non-linear combination of rules in the training set to estimate age based on the respective features. As noted above, training was repeatedly performed on a subset of the training cases, yielding "weak learners" that were later combined to achieve the final prediction. Here we employed the "TreeBagger" in the MATLAB "statistics and machine learning toolbox" (Number of trees: 25.000, Minimum Leaf Size: 1, Predictors to sample: all, Surrogate Splits: on, Predictor Selection: Interaction-Curvature).

Each of these models based on a subset of the training subjects was then applied to predict the age of a held out, "new" subject, that is, a case that was not part of the training set. Technically, this was achieved by out-of-bag prediction. That is, each subject's prediction was based on only those trees that were constructed from subsamples that did not include this particular individual. This approach yielded an out-of-sample prediction of the age for each subject in the current sample that did not use any information about that particular person when training the model.

As a measure of prediction accuracy, we computed the mean absolute error (MAE). We then computed the mean (averaged across subjects) absolute differences between the predicted and the true chronological age for each subject. In addition, we also computed the Pearson (linear) correlation coefficient between the true chronological age and the predicted age. These analyses were performed for all age-groups as well as for the subgroup of individuals with ages under 28 years.

Finally, to identify the most relevant features of each model, we assessed the predictor importance. These are estimates by a permutation measure reflecting how influential a variable is for predicting the response. If a predictor is influential, then permuting its values should affect the model error. If a predictor is not influential, then permuting its values should have little to no effect on the model error. The normalized predictor importance, reflecting the increase in model loss by permuting this predictor, hence corresponds to the relevance of the respective feature.

3 | RESULTS

3.1 | Univariate models for molecular methods

Among the univariate models for the molecular methods, the protein markers (D-Asp, Pen) exhibited the highest correlations (r = 0.96 and 0.94, respectively) and the lowest MAEs (2.93 and 3.41 years; 2.25 and 2.87 years in individuals under ages of 28 years, Figure 1, Table 3).

The DNAm marker DDO exhibited very poor results (r = 0.31, MAE = 24.74 years, Figure 1), also in the age-group of under 28 years (r = 0.2; MAE = 16.44, Table 3). The other DNAm markers delivered much better results (r = 0.71–0.81, MAE = 5.87–7.08 years; r = 0.3–0.43, MAE = 7.18–10.52 years in individuals with ages under 28 years, Figure 1, Table 3).

3.2 | Multivariate models

3.2.1 | D-Asp & Pen

The combination of the two protein markers (D-Asp and Pen) resulted in a slightly lower MAE (2.68, 1.96 years in individuals with ages under 28 years, Figure 2, Table 4) compared to the univariate D-Asp model (2.93, 2.25 years, Table 3, respectively). Both parameters were equally important for the model, and only in the group of individuals with ages under 28 years, D-Asp was slightly more important (Figure 2).

3.2.2 | D-Asp & Pen & DNAm (5 markers)

The model comprising the two protein markers and all DNAm markers delivered less precise results (MAE = 3.52 years, 2.98 years in individuals with ages under 28 years) than the model based solely on the two protein markers; the protein markers were most important for this model (Figure 2, Table 4).

3.2.3 | DNAm based on the combination of five CpGs

A model based solely on the five DNAm markers resulted in a MAE of 4.14 years (3.85 years in individuals under 28 years of age). The most important markers in our model were RPA2, EDARADD, and PDE4C.

