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FILIPA BARBOSA BASTOS

Ferramentas Epigenéticas Usadas para Estimativa de Idade

Epigenetic Tools in Age Estimations

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Epigenetic Tools in Age Estimations

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutor Luís Manuel Souto de Miranda, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro

Dedico este trabalho aos meus avós, que percorreram um longo caminho comigo sem poderem ver-me atravessar esta meta. Aos meus pais, que me apoiaram e a quem devo a oportunidade de frequentar mais um grau académico. Ao meu irmão e amigos por todo o apoio e confiança que depositaram em mim. Ao meu namorado que me ajudou em todos os contratempos, apoiou ao longo de todo o percurso e me deu força para continuar. À Dra. Ana Cristina Silva e equipa do Serviço de Patologia Clínica do CHEDV que me ajudaram sempre a conciliar os estudos com o trabalho.

Por fim, um agradecimento ao meu orientador, por todo o acompanhamento prestado a este trabalho e pela confiança depositada em mim.

o júri

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palavras-chave

Epigenética, metilação do DNA, dinucleótidos CpG, estimativa da idade, fenótipagem de DNA forense

resumo

O presente trabalho pretende explorar o estado atual das metodologias usadas para a estimativa da idade em contexto forense e através de marcadores epigenéticos, nomeadamente a metilação do DNA. A estimativa da idade é já um conceito conhecido e praticado nas ciências forenses, a partir de métodos dentários, ósseos e citológicos. A epigenética abriu novos horizontes para a estimativa de idade através de métodos moleculares, particularmente o estudo de alterações nos padrões de metilação do DNA. A metilação do DNA ocorre principalmente em citosinas que são seguidas de guaninas (dinucleótidos CpG) e que frequentemente se encontram em regiões ricas nestes dinucleótidos (ilhas CpG). Utilizando determinados dinucleótidos CpG como marcadores, é possível estimar a idade de uma pessoa através do padrão de metilação detetado. A principal aplicação desta metodologia é em casos onde uma amostra de um possível perpetuador é encontrada na cena do crime e esta amostra não tem correspondência em bases de dados nacionais de DNA. Nestes casos, reduzir os possíveis suspeitos àqueles que se inserem numa determinada faixa etária, em combinação com outras características externas visíveis que também podem ser previstas a partir da mesma amostra (ex. cor dos olhos e do cabelo), pode ser muito útil no decorrer de uma investigação. As metodologias para a estimativa da idade que têm por base padrões de metilação do DNA, apesar de serem uma melhoria às que já são utilizadas, tem limitações que devem ser abordadas antes da sua implementação como práticas forenses. Todas as questões éticas relativas a este tema ainda são motivo atual de discussão e a legislação, na maioria dos países, não existe.

keywords

Epigenetics, DNA methylation, CpG dinucleotides, age estimations, Forensic DNA Phenotyping.

abstract

This work seeks to explore the current state-of-the-art regarding the epigenetic methodologies used for Forensic Age Estimations, especially DNA methylation. Age estimation is an already known and practiced concept in the forensic field through dental, skeletal, and cytological methods. Epigenetics opened new horizons in this field with molecular methods for the study of changes in DNA methylation patterns. DNA methylation occurs in cytosines that are immediately followed by guanines (CpG dinucleotides) and frequently are within CpG rich regions (CpG islands). Using a selected set of CpGs as markers, it is possible to estimate someone's age through the detected methylation pattern. The major application of this technique is in cases where a sample from the perpetrator (or possible perpetrator) is found on the crime scene and this sample doesn't have a match on nation DNA databases. In these cases, limiting the pool of suspects to a certain age group, in combination with other externally visible traits that can be predicted from the sample (e.g. eye and hair color), could be very useful in the course of an investigation. Epigenetic age estimation tools, despite being an improvement to the currently used age estimation methodologies, have limitations that should be addressed before implementing these tools in forensic practices. All the ethical and societal issues surrounding this theme are a current motive for discussion and legislation relating to the use of these tools, in most countries, is inexistent.

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1. Epigenetics

1.1 Introduction

Epigenetics is the study of heritable alterations, not in the DNA sequence but in the regulation of gene expression. Every cell in the human body has nearly the same genome even though they have different structures and functions. This is possible due to epigenetic alterations that regulate gene expression in the different cell types in a way that each cell only translates the genes necessary to its function (NIH, 2020); the multitude of these alterations is called the epigenome.

C.H. Waddington, an embryologist, geneticist, and philosopher of science, introduced the notion of epigenetics in the 1940s, to describe the studies of the developmental processes that connect genotype with the phenotype (Waddington, 2012). Since then, there has been a growing interest in epigenetics in many fields. When searching for "Epigenetics" in the database SCOPUS, from 1956 and until 2020, there are over 76 000 results. Since the early 2000s, the number of published documents increased exponentially and the results are distributed by many areas,

