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Age Estimation with DNA: From Forensic DNA Fingerprinting to Forensic (Epi)Genomics: A Mini-Review

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Abstract
Forensic genetics developed from protein-based techniques a quarter of a century ago and became famous as "DNA fingerprinting," this being based on restriction fragment length polymorphisms (RFLPs) of high-molecular-weight DNA. The amplification of much smaller short tandem repeat (STR) sequences using the polymerase chain reaction soon replaced RFLP analysis and advanced to become the gold standard in genetic identification. Meanwhile, STR multiplexes have been developed and made commercially available which simultaneously amplify up to 30 STR loci from as little as 15 cells or fewer. The enormous information content that comes with the large variety of observed STR genotypes allows for genetic individualisation (with the exception of identical twins). Carefully selected core STR loci form the basis of intelligence-led DNA databases that provide investigative leads by linking unsolved crime scenes and criminals through their matched STR profiles. Nevertheless, the success of modern DNA fingerprinting depends on the availability of reference material from suspects. In order to provide new investigative leads in cases where such reference samples are absent, forensic scientists started to explore the prediction of phenotypic traits from the DNA of the evidentiary sample. This paradigm change now uses DNA and epigenetic markers to forecast characteristics that are useful to triage further investigative work. So far, the best investigated externally visible characteristics are eye, hair and skin colour, as well as geographic ancestry and age. Information on the chronological age of a stain donor (or any sample donor) is elemental for forensic investigations in a number of aspects and has, therefore, been explored by researchers in some detail. Among different methodological approaches tested to date, the methylation-sensitive analysis of carefully selected DNA markers (CpG sites) has brought the most promising results by providing prediction accuracies of ±3–4 years, which can be comparable to, or even surpass those from, eyewitness reports. This mini-review puts recent developments in age estimation via (epi)genetic methods in the context of the requirements and goals of forensic genetics and highlights paths to follow in the future of forensic genomics.

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The First Quarter Century of Forensic DNA Fingerprinting

With the development of the famous "DNA fingerprinting" methods (restriction fragment length polymorphism), Alec Jeffreys provided the first molecular genetic tools to genetically differentiate between humans, which initiated the replacement of protein-based methods in forensic testing [1]. Earlier multi-locus DNA fingerprints were soon replaced by more specific single-locus DNA detection assays with simplified patterns that were easier to interpret. The increased differentiation relative to protein-based polymorphisms came with the large number of observed variable number of tandem repeat loci (also known as minisatellites), comprising alleles that contain repetitive nucleic elements of half a dozen to several thousand nucleotides per repeat motif. The overall DNA fragment sizes were relatively large (up to 20 kbp) and could therefore be analysed only from large amounts of high-quality DNA (in the order of 100 ng).

The discovery of simple repeat sequences, also known as short tandem repeats (STRs, microsatellites), again revolutionized the forensic field [2]. Their smaller repeat motif size (2–6 bp) resulted in shorter overall fragment lengths (<500 bp) that could be detected even in degraded (i.e., highly fragmented) DNA, which is often present in forensically relevant samples. More importantly, STR alleles could be amplified via the polymerase chain reaction (PCR), which increased the sensitivity of DNA fingerprinting by 3 orders of magnitude and allowed useful STR genotypes from as little as 100 pg (approximately the equivalent of 15 cells). Furthermore, multiple STR loci could be analysed simultaneously in so-called multiplex PCRs to save precious evidentiary samples and speed up the analytical process. At the same time, improved chemistry and detection devices allowed the parallel analysis of up to 30 STR loci with electrophoretic detection methods and, more recently, up to 200 or more loci including single nucleotide polymorphisms (SNPs) with massively parallel sequencing (MPS) technology. The combined probability of identity (that 2 random individuals show the same genotype) of modern STR multiplex kits, e.g., GlobalFiler (Thermo Fisher Scientific) or PowerPlex Fusion (Promega), regularly reaches levels below 10–24.

These developments led to an explosion in the number of crime scene samples amenable to DNA analysis and also allowed typing of samples invisible to the human eye, with the possibility of successful results. STR typing has also been used to identify unknown individuals by their skeletal remains, as this technology provides useful results with DNA extracted from bones and teeth [3]. To communicate results between laboratories and across borders, STR allele annotation conventions were based on the repeat number of representative, previously sequenced allelic categories. This led to the establishment of a highly discriminatory code that can be translated into a simple array of numbers and is easily and relatively unambiguously reported in expert opinions, exchanged between laboratories and stored in databases. This, in turn, triggered the development of intelligence-based criminal investigations: DNA fingerprints were not only used to confirm the authenticity of a perpetrator's sample found at the crime scene by direct comparison; but the same DNA fingerprint could also be used to search datasets of STR genotypes in an attempt to find a perpetrator that could not be identified by eyewitness or other evidence.

