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Molecular methods for age estimation

The current state of the art in relation to specific demands of forensic practice

Molecular methods of age estimation and the demands of forensic practice

Migration in a globalized world has increased the need for accurate methods for age estimation in forensic practice during the last decades in diverse fields of application, especially for age estimation in living individuals as well as for age estimation in the identification of unknown deceased of unknown donors of traces. The need for suitable methods in different contexts has further stimulated research. Various morphological and molecular approaches have been proposed by many publications of different quality. Not infrequently it remains open which methods have already reached or will reach a level of development that will allow their use in practice. Today, it may be challenging for the forensic practitioner to keep track of suitable methods and to find the optimal method for a single case with its specific questions, conditions and requirements.

Already 20 years ago, a paper titled "Age estimation: the state of the art in relation to the specific demands of forensic practice" addressed this problem [1]. This paper has been highly cited since it has given orientation by 1) defining the specific demands that have to be fulfilled by methods for forensic age estimation and by 2) identifying the methods that could fulfil these demands.

In short, these defined criteria were [1]:

A. "The methods must be transparent and provable, the underlying data must have been presented to the scientific community, as a rule by publication in peer-reviewed journals …"
B. "Clear information concerning the accuracy of age estimation by the method should be available. Accuracy must have been tested by using valid statistical procedures and described in clearly defined terms …"
C. "The methods need to be accurate enough to fulfil the specific demands of the single case to solve the underlying questions …"
D. "In cases of age estimation in living individuals principles of medical ethics and legal regulations have to be considered …."

Twenty years ago only one molecular method, namely age estimation based on the D-aspartic acid content of dentine, could withstand the strict selection criteria cited above [1]. Since then, the scientific field of molecular approaches for age estimation has been rapidly expanding, not least because of the discovery of DNA methylation as a biomarker of aging and age. These molecular methods have been attracting much scientific attention since they can be superior to traditional morphological anthropological methods by promising much more accurate results at least in adult age. Moreover, as laboratory methods that quantify certain parameters, molecular methods mostly offer more convincing possibilities for standardization, validation and quality assurance than the traditional morphological methods, which is an important aspect with respect to the demands on modern forensic disciplines. Finally, some molecular methods (especially those based on DNA methylation) address more fields of application; they can be applied not only in the traditional field of age estimation for the identification of unknown deceased but also for the analysis of traces and noninvasive age estimation in living persons.

Although a lot of research will still be necessary for optimization and validation of many molecular methods, it is evident that they already play or will play an increasing role in different fields of application [2].

The aim of this paper is to give orientation regarding the current state of the art of molecular methods for age estimation with respect to the criteria cited above and to the different application fields in forensic practice. It is not intended to be an all-encompassing review but rather a practical guide for those colleagues who ask themselves which of the multitude of published molecular approaches for age estimation can already be used in casework or could be used in the near future.

Which molecular methods have a very high potential to fulfil the specific demands of forensic practice?

In the last decades diverse molecular approaches for age estimation have been proposed, e.g. the analysis of DNA methylation, posttranslational protein modifications, telomere length, mitochondrial DNA deletions and signal

Table 1 Eligible and promising molecular approaches for different fields of application