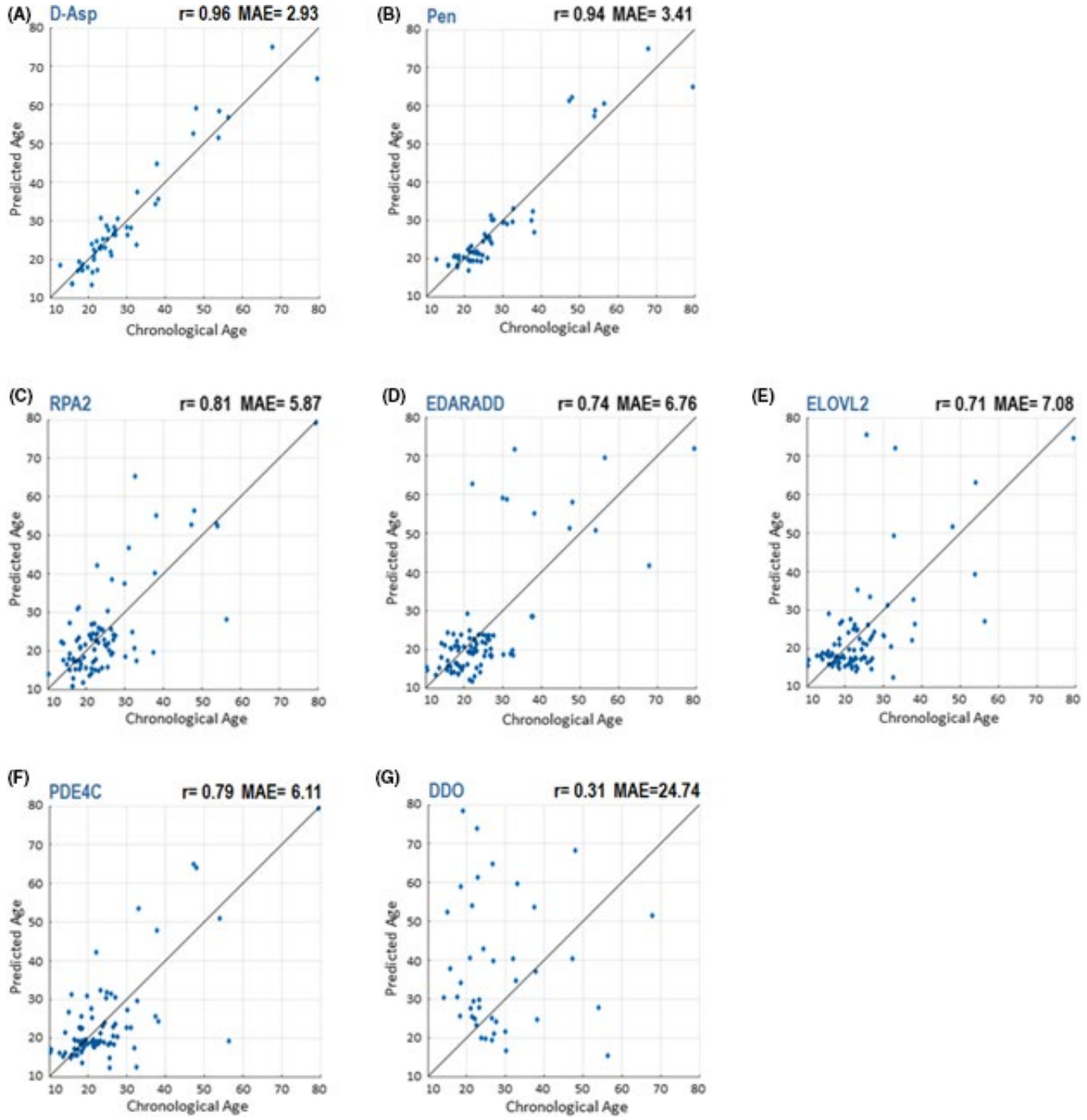

FIGURE 1 Age estimation by univariate models based on the protein markers D-Asp (A) and Pen (B) and on the DNAm at CpGs in the genes *RPA2* (C), *EDARADD* (D), *ELOVL2* (E), *PDE4C* (F), and *DDO* (G): Predicted ages plotted against chronological ages (*r*, correlation coefficient, MAE, mean absolute error in years). The line marks the theoretical position of values in case of identity of estimated and chronological age [Colour figure can be viewed at wileyonlinelibrary.com]

DDO played a relevant role only in individuals under 28 years of age (Figure 2, Table 4).

3.2.4 | DNAm & third molar mineralization

A combination of the third molar mineralization stages with all DNAm markers resulted in an improvement of the sole DNAm model (MAE = 2.81 years vs. 3.85 years for individuals under 28 years). Tooth mineralization was most important for the model (Figure 2, Table 4).

4 | DISCUSSION

It was not the aim of this study to develop a "ready to use" model for age estimation or to compare different methodological approaches,

TABLE 3 Univariate models: numbers of samples (*N*), correlation coefficients (*r*), and mean absolute error (MAE, in years) for all age-groups and for individuals with ages under 28 years

	Univariate models					
	All ages			Ages under 28 years		
	N	*r*	MAE	*N*	*r*	MAE
D-Asp	66	0.96	2.93	46	0.78	2.25
Pen	66	0.94	3.41	46	0.71	2.87
EDARADD	83	0.74	6.76	68	0.37	8.68
DDO	86	0.31	24.74	70	0.2	16.44
ELOVL2	88	0.71	7.08	71	0.3	10.52
RPA2	88	0.81	5.87	71	0.43	7.18
PDE4C	87	0.79	6.11	70	0.38	7.87

but to explore which parameters may contribute to multivariate models of age estimation to which extent and which combination of markers may be promising.