Finding the Perpetrator with Intelligence: Mass Screenings and National DNA Databases

One of the first applications of Jeffreys' DNA fingerprinting helped solve the murders of two 15-year old girls in 1987 [4]. The identical restriction fragment length polymorphism profile analysed in the semen stains that were found on both victims matched the one of Colin Pitchfork, who was later found guilty of the crimes. The two important elements characterizing this legendary case are the exoneration of an earlier suspect whose DNA fingerprint did not match the semen stains and a DNA screening test of more than 5,000 male individuals in the search for the perpetrator. It is one of the strongest features of DNA analysis that a suspect is excluded from being the donor of a sample when the respective DNA fingerprints do not match. In turn, DNA-based mass screens, where individuals matching investigative criteria are systematically invited to provide a reference sample, can be effective in finding the perpetrator when other evidence is missing. Although DNA mass screens are usually performed on a voluntary basis, their success rate is high, since close relatives of the actual perpetrator that participate in such testing regimes can often guide the investigations by so-called familial searches. The success of DNA mass screens led to the development of more systematic intelligence-led DNA investigations by comparing STR profiles from unsolved crime scenes to those of other crime scenes, suspects and convicted offenders.

STR profiles have been stored and searched in "national DNA (intelligence) databases," which are usually curated by the court or the police. The first national DNA database was inaugurated by the British Home Office in 1995, and the Austrian DNA database of the Ministry of the Interior was the second to become effective in 1997, in the same year as the Netherlands and Slovenia started their databasing projects. National DNA databases are effective tools to solve and prevent crime by creating new evidentiary leads in cases where matching STR profiles link crime scenes to suspects/convicted felons or to other crime scenes. Worldwide DNA databases may in the near future contain some 100 million STR profiles with China (55 M), Europe (>12 M) and the USA (15 M) contributing the largest proportions. Intelligence-led evidence to combat crime is used worldwide today, as typically between 1 in 2 and 1 in 5 profiles result in a match and, therefore, provide new investigative leads to solve a crime. The framework and administration of national DNA databases follow the legal policies of the country, and agreed conventions were put in place that regulate the exchange of profile information between countries, e.g., through the European Prüm treaty that regulates exchange of STR profiles between its member states.

Limitations of Confirmatory Forensic DNA Testing and New Avenues to Aid Solving Crime

Despite their enormous power of discrimination, STR profiles can only provide assistance in solving a crime when the genetic information of the perpetrator is available for comparison. Typically, suspects that come to the attention of the police are DNA tested in order to compare their STR profiles to the genotypes obtained from the crime scene samples. In cases without suspects, national DNA databases can provide new evidentiary leads by linking an individual to a crime scene based on matching STR data. In the remaining cases, highly informative STR profiles may be available from evidentiary samples, but due to a lack of links, they are not immediately useful. In an attempt to provide investigative leads of non-matching STR profiles, the suitability of STR genotypes to infer the geographic ancestry of the donor has been evaluated. Pereira et al. [5] developed PopAffiliator, an online calculator to individually assign a 17 autosomal STR profile to 1 of the 3 major population groups, Eurasian, East Asian and sub-Saharan African, with an accuracy of 86%. Later developments included new algorithms to improve the result, leading to a system that can provide useful guidance in cases where little is known about the provenance of the stain donor (http://cracs.fc.up.pt/~nf/popaffiliator2/). More precise inference of the geographic background of an unknown DNA sample has been achieved with the analysis of ancestry-informative SNP markers whose bi- or tri-allelic distributions across populations are indicative of continental-scale geographic backgrounds. With the emergence of MPS techniques, larger ancestry-informative SNP marker PCR multiplexes were developed that can, for example, be used to differentiate between 5 major sub-continental populations (Africa, Europe, East Asia, Native America and Oceania) using 128 markers [6].

