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ORIGINAL ARTICLE

Analysis of advanced glycation end products (AGEs) in dentine: useful for age estimation?

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Abstract Ageing of the human organism results in the accumulation of modified molecules. Some of these molecular changes may be used for age estimation, as already shown for aspartic acid racemization (AAR). Another example for an accumulation of damaged molecules is advanced glycation end products (AGEs). We examined, (1) if the correlation between the concentration of AGEs (pentosidine) in root dentine and age is close enough to be used as basis for age estimation, and (2) if the combined analysis of AGEs and AAR in dentine may be a useful approach to rule out or to detect relevant effects of confounding factors in age estimation. We determined the pentosidine content of root dentine samples of 64 healthy teeth as well as in carious, "pink", diabetic and heated teeth, and in teeth after different storage times. In 23 teeth, the extent of aspartic acid racemization (AAR) was determined in parallel. We observed a close relationship between the concentration of pentosidine in dentine and chronological age ($r = 0.94$) in healthy teeth. The analysis of pentosidine in dentine can theoretically be used as a basis for age estimation in healthy teeth of non-diabetic individuals; diabetic individuals may exhibit very high pentosidine levels in dentine. This finding limits the application of this method, since information regarding the question if an unidentified person suffered from diabetes mellitus or not are missing in most cases. Moreover, the method is not suitable to identify or rule out the influence of confounding factors in age estimation based on AAR, since both methods are sensible to the most relevant confounding factors (caries, heat).

Keywords Age estimation · Advanced glycation end products · Pentosidine · Aspartic acid racemization · Root dentine

Introduction

Ageing of the human organism produces molecular changes that can be used for age estimation. Examples for such changes are DNA methylation and other modifications of DNA, or posttranslational protein modifications as the accumulation of D-aspartic acid residues (aspartic acid racemization, AAR) and advanced glycation end products (AGEs) [1, 2].

AGEs, heterogeneous and complex groups of compounds, are generated by the Maillard reaction, an irreversible non-enzymatic reaction of reducing sugars with free amino groups on proteins, lipids or nucleic acids [3]; well investigated AGEs are e.g. pentosidine, *N*-carboxymethyllysine (CML), pyralline and furosine [4–6]. AGEs accumulate in permanent and long-living proteins during lifetime. This has been demonstrated for pentosidine, CML, *N*-carboxyethyllysine (CEL) and/or furosine in dentine [7], crystalline lens [8], articular cartilage [9], rib cartilage, intervertebral disc [10] and skin collagen [11].

Rib cartilage and dentine have already been proposed to be used for age estimation based on the accumulation of AGEs. Pilin et al. (2007) suggested that colour changes of rib cartilage caused by AGE accumulation may be useful for age estimation [10]. Miura et al. (2014) compared six caries-free third molars of young (around 20 years) and old (around 70 years) individuals regarding mechanical characteristics, fluorescence

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spectra and immunohistochemical analyses of demineralized dentine sections. They described an accumulation of CML in old dentine, especially in the collagen fibrils around dentinal tubules; therefore they concluded that the extent of accumulated AGEs in dentinal collagen may be a basis for age estimation. This study was primarily focused on the question, if AGEs are generally formed in dentinal collagen and if there is any possible influence of AGEs on dentinal physiology [7]; it remains unclear, if the relationship between the content of AGEs in dentine and age is close enough to serve as basis for age estimation. Pentosidine, the AGE examined in our study, is a fluorescent AGE that is able to cross-link proteins between free amino groups of lysine and arginine [12].

One of the already established and most precise methods for molecular age estimation is based on AAR in dentine [13]. AAR results in an irreversible and age-dependent accumulation of D-aspartic acid residues in long-living and permanent proteins during lifetime, which can be used as basis for age estimation. Though this method is very robust, few confounding factors may at least theoretically influence the precision of the method, e.g. protein degradation caused by caries [14, 15], contaminating proteins in cases of "pink teeth" [16] and high temperatures in cases of burnt bodies [17]. Relevant effects of such confounding factors in the single case may be ruled out or detected by the combination of age estimation based on AAR in dentine samples with the analysis of the identical samples by another, completely independent method. The additional detection of AGEs may be a possible approach to improve age estimation based on AAR in cases in which confounding factors cannot be excluded. Cabo et al. (2017) already assumed that use of AGEs for age estimation of burnt bodies may have an advantage compared to heat-affected methods, e.g. based on AAR [18].