Field of application	Eligible/promising molecular approaches	Analyzed tissues/ parameters	Information about accuracy in years according to the literature		R	Literature	Remarks
Corpses or body parts	*If teeth are available*						**14C**- bomb pulse dating: information about accuracy applies to individuals born after 1950 until the present; future implications of the flattening of the bomb pulse curve have to be clarified. *In cases with advanced putrefaction*: **D-Asp and Pen:** analysis of dentine or usage of multivariate models are recommended. **DNAm:** so far only few data; obviously applicable as long as a sufficient amount of DNA is available [46]. *In burnt remains:* **D-Asp and Pen** levels may be falsely high if heat affected tissues are analyzed
	14C-bomb pulse dating	Enamel	1.4-1.8	(MAE)	0.99	[31–33]*	
	D-Asp	Dentine	±4 1.26 2.19–2.93	(95% PI) (SEE) (MAE)	0.93–0.99	[13]*; [14, 47]* [48, 49]*; [50]*	
	Pen	Dentine	±9.4 3.41–6.03	(95% PI) (MAE)	0.94	[19, 20]*; [49]*	
	DNAm	Pulp (13 CpGs)	2.25	(MAD)	0.96	[51]*	
		Dentine (7-9 CpGs)	4.86 5.08	(MAD) (MAE)	0.76–0.85	[52]*; [53]	
	Multivariate models	Relative telomere length and DNAm (9 CpGs in dentine)	5.04	(MAE)	0.77	[53]	
		D-Asp, Pen (in dentine), DNAm (5 CpGs in buccal swabs)	2.68–3.59	(MAE)	0.92–0.96	[49]*	
	If teeth are not available						
	D-Asp	Bone (osteocalcin)	±6 2.8	(95% PI) (SEE)	0.99	[54]*	
		Different soft tissues, partly after purification of proteins (e. g elastin)	n. s.	–	0.92–0.97	[8]*; [55]* [56–59]	
	Pen	Intervertebral disc	n. s.	–	0.92	[60]	
		Articular cartilage	n. s.	–	0.93	[58]	
	DNAm	Bone (6 CpGs)	2.56 3.4	(MAD) (MAE)	0.96, n. s.	[28]*; [30]*	
		Blood (4 CpGs)	5.36	(MAD)	0.89	[61]*	
		Buccal swabs (1 CpG)	n. s.	–	0.95	[46]	
	Multivariate models	D-Asp, Pen in intervertebral discs and epiglottis	4.0–6.3	(MAE)	0.86–0.95	[21]	
Ancient skeletal remains	**Pen**	Dentine	3.92	(MAE)	0.94	[40]*	**Pen** seems to be stable over very long postmortem intervals up to thousands of years
Living individuals	*(Rare) cases with available teeth after extraction for medical reasons*						According to the AGFAD guidelines [62]
	D-Asp	Dentine	±4	(95% PI)	0.98–0.99	[47]*	
	All other cases						–
	DNAm	Blood (2–13 CpGs)	3.16–5.17 3.93–6.18 4.1–4.5	(MAD) (RMSE) (MAE)	0.78–0.97, partly n. s.	[23]*; [29, 30, 63, 64]* [65]*; [66, 67]* [68]*; [69]	
		Saliva (5 CpGs)	3.55 4.52	(MAD) (RMSE)	0.95	[29]	
		Buccal swabs (5 CpGs)	4.29 5.29 3.7	(MAD) (RMSE) (MAE)	0.93, n. s.	[29, 30]	
Traces	**DNAm**	Blood (5 CpGs)	4.8–5.0	(MAD)	n. s.	[70]	Minimal recommended amount of DNA for evaluable and reliable results: ≥ 10 ng [24, 71]
		Blood (12 CpGs)	4.1 4.9	(MAE) (RMSE)	n. s.	[69]	
		Semen (3 CpGs)	4.8 5.8	(MAD) (RMSE)	0.91	[72]*	
		Saliva (7 CpGs)	3.15	(MAD)	0.95	[73]*	

14C carbon-14, *D-Asp* D-aspartic acid, *Pen* pentosidine, *DNAm* DNA methylation, *MAD* mean absolute deviation, *MAE* mean absolute error, *SEE* standard error of estimation, *PI* prediction interval, *R* correlation coefficient, *n. s.* not specified, *RMSE* root mean square error, *AGFAD* working group for forensic age diagnostics. The listed measurements of accuracy mostly relate to the entire age range; exceptions (i.e. only adult ages) are indicated by an asterisk (*)

joint T-cell receptor rearrangement excision circles (sjTRECS) [2–6]. Some of these parameters are so closely related with age that they have been referred to as "molecular clocks".

So far, several approaches have not made it from basic science to practical use. For some methods, there are only initial experiences and still insufficient data, and they have not yet been properly validated. Other methods do not provide the accuracy of age estimates that is needed. It may be very difficult to get satisfying information about the accuracy that can be expected when a method is applied in the field. The accuracy is described very differently, e.g. by correlation coefficients (R), by standard errors of estimates (SEE), by prediction intervals (PI), by mean absolute deviations or by mean absolute errors (MAD, MAE). Therefore, it is difficult to compare the accuracy of different methods.