Recent developments are investigating the use of microhaplotypes, short arrays of linked SNPs within DNA regions of <200 bp [7]. In this context, it is important to note that uni-parentally inherited markers (mitochondrial DNA and the non-recombining part of the Y-chromosome) contain phylogenetic information that can be used to infer the ancestries of the maternal/paternal background of a DNA sample. Such tests are increasingly used in forensic practice to reduce the pool of potential suspects and, thus, aid to triage police investigations.

Closely related to geographic ancestry is another promising forensic genetic avenue to provide investigative leads in criminal investigations: the inference of externally visible characteristics (EVCs) through DNA. This field is also known as "forensic DNA phenotyping" and aims to analyse DNA extracted from unknown crime scene stains to provide evidence for appearance prediction of the stain donor [8]. Much of the technological elements and statistical methods stem from investigations on inherited diseases in medical genetics, where causative genes are being identified, e.g., through genome-wide association studies, from which candidate markers are extracted and tested in forensically applicable tools. EVCs related to pigmentation were the first successfully applied forensic genetic markers for the prediction of eye, hair and skin colour, which may relate to the fact that they seem to be less genetically complex and less prone to environmental factors than other phenotypic traits, such as body height [8]. The societal, ethical and ethnological impact of a broader implementation of ancestry and EVC tests in forensic practice is currently under debate. Another, less intensively debated but equally important predictive application in forensic genetics is the estimation of the chronological age of a sample donor using DNA.

Markers and Methods to Estimate Human Chronological Age

The estimation of the chronological age of the donor of an unknown sample plays an important role in forensic investigations. It provides investigative leads in the search for an unknown perpetrator for various reasons: it is essential for human identification from skeletal remains; the applicability of law depends on the age of the person in question, and it can be critical in immigration cases where the identity and age of individuals are unclear. Previously established age classification schemes involved morphological analyses of skeletal features [9], which are still essential for the investigation of human remains; however, they are only applicable to forensic cases that involve solid tissues, like bones and teeth, while results from these tests can be ambiguous [10]. This limitation also applies to chemical methods, such as combined aspartic acid racemization and radiocarbon analyses, which provide precise age estimates [11] but require the presence of preferably dental specimens.

The main advantage of molecular genetic methods for age estimation is that they can principally be applied to any tissue containing DNA that also serves as a source for forensic DNA fingerprinting. Mitochondrial DNA was one of the first genetic markers to be investigated in this respect, as its nucleotide sequence is known to change in aging individuals, including a higher load of point heteroplasmies and an increasing number of large-scale deletions (e.g., [12]). The mechanisms are not yet well understood. There are technical difficulties, and the association of these modifications with age is only weak. In nuclear DNA, telomere sequences are known to shorten with every cell division; however, its application to age estimation does not seem to be straightforward. Earlier studies found large prediction errors (e.g., 22 years in [13]), while categorical approaches using age groups separated by 20 years found high prediction accuracies by quantifying particular T-cell-specific DNA rearrangements in blood (e.g., [14], mean absolute deviation [MAD] ±9.7 years). Nevertheless, more accurate techniques allowing a precise age estimate are desirable in forensic applications and would be severely limited with these techniques alone.

A promising method for more accurate age prediction arose from the field of epigenetics, where a significant change of global DNA methylation levels was observed to be associated with increasing age in epigenome-wide association studies [15]. The epigenetic code of an individual undergoes dynamic alteration during lifespan in response to environmental factors (in a broad sense) in order to modulate gene expression. Also, gene expression is known to correlate with human age [16], but both DNA methylation and mRNA genome-wide techniques to capture and quantify this information require large amounts of nucleic acids [17] that are usually not present in typical forensic specimens. In order to estimate age with these markers in forensic samples containing only small amounts of DNA (<100 ng), targeted mRNA and CpG site assays were developed, some of which are described in more detail below.

Based on genome-wide expression microarray data from 222 genes that are differentially expressed with age, Zubakov et al. [18] investigated a set of 9 mRNA markers, of which 8 (*NRCAM, ABLIM1, LRRN3, NELL2, NOG, CCR7, AK5* and *SLC16A10*) were down-regulated with age and 1 (CFH) was selected as the most age-correlated marker among all up-regulated genes. The authors performed a qPCR assay on DNA extracted from blood, normalized by 2 house-keeping genes (*ACTB* and *GAPDH*), and observed an overall smaller correlation with age (R^2: 0.041–0.46) compared to the initial microarray screening (R^2: 0.36–0.84). The most promising candidate was *NRCAM*, a gene that showed the highest age correlation in the screening dataset ($R^2 = 0.84$) and a reasonable age correlation in the validation dataset ($R^2 = 0.46$). The age prediction model based on all 9 mRNA markers resulted in an overall R^2 of 0.523 (MAD ±9.2 years).