We examined,

- if the correlation between the concentration of AGEs (pentosidine) in root dentine and chronological age is close enough to be used as basis for age estimation, and
- if the combined analysis of AGEs and AAR in dentine may be a useful approach to rule out or to detect relevant effects of confounding factors in age estimation.

Materials and methods

The contents of pentosidine and D-aspartic acid were determined in root dentine samples of third molars with known ages.

The teeth were extracted by dentists due to medical indications. After extraction, the teeth were washed with water, dried and stored at − 20 °C (for up to 8 years). Table 1 gives an overview over the analyses performed.

The crowns of the analysed carious teeth were extensively destroyed (ICDAS-Code 5-6, International Caries Detection and Assessment System); their roots were at most slightly involved. Carious lesions of the roots were removed according to clinical standards; therefore, the samples themselves were caries-free.

The "pink teeth" exhibited the characteristic reddish/pink colour of dentine due to a "contamination" of dentinal tubules with haemoglobin, which obviously resulted from an insufficient washing after the extraction and the subsequent storage of the teeth.

Heating experiments

To examine the influence of heat, four third molars were placed in Pyrex tubes with 500 μl double distilled water and heated at 130 °C for 1.5 h using an aluminium heating block (type 51388101, Liebisch, Bielefeld).

Preparation of root dentine samples

Root dentine was prepared with water-cooled instruments as described by Ritz-Timme (1999) [13]: After removal of soft tissue, the root was separated from the crown, and the distal third of the root was cut. Cementum was removed, and the root canal was cleaned by extirpation of the dental pulp. The quality of preparation was evaluated using ultraviolet light (wavelength of 366 nm).

The prepared roots were washed at 4 °C, successively with each 7 ml 15% sodium chloride (for 1 h), ethanol/ether (vol. 3:1; for 15 min) and 2% sodium dodecyl sulphate (for 1 h). Between these steps, the roots were washed with double distilled water. After washing, they were lyophilized for 24 h and stored at − 20 °C until further processing.

The lyophilized roots were pulverised by a hydraulic press (Mod. 8, Gr. III, ø 25 mm, P/O/Weber, Remshalden) at 20 kN.

Analysis of AGEs (pentosidine)

Pentosidine concentrations were determined by high-performance liquid chromatography (HPLC), basically as described by Odetti et al. (1992) [19].

Dentine samples of 50 mg were transferred into Pyrex tubes and hydrolysed in 1 ml 6N HCl for 18 h at 110 °C. Samples were dried overnight using a desiccator.

In a next step, samples were dissolved in 1 ml 0.01 M heptafluorobutyric acid (HFBA, Thermo Scientific, Rockford, IL, in HPLC-water, HiPerSolv Chromanorm, VWR International), then filtrated over 0.45-μm pore diameter syringe filters (ø 25 mm, VWR International) and dried overnight in a desiccator again.

Dried samples were dissolved in 350 μl pyridoxine-HFBA (pyridoxine 2.068815 μmol/ml in 0.01 M HFBA). Pentosidine

Table 1 Overview over the analyses performed: numbers, donor ages and storage times of teeth, numbers of analyses. *AGEs* advanced glycation end products, *AAR* aspartic acid racemization

	Numbers of teeth	Donor ages	AGEs (pentosidine), numbers of analyses	AAR, numbers of analyses	Storage time	
					< 1 year	5–8 years
Healthy teeth	64	15–65 years	64	8	18	50
"Pink teeth"	4	20–36 years	4	2		
Teeth with carious lesions	9 (incl. 1 diabetic tooth)	22–58 years	9	9		
Heated teeth	4	20–36 years	4	4		

(Cayman Chemical) was used as external standard (pentosidine 0.03303 nmol/ml in 0.01 M HFBA).