To investigate, which significance DNAm, D-Asp, Pen, and tooth mineralization stages may have in multivariate models and which combinations of markers offer added value, we analyzed these parameters in parallel, derived diverse multivariate models for age estimation, and identified the most relevant features of each model by assessment of the predictor importance. The choice of the patient sample (oral surgery clinic) enabled the analysis of several molecular and morphologic parameters in every single individual, since OPTGs, buccal swabs, and extracted teeth were available. However, the choice of this sample was associated with the problem that it consisted mainly of young individuals, and older individuals were under-represented. Despite this

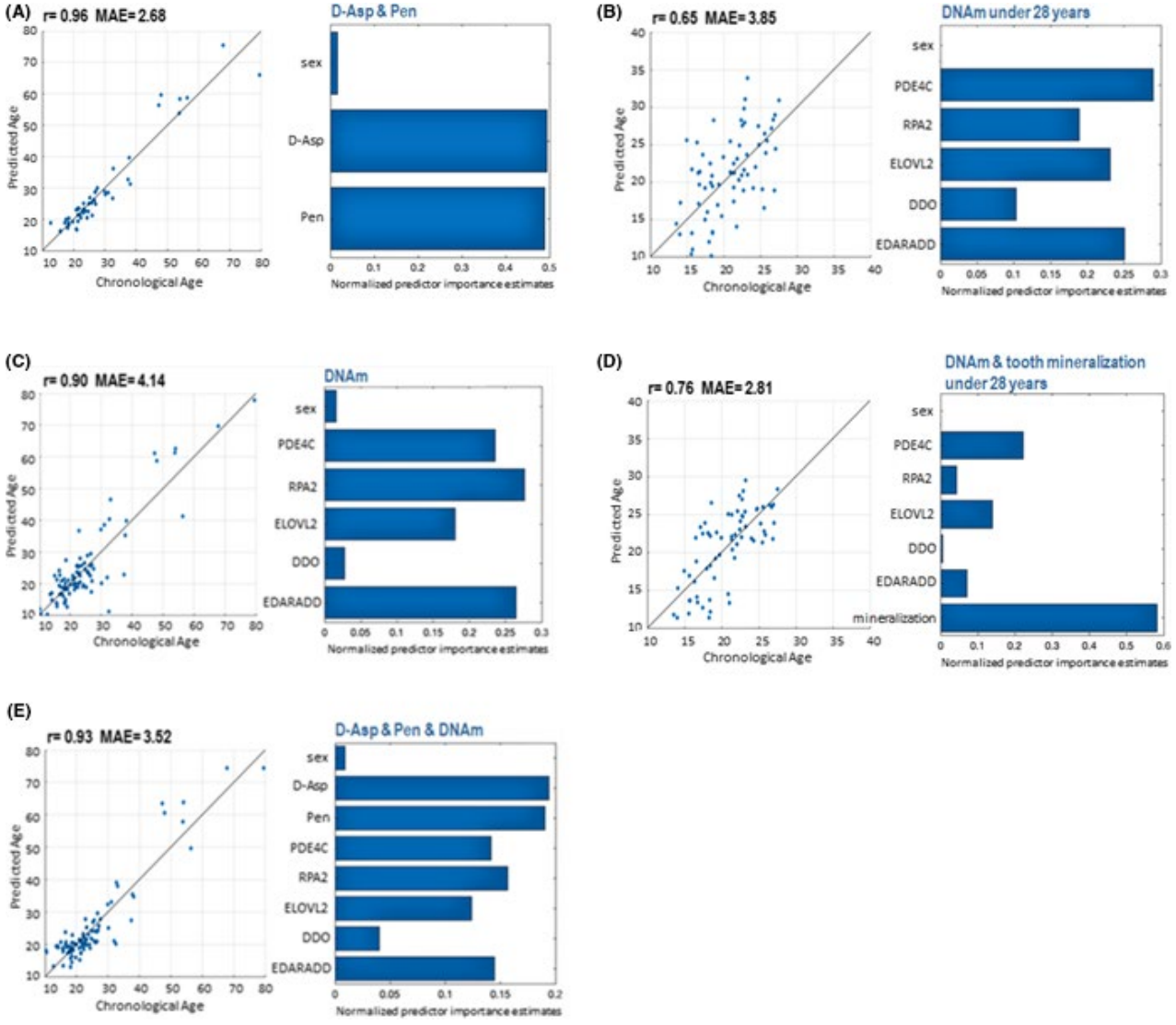

FIGURE 2 Age estimation by multivariate models based on D-Asp and Pen (A), the DNAm markers *PDE4C*, *RPA2*, *ELOVL2*, *DDO*, and *EDARADD* for individuals with ages under 28 years (B) and for all age-groups (C), tooth mineralization stages and the DNAm markers *PDE4C*, *RPA2*, *ELOVL2*, *DDO*, and *EDARADD* for individuals with ages under 28 years (D), and D-Asp, Pen, and the DNAm markers *PDE4C*, *RPA2*, *ELOVL2*, *DDO*, and *EDARADD* (E): Predicted ages plotted against chronological ages (*r*, correlation coefficient, MAE, mean absolute error in years) and normalized predictor importance estimates. The line marks the theoretical position of values in case of identity of estimated and chronological age [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 4 Multivariate models: numbers of samples (*N*), correlation coefficients (*r*), and mean absolute error (MAE, in years) for all age-groups and for individuals with ages under 28 years

	Multivariate models					
	All ages			Ages under 28 years		
	N	*r*	MAE	*N*	*r*	MAE
D-Asp & Pen	66	0.96	2.68	46	0.82	1.96
DNAm (5 markers)	88	0.90	4.14	71	0.65	3.85
Third molar mineralization & DNAm (5 markers)				69	0.76	2.81
D-Asp & Pen & DNAm (5 markers)	50	0.93	3.52	35	0.74	2.98
D-Asp & DNAm (5 markers)	50	0.92	3.59	35	0.73	2.96
Pen & DNAm (5 marker)	50	0.92	3.55	35	0.73	3.22
D-Asp & Pen & DNAm (5 markers) & third molar mineralization				82	0.83	2.22

limitation, the presented data allow some conclusions regarding further strategies for the development of age estimation methods for postmortem age estimation as well as for age estimation in living individuals.

Postmortem age estimation:

- If teeth (dentine) are available, the protein markers D-Asp and Pen are very informative for age estimation in adult age. The high potential of age estimation based on D-Asp in dentine has already been proven by many groups, by data revealing MADs of 0.72–3.4 [22–25]. In our explorative study, the additional analysis of age-associated DNAm changes did not improve the accuracy of age predictions.