Currently, the most commonly pursued approach for analysing CpG sites is sequence analysis of bisulfite-converted DNA, during which single-stranded genomic DNA is treated with sodium bisulfite that deaminates unmethylated cytosine to uracil, while methylated cytosine remains unaffected. With increasing age, not only genome-wide DNA hypomethylation has been observed but also regional DNA hypermethylation of CpG islands (e.g., [19]). A large body of human methylation pattern data has been established with the Illumina Infinium HumanMethylation450 BeadChip [20], which are often directly accessible from public databases that provide a rich resource for epigenetic studies to develop age-predictive tests. These data were based on human blood as source tissue, and the following studies based their findings also on blood (except where stated otherwise). Using such data, a quantitative prediction model was developed based on 71 markers that gave a correlation of 0.96 with the true chronological age with an average error of 3.9 years [21]. Bocklandt et al. [19] investigated the promoters of *EDARADD*, *TOM1L1* and *NPTX2* and found that 2 CpG markers alone explained 73% of the age variance and predicted the chronological age with an accuracy of

±5.2 years in saliva. In a total of 13 different cell types, Koch and Wagner [22] developed a model based on 5 CpG sites with a mean error of 9.3 years, whereas Garagnani et al. [23] described one of the most promising markers for age determination, *ELOVL2*, with a Spearman correlation coefficient of 0.92 in blood.

Weidner et al. [24] analysed 3 CpG sites in *IASPA*, *TGA2B* and *PDE4C* with pyrosequencing and found a strong correlation with age in blood (MAD ±5.4 years). Two CpG sites in *ELOVL2* were targeted by Zbiec-Piekarska et al. [25], which resulted in an MAD of ±5.03 years in blood. In a follow-up study, the same group was able to decrease the MAD to ±3.40 years by adding CpG sites in *C1orf132*, *FHL2*, *KLF14* and *TRIM59* [26], which is generally in concert with the findings of Park et al. [27], who reported an MAD of ±3.16 years in *CCDC102B*, *ELOVL2* and *ZBF423*. Zbiec-Piekarska et al. offer an open-access online tool for age estimation that is freely accessible (http://www.agecalculator.ies.krakow.pl).

Other forensically oriented studies started to appear that built upon the earlier reports, and some of these discovered additional markers to target specific CpG markers in blood [18, 25–34], saliva [35], semen [36] and teeth [28, 37]. Many of these studies investigated large enough sample sizes and also validated their developed models in independent cohorts to provide a more robust prediction tool that better describes the benefits and limitations of currently available markers and techniques.

Freire-Aradas et al. [29] investigated the methylation patterns of 177 CpG sites from 22 candidate genomic regions [20] in 725 individuals of European descent (18–104 years) and established a novel age prediction model based on a multivariate quantile regression analysis of the 7 highest age-correlated loci in *ELOVL2*, *PDE4C*, *FHL2*, *ASPA*, *CCDC102B*, *C1orf132* and *chr16:85395429*. They found a significant progression of DNA methylation increasing with age in *ELOVL2*, *PDE4C* and *FHL2* and reduced methylation in *ASPA*, *CCDC102B*, *C1orf132* and *chr16:85395429*. The predictive accuracy tests of the model yielded an MAD of ±3.1 years (the calculated percentage of prediction error relative to the age was 6.3%). The authors offer an open-access online tool that indicates the predicted age and intervals using the 7 markers (Snipper forensic classification website; http://mathgene.usc.es/cgi-bin/snps/processmethylation.cgi). This model was then independently tested in 46 monozygotic twin pairs (42–69 years), which produced 83.7% correct predictions with an MAD of ±4.2 years [29]. The authors observed outliers in 4 twin samples that did not fall within the values expected from the training set evaluations and discuss different tissue sources (cultivated cell lines instead of peripheral blood) as a potential reason. It was further demonstrated that linear regression models produce higher prediction intervals (MAD ±3.16 years) than quantile regression models do (MAD ±3.07 years) that adjust relative to age.