Samples were injected into an HPLC system (HPLC 1100 Series, Agilent, CA). Separations were made with a semi-preparative column (Onyx Monolithic, Semi-Prep C18, Phenomenex, CA). A linear gradient program of 10–85% acetonitrile (LiChrosolv, Merck KGaA, Darmstadt) from 0 to 32 min with 0.1% HFBA as a counterion was used. The fluorescence detector was set at an excitation-emission wavelength of 335/385 nm. Pentosidine could be identified by its retention time.

The pentosidine concentrations of 63 analysed healthy teeth of non-diabetic individuals were used to describe the relationship between pentosidine concentration and age by linear regression. One healthy tooth was excluded, because it was identified as a sample with wrong information regarding the donor age (see below). The resulting equation was used for age estimation in carious (incl. diabetic), "pink" and heated teeth; a 95% prediction interval was calculated for age estimation in healthy teeth [20].

Analysis of AAR

In 23 teeth (8 healthy teeth, 9 carious teeth (incl. the diabetic tooth), 2 "pink" and 4 heated teeth), the extent of AAR was determined by gas chromatography (GC) [13] in parallel to the analysis of pentosidine.

Twenty milligrams of dentine was hydrolysed in 1 ml 6N HCl at 100 °C for 6 h and then dried overnight in a desiccator. The hydrolysates were esterified with 1 ml isopropanol and 0.1 ml sulfuric acid (97%, *w*/*w*) at 110 °C for 1 h, then dried by a nitrogen stream and alkalinized with 1 ml 4N ammoniac, extracted with 1 ml dichloromethane and dried by a nitrogen stream again. The samples were acetylated with 0.05 ml trifluoroacetic anhydride in 1 ml dichloromethane at 60 °C for 1 h and dried by a nitrogen stream again.

After derivatization, amino acids were separated by GC (Shimadzu GC-2014, chiral capillary column: Chirasil-L-Val, Varian, CA). The ratio of D- and L-aspartic acid (D/L) was calculated and used for age estimation based on AAR according to Ritz-Timme (1999) [13].

Results

AGEs (pentosidine) in dentine

Healthy teeth of non-diabetic individuals

A close correlation between the concentration of pentosidine in healthy dentine and chronological age was observed ($r = 0.94$, Fig. 1a) that could be described by the following equation:

$$\text{age} = 4.342 + 314.445 * \textit{pentosidine concentration}$$

A 95% prediction interval of ± 9.4 years (for all examined concentrations) was calculated for age estimation.

Carious teeth

Compared to healthy teeth, the pentosidine levels of dentine samples from caries-affected teeth exhibited a slightly greater scattering (Fig. 1b). Using the equation derived from the data of the healthy teeth for age estimation, deviations of − 14 to + 9 years from real age were determined for these caries-affected teeth.

Diabetic tooth

The tooth of the individual with diabetes mellitus exhibited a considerably higher concentration of pentosidine than all other teeth of the same age (Fig. 1b), resulting in a deviation between estimated and real age of + 23 years.

"*Pink teeth*"

The analysis of samples from three "pink teeth" resulted in age estimates differing between − 1 to + 2 years from the real ages. One "pink tooth" exhibited a deviation of + 6 years between estimated and real age (Fig. 1b).