When reviewing papers proposing molecular methods for age estimation under consideration of the defined demands (A-D), there are essentially the following molecular approaches that have a very high potential to meet these demands or already fulfil them, and that are already applied or can be expected to be ready for use in the near future:

- The accumulation of D-aspartic acid (D-Asp) with increasing age is the result of a spontaneous, non-enzymatic conversion of L-asparagine and L-aspartic acid residues into their D form [5, 7]. It has been described for long-lived proteins in numerous tissues [8–12]. This approach is already established in forensic practice, especially in the analysis of dentine (e.g. [13–16]).
- The age-dependent accumulation of pentosidine (Pen), an advanced glycation end product (AGE), is the result of complex non-enzymatic posttranslational protein modifications [17, 18]; Pen accumulates in long-lived proteins in diverse tissues, such as dentine, bone, intervertebral discs and epiglottis [19–22].
- DNA methylation (DNAm) at certain sites in the genome are closely related to a person's age. A large number of age-dependent DNAm markers have already been identified in diverse tissues and body fluids, and various models for age estimation have been proposed (e.g. [23–30]).
- Age estimation based on bomb pulse derived carbon-14 (14C) dating is more a physicochemical than a molecular method in a narrower sense. Nonetheless, this very interesting approach addresses age-dependent changes on a molecular level and is therefore listed here. It uses the rise of 14C in the atmosphere due to the above-ground nuclear bomb tests between 1955 and 1963 and its exponential drop after the tests were stopped. The amount of incorporated 14C in human tissues enables the estimation of the time of birth (and therefore an indirect estimation of age) of individuals born after ca. 1950; very low errors have been reported especially for the analysis of enamel [2, 31–34].

All in all there have been many peer reviewed papers regarding these four approaches (demand A), but the question of the achievable accuracy (demand B) cannot be finally answered for all methods that have been proposed on their basis. Age estimation based on D-Asp is the oldest of the four approaches; D-Asp analysis in dentine has been evaluated over decades by many groups over the world (e.g. [10, 14–16, 35–37]). The approach seems to be robust against non-age-related factors, such as life style, diseases or biographic origin [38, 39]; however, data about D-Asp in other tissues than dentine are still comparatively rare. In the case of Pen, there are still too few studies for a final evaluation; however, it seems to be very useful in combination with other approaches and in ancient cases [21, 40]. Also, for DNAm there are still open questions (e.g. regarding non-age-related factors that may influence DNAm [3, 41–43]) that have to be answered, before final statements about the accuracy of all derived methods can be made. Further research is also required with respect to the 14C approach; in particular, the future implication of the flattening of the bomb pulse curve due to the slow return to the pre-bomb pulse 14C levels on the errors in age estimation has to be clarified [34].

Nevertheless, it can safely be assumed that these four molecular approaches will sooner or later fulfil the demand B (clarity about the accuracy of age estimation). The question if the accuracy of a method is sufficient in a single case (demand C) depends on the application field and can be easily answered if there are clear data regarding the accuracy. Demand D (ethical issues of age estimation in living individuals) can be optimally addressed by DNAm, since epigenetic age estimation can be performed by analysis of buccal swabs or blood samples.

Which of these approaches (D-Asp, Pen, DNAm, 14C) are suitable or promising for which fields of application?

Table 1 gives an overview over eligible and promising molecular approaches for different fields of application (corpses or body parts in forensic cases, ancient skeletal remains, age estimation in living individuals, and estimation of the age of an unknown donor of a trace).

The compilation of literature in **Table 1** does not claim to be complete but includes exemplary and current articles with relevant information regarding the approaches and their achievable accuracy. The statistical robustness of data regarding the accuracy varies widely; nevertheless, the extracted information listed in **Table 1** allows at least a rough overview of the potential of the different approaches. Particular consideration was given to work recording correlation coefficients (R) above ca. 0.9 as well as SEEs, MAEs and MADs below ca. 5 years or 95% prediction intervals (PI) below ca. 10 years as an indication that the minimum required accuracy can be achieved to fulfil the demands of forensic casework [1, 44]. Due to complex aging processes with an accumulation of interfering confounding factors with age, the accuracy of nearly all models for age estimation becomes worse with increasing age.

The extent to which the methods can already be used in forensic practice depends on the level of development achieved by specialized laboratories. Therefore, in special cases it is always worth contacting a corresponding laboratory and discussing the question of whether a method should be applied under the special conditions of the single case.