The above-mentioned studies used relatively large amounts of input DNA (200 ng to 5 μg) and Pyrosequencing, SNaPshot or EpiTYPER techniques that are generally known and successfully applied in the forensic genetic field, albeit not the mainstream technologies. With the emergence of MPS in the forensic community, more recent research is going to focus on this new method [38]. Based on a selection of age-informative CpG sites using publicly available methylation data from 1,156 whole blood samples (2–90 years), Vidaki et al. [31] applied stepwise regression for variable selection and identified 23 CpG sites that provided an age prediction accuracy of 0.92 (MAD ±4.6 years). The application of machine learning and a generalised regression neural network model improved the age prediction accuracy in their training dataset (MAD ±3.3 years) and a blind test study of 231 individuals (MAD ±4.4 years). The machine learning approach used 16 CpG sites with the top 3 age predictors found in genes *NHLRC1*, *SCGN* and *CSNK1D*. The model was further tested in 106 monozygotic twins (MAD ±7.1 years) and a cohort of 1,011 disease state individuals (MAD ±7.2 years). The markers and the model that were established in blood samples were tested in 265 saliva samples with high accuracy in the training set (MAD ±3.2 years) and blind study (MAD ±4.0 years). The authors developed an MPS-based assay to quantify the methylation status of the selected 16 CpG sites and found a lower prediction accuracy compared to the original model (MAD ±7.5 years), which is likely to be due to technical reasons. However, the authors for the first time demonstrated that age prediction could be offered in a technological format (MPS) that may become amenable to the modern forensic genetic laboratory workflow.

Forensic Relevance and Outlook

The forensically oriented methylation-based studies to estimate chronological age demonstrate that sequence analysis of bisulfite-converted DNA currently seems to be the most promising technique, albeit one that still requires higher DNA amounts than available in typical forensic samples. Methylation studies outside the forensic field use 200 ng and more input DNA, while a typical fo-

rensic sample contains <10 ng of DNA. Future research will need to focus on lowering the DNA input required for methylation analysis, and standardization of protocols and methods will be required to enable broader use of this technology in routine forensic work. One initiative that has these goals in mind (among others) is the European Union's Horizon 2020 Research and Innovation Programme project VISAGE (Visible Attributes through Genomics; www.visage-h2020.eu). The agreement on, and development of, a standard set of age-informative methylated markers is a logical consequence of the research trends in forensic genetics so far. Harmonization is key in this practical discipline and has been introduced at different levels of routine laboratory work, including DNA extraction, quantitation and downstream genotyping/haplotyping. The selection of core markers will require additional research to address questions that are still open. In that respect, it is still unclear, due to limited studies, how the best methylation markers will perform in different forensically relevant tissues, such as blood, epithelial cells, semen, hairs, bones and teeth; as different tissues and cell types are known to demonstrate different methylation patterns. Furthermore, detailed studies are needed to establish if methylation patterns remain stable over longer time periods (as suggested in [25]), as crime scene samples typically display different degrees of decomposition due to varying times and conditions prior to their collection. A number of forensic anthropological studies deal with the identification/genetic analysis of the remains of historic individuals that date back to (early) medieval times. Solid tissue samples of that age are known to display damage patterns that are useful to confirm the authenticity of the genetic result [39] but may pose difficulties for methylation analysis given that deamination processes act naturally in those ancient samples as well. Sex and broadly defined age groups are variables that may influence the prediction accuracy. While there are contradicting results reported for the effect of sex, most researchers agree that it is important to have improved estimates for the younger age groups, both infants and adolescents, which are more critical in legal decisions and in a large proportion of cases are forensically more relevant.

Most of the research thus far focussed on European/West Eurasian populations with some exceptions. The variability of methylation-based age estimation needs to be investigated on geographically broad and representative datasets in order to define the characteristics and limitations of such tests, as different populations have different health conditions, lifestyles and exposure to climatic and environmental factors that may affect the estimates. Studies have started to appear that analyse suggested marker sets and models developed in other populations, e.g., Cho et al. [40] have investigated CpG markers, proposed and originally tested in a Polish population sample [26], in a dataset of Koreans. The authors of this study report relatively consistent age prediction results of the earlier developed models, but some markers required adjustment with the new data. These findings confirm that forensic genetic laboratories need to individually evaluate and validate methods with their specific methodology and based on their relevant populations.

This closes the circle of the traditionally established procedure in forensic genetics to standardise and harmonise markers, techniques and interpretation methods as critical parameters for reliable genetic analyses. In turn, the forensic focus on finding and validating markers showing strong, robust and reproducible associations with chronological age across varied individuals may feed back into more fundamental research into the ageing process.

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