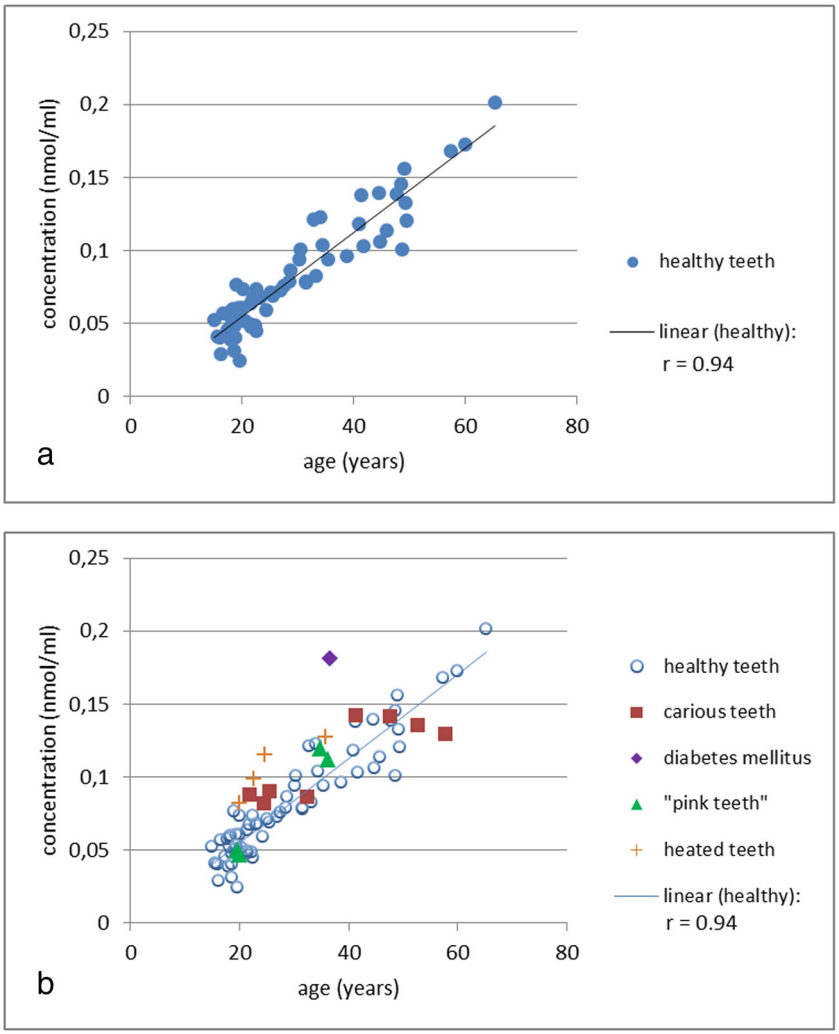

Fig. 1 **a** Concentration of pentosidine (nmol/ml) in root dentine of 63 healthy teeth from non-diabetic individuals (black circles): relatively close correlation between pentosidine levels and ages ($r = 0.94$). **b** Concentration of pentosidine (nmol/ml) in root dentine of teeth bearing the risk of confounding factors for age estimation, as compared to the results for healthy teeth: 9 carious (black square) (incl. 1 diabetic (black diamond)), 4 "pink" (black up-pointing triangle) and 4 heated teeth (plus sign); data for healthy teeth (Fig. 1**a**) and the corresponding regression line (white circle). The 9 caries-affected teeth exhibit a relatively large scattering; especially the one with diabetes mellitus deviates up. The "pink teeth" do not deviate considerably. The heated teeth present a relatively high pentosidine concentration

Teeth exposed to heat

After heating, the teeth presented relatively high pentosidine levels (Fig. 1b). The estimated ages deviated + 7 to + 15 years from real age (only false high age estimates).

Different storage times

There was no hint to a relevant influence of storage times between < 1 and 5–8 years. Both the correlation coefficients ($r = 0.9374$/$r = 0.9418$) and the slopes of the trendlines for the two groups were nearly identical.

Additional analysis of AAR in selected cases

Healthy teeth of non-diabetic individuals

Dentine samples from healthy teeth exhibited a close relationship between AAR and age ($r = 0.97$, Fig. 2). Using the AAR equation published by Ritz-Timme (1999) [13], the estimated ages deviated − 4 to + 6 years from the real ages.

Carious teeth

Age estimates based on AAR revealed deviations of + 2 to + 11 years between estimated age and real age (only false high age estimates).

Diabetic tooth

The diabetic tooth did not reveal a relevant deviation of AAR, as compared to the teeth of the non-diabetic donors (Fig. 2). It exhibited a deviation between estimated and real age of + 5 years. This finding contrasts the considerably high concentration of pentosidine (see above).

"*Pink teeth*"

Age estimation based on AAR in samples from "pink teeth" revealed only small deviations (− 1 and − 3 years between estimated and real ages, Fig. 2).