From **Table 1** it becomes very clear how inconsistently the information on accuracy is reported in the literature. In future, researchers should agree on procedures that allow a direct comparison between methods and clear statements about the accuracy in individual cases. This is not only important for research, but also for practical use in casework; those who have to work with the result of an age estimation must get a clear statement about the error in every individual case.

Final remarks

This paper aims to give some orientation regarding the state of the art in the field of molecular age estimation. In view of the extensive research activities in this field, this overview may no longer be up to date when it is published. Nevertheless, it seemed sensible to give such an overview, not least because some methods are already being used or will soon be implemented.

Another point that could be supported by this overview is the promotion of interdisciplinarity in the field of age estimation, in research and practice as well. Due

Abstract · Zusammenfassung

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Molecular methods for age estimation. The current state of the art in relation to specific demands of forensic practice

Abstract

With the increase of globalization and migration, the topic of age estimation has become more and more important in diverse fields of application, especially for age estimation in living individuals as well as for age estimation in the identification of unknown deceased and of unknown donors of a trace. Especially in the last decade, the traditional spectrum of morphological methods has been expanded to numerous new approaches based on the use of age-dependent molecular changes. Articles in this field have been and are being published in quick succession but not all approaches can (already) meet the demands of forensic practice. It may be a challenge for the forensic practitioner to keep track of suitable methods and to find the optimal method for a single case with its specific questions, conditions and requirements. This overview is intended to provide orientation on the question of which molecular approaches can already be used or will be applicable in the foreseeable future in different application fields. The focus is on the accumulation of D-aspartic acid and pentosidine, DNA methylation and the use of the bomb pulse-derived carbon-14 (14C).

Keywords

Age estimation · State of the Art · DNA methylation · Posttranslational protein modifications · Carbon-14 (14C)

Molekulare Methoden zur Lebensaltersschätzung. "The State of the Art" unter Berücksichtigung der spezifischen Anforderungen der forensischen Praxis

Zusammenfassung

Mit Zunahme von Globalisierung und Migration hat das Thema Lebensaltersschätzung in den forensischen Wissenschaften mehr und mehr an Bedeutung gewonnen (bei Lebenden als auch bei nicht identifizierten Leichen oder im Zuge der Identifizierung eines unbekannten Spurenverursachers). Das traditionelle Spektrum morphologischer Methoden wurde insbesondere im letzten Jahrzehnt um zahlreiche neue Ansätze erweitert, die auf der Nutzung altersabhängiger molekularer Veränderungen basieren. In rascher Folge wurden und werden Beiträge in diesem Feld publiziert – aber nicht alle Ansätze können die Anforderungen der forensischen Praxis (bereits) erfüllen. Für den forensischen Praktiker kann es zur Herausforderung werden, die Übersicht über geeignete Methoden zu behalten und die optimale Methode für den konkreten Einzelfall mit seinen spezifischen Fragestellungen, Bedingungen und Voraussetzungen zu finden. Diese Übersicht will Orientierung zu der Frage geben, welche molekularen Ansätze unter welcher Fragestellung bereits einsetzbar sind oder in absehbarer Zeit einsetzbar sein werden. Im Fokus stehen dabei die Akkumulation von D-Asparaginsäure und Pentosidin, die DNA-Methylierung und die Nutzung des bei Atombombenversuchen freigesetzten Radiocarbons (14C).

Schlüsselwörter

Lebensaltersschätzung · "State of the Art" · DNA-Methylierung · Posttranslationale Proteinmodifikationen · Radiocarbon (14C)

to the different nature of the approaches and their different fields of application, very differently qualified scientists (such as morphologists and molecular geneticists) are involved in research regarding this topic. It has already been shown that multivariate approaches using several biological levels (morphology, proteins, DNAm) may produce better results than univariate approaches [21, 26, 45]. Important impulses can be expected by interdisciplinary approaches that require a look beyond the boundaries of the subdisciplines of (forensic) sciences.

This overview focuses only on molecular methods for age estimation and neglects morphological approaches, whose importance should by no means be negated. It will be exciting to see how the repertoire of methods will develop in the future and how it will be used as best as possible in practice.

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Declarations

Conflict of interest. P. Boehme, A. Reckert, J. Becker and S. Ritz-Timme declare that they have no competing interests.

Ethical standards. For this article no studies with human participants or animals were performed by any of the authors.

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