- This does not account for situations in which teeth are not available. Age estimation based on D-Asp and Pen in other tissues produces less accurate results, as compared to dentine [26,27,29,30]. It has already been shown that the combination of D-Asp and Pen data from several complex tissues in multivariate models results in a significant improvement of age estimation based on these protein markers [28]. The introduction of DNAm markers into such models should open new possibilities for a further improvement of postmortem age estimation, especially if teeth are not available.
- The improvement of age estimation by combining third molar mineralization stages with DNAm markers emphasizes the potential of morphological information in age estimation when included in multivariate models. Possibly the information from the tooth mineralization stages led to a pre-structuring of data with positive effects for age estimation. Similarly, also skeletal findings may add a significant contribution to multivariate approaches for postmortem age estimation.

Age estimation in living individuals:

- So far, there are only few studies regarding the application of multivariate models to age estimation in living persons. However, a combination of morphological findings (skeletal and dental development) and DNAm markers in multivariate models has already been proposed for children [31]. This concept has not been tested for adolescents yet. Our data demonstrate an improvement of age estimation by combining third molar mineralization stages with DNAm markers and therefore seem to support this approach, at least at a first glance. However, some samples revealed a high deviation of chronological and estimated age. Though the MAE was 2.81 years for the model "DNAm & tooth mineralization" (young individuals with ages under 28 years), substantial errors of up to 15 years in single cases were observed (Figure 2). As long as future research cannot eradicate such errors, this approach will not be applicable to age estimation in living individuals. Still, better results might be obtained by including further parameters like information about the skeletal development (hand, clavicula) and/or other DNAm markers.

Our findings confirm that the combination of parameters in multivariate models may be very useful. However, the combination of numerous parameters does not necessarily lead to better results. It is a task for future research to identify the best approach and the best choice of parameters for the different requirements in forensic practice.

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CONFLICTS OF INTERESTS

W.W. is cofounder of Cygenia GmbH (www.cygenia.com) that may provide service for the analysis of epigenetic age.

ETHICS APPROVAL

All procedures performed in studies involving human tissue were in concordance with the ethical standards of the institutional and/ or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards (approved

by Ethics Committee at the Medical Faculty of Heinrich-Heine University: HEBE-project, study number 5049). This article does not contain any studies with animals performed by any of the authors.

CONSENT TO PARTICIPATE

Informed consent was obtained from all individual participants included in the study and/or from legal guardians.

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