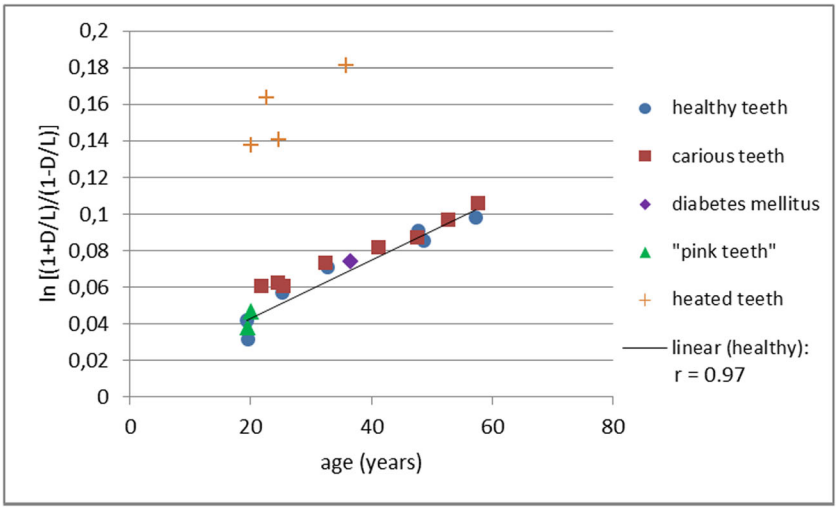

Fig. 2 Extent of aspartic acid racemization (as ln [(1 + D/L)/(1 − D/L)]) in root dentine of teeth bearing the risk of confounding factors for age estimation, as compared to the results for healthy teeth: 8 healthy (black circle), 9 carious (black square) (incl. 1 diabetic (black diamond)), 2 "pink" (black up-pointing triangle) and 4 heated (plus sign) teeth. There is a relatively close correlation between AAR values and ages. The correlation coefficient for healthy teeth is $r = 0.97$. The heated teeth deviate up considerably

Teeth exposed to heat

Age estimation based on AAR resulted in deviations of + 59 to + 75 years between estimated ages and real ages. There was a much higher influence of heat, as compared to age estimation based on the concentration of pentosidine (Figs. 1b and 2).

Findings in a case with obviously wrong information about the donor age

One healthy tooth exhibited an extremely high deviation from the trendline in both (!) methods. The donor age was firstly declared to be 13 years. The sample exhibited a very high D-aspartic acid content as well as a very high pentosidine level, resulting in estimated ages of 46 and 45 years, respectively.

Discussion

AGEs (pentosidine) accumulate in healthy root dentine of non-diabetic individuals with increasing age

This study was primarily focused on the question, if analysis of AGEs in dentine is an interesting approach for age estimation in forensic case work. Since our study merely aimed at proving the principle, we only used one tooth type (wisdom teeth).

Our results for 63 healthy teeth confirm that there is a strong correlation ($r = 0.94$) between the accumulation of pentosidine in healthy root dentine from non-diabetic individuals and chronological age.

In theory, pentosidine can be used as basis for forensic age estimation using healthy teeth. The results also suggest that storage times of up to 8 years (at − 20 °C) and post-mortem changes in the sense of "pink teeth" do not influence pentosidine levels. The 95% prediction interval of ± 9.4 years for age estimation should be treated with caution, since only 63 samples were used to calculate it. In addition, the age distribution of these samples was not uniform; more samples of young ages were examined (mostly 18–25 years, only 3 samples older than 50 years, Fig. 1a).

Even if the prediction intervals for healthy teeth would be suitable for sufficiently precise age estimation, the application of age estimation based on pentosidine levels in dentine to forensic case work may be difficult because of some confounding factors identified by our data.

Age estimation based on the accumulation of AGEs in dentine may be affected by the confounding factors diabetes mellitus, caries and heat

The one diabetic tooth in our sample set exhibited (expectedly) a very high pentosidine level (Fig. 1b), which would have resulted in a falsely high age estimate (+ 23 years). Despite the fact that we could analyse only one diabetic sample (which, in addition, was caries-affected), there is not any doubt about the impact of diabetes mellitus on the results of age estimation based on AGEs. Elevated blood sugar levels in patients with diabetes mellitus cause an increased production of AGEs. Therefore, excessive amounts of AGEs can be detected in many tissues of diabetic individuals [21], such as in skin collagen [11], blood vessel walls and interstitial connective tissue [22]. Undoubtedly, the analysis of samples from individuals with diabetes mellitus can result in false high age estimates. In cases of an unidentified deceased (one of the classical fields with an indication for age estimation) it is—of course—not known, if the deceased suffered from diabetes mellitus or not. This is a relevant limitation for the applicability of age estimation based on AGEs. In contrast, the analysed diabetic tooth did exhibit a "normal" AAR value (Fig. 2).

Compared to healthy teeth, the caries-affected teeth exhibited a slightly greater scattering of their pentosidine values

(Fig. 1b). Higher levels of AGEs in caries-affected teeth have already been described and discussed in a clinical context. Kleter et al. (1998) analysed 20 carious third molars and determined higher AGE levels in carious sections than in healthy sections [23]. Armstrong et al. (1964) supposed that the Maillard reaction is responsible for the increased resistance of dentine collagen against collagenolytic breakdown and for browning of carious lesions [24]. We observed a slightly greater scattering of the pentosidine values in the caries-affected teeth though the visible carious lesions had been removed according to clinical standards. This may indicate caries-induced molecular changes even in distant tissue regions that appear clinically unaffected.

The results for the heated teeth indicate a heat-induced increase of the pentosidine concentration (Fig. 1b). This finding could be expected, since high temperatures accelerate the formation of AGEs, which can be observed also during food production and cooking [21]. Thus, the method may result in high deviations between estimated and true age when applied to burnt bodies.

The combined analysis of AGEs and AAR in dentine samples is a useful approach for molecular age estimation—but only theoretically

Age estimation based on AAR in dentine is one of the most precise methods for molecular age estimation. The additional analysis of pentosidine in dentine samples could make sense, if relevant effects of confounding factors may be ruled out or detected by this procedure.

There are only few confounding factors that may have an impact on the quality of age estimation based on AAR in dentine. It has been described that the "contamination" of dentinal tubules with haemoglobin in cases with "pink teeth" may have an impact on the results (false low age estimates); this impact may be reduced by adequate washing [16]. In our cases, age estimation with both methods (AGEs and AAR) did not reveal strikingly high deviations between estimated and real ages. However, it must be taken in mind that we did only analyse four and two "pink teeth", respectively. In forensic practice, the most important confounding factors for age estimation based on AAR are caries [14, 15] and heat (in cases of burnt bodies) [17]. According to our results, age estimation based on AGEs cannot provide more precise results than age estimation based on AAR in cases of carious and heated teeth; caries and heat do have an influence on both methods (Figs. 1b and 2). Thus, the combined analysis of AGEs and AAR in dentine samples is only theoretically a useful approach for molecular age estimation, since it will not help to overcome the influences of the most relevant confounding factors.

However, the combined analysis of pentosidine and AAR enabled us to identify wrong information about the donor age of one tooth. In this case, the donor age was firstly declared to be 13 years. Age estimation based on AAR revealed an age of 46 years. The accuracy of the AAR method is very high; in our hands, it works with 95% prediction intervals of ± 4 years [13], and the results of other groups are very similar [25, 26]. Thus, the reasonable suspicion of having wrong information regarding the donor age arose. The analysis of AGEs confirmed this suspicion since it revealed an age of 45 years—a nearly identical result as compared to that of the AAR analysis. Researchers dealing with the analysis of teeth extracted by dentists know about the risk of getting wrong information about the donor ages. Despite all efforts for quality assurance, this risk is reality and has to be considered. In future, we will use the tool of the combined analysis of pentosidine and AAR to identify wrong donor age data in any suspicious case.

Compliance with ethical standards

Ethical approval All procedures performed in studies involving human participants were in accordance 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.

This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare that they have no conflict of interest.

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