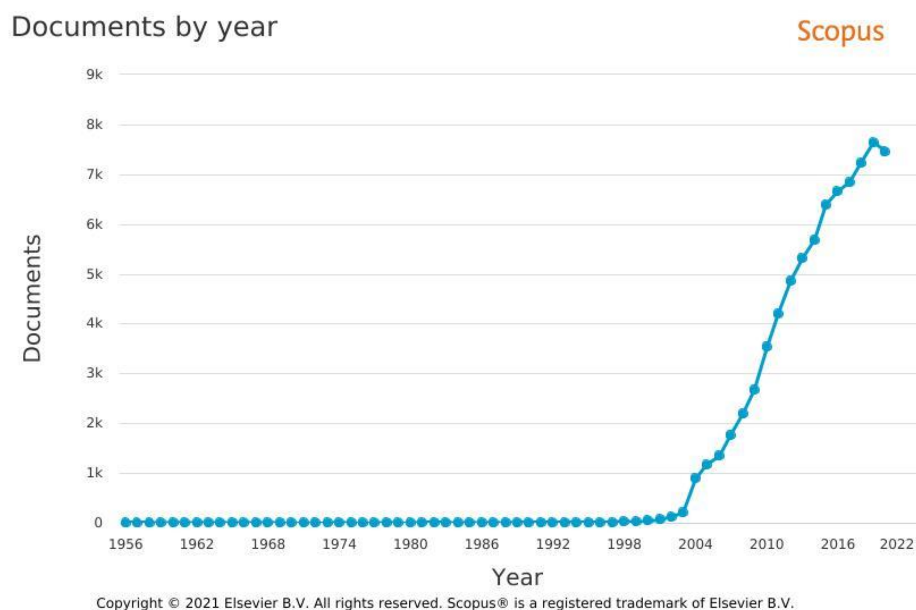

Figure 1: Graphical representation of the results obtained when searching "Epigenetics" in the SCOPUS database. This graphic represents the number of publications by year. Copyright © 2021 Elsevier B.V.

predominantly in “Biochemistry, Genetics, and Molecular Biology”, and “Medicine” with great focus on its association with cancer.

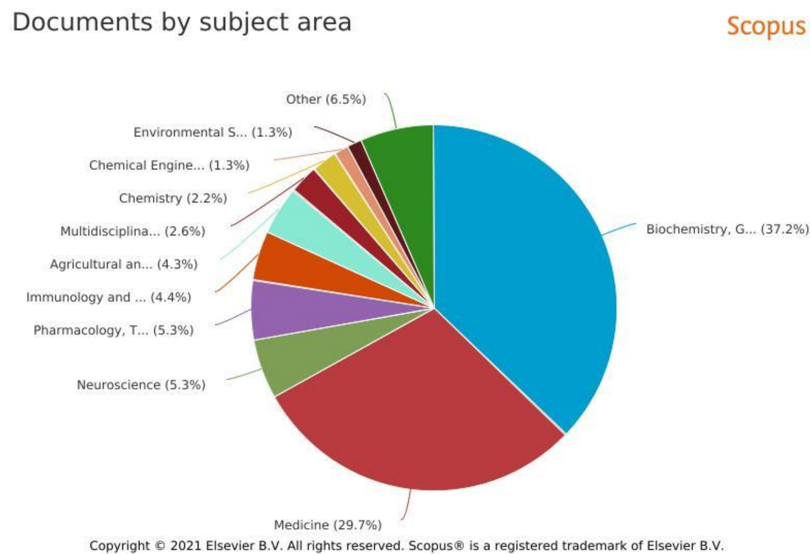

Figure 2: Pie chart of the results obtained when searching “Epigenetics” in the SCOPUS database divided by subject area. Copyright © 2021 Elsevier B.V.

Among the results are investigation articles. review articles, and book chapters on the relationship of epigenetics with depression, obesity, cardiovascular pathologies, sporting performance, cancer, autism spectrum disorder, and many others. These examples are regarding humans, but many others can be found regarding other animals, plants, and bacteria.

Forensic researchers also have a growing interest in epigenetics, especially in the study of DNA methylation patterns, that can help in a criminal investigation by giving clues on the appearance, age, and lifestyle of the person to whom a sample found on a crime scene belongs to (Sabeeha & Hasnain, 2019).

1.2 Epigenetic alterations

An epigenetic alteration is a change that occurs in the chemical structure of DNA without changing its sequence. These alterations are responsible for the regulation of gene expression, X-chromosome silencing, and imprinting of genes. Different types of cells and tissues require the expression of different genes, and these requirements are not constant throughout development. This means that epigenetic

alterations have to be highly dynamic to accommodate every cell's request and at any moment.

Here, we explore three categories of epigenetic modifications with a greater focus on DNA methylation due to its value in the forensic field, but also because it is a more widely studied modification and very present in mammals.

1.2.1 Histone modifications

The nuclear DNA in eukaryotic cells, if extended, would be a string of approximately 2 meters long. For this string to fit inside the nucleus of a cell, it has to be properly organized. Histones are proteins to which the DNA wraps around so that it is condensed enough to fit inside the cells. This pairing is called chromatin. Chromatin not only allows the packing of 2 meters of DNA in each cell but also regulates gene expression since this structure affects the accessibility of DNA to the proteins responsible for the reading and copying of the nucleotide sequence. Changes in this structure regulate gene expression.

Many post-translational histone modifications are known to affect chromatin structure, such as acetylation, methylation, phosphorylation, ubiquitylation, and sumolation. These modifications may occur in the different histones that compose an octamer and are involved in a variety of biological functions, such as transcriptional activation and silencing, DNA repair, DNA replication, histone deposition, inactivation of the X chromosome, mitosis, spermatogenesis, etc.

Histone modifications have been associated with cancer. For example, a change in the histone methylation patterns may lead to the silencing of tumor suppressor genes and be implicated in cancer progression (Sharma et al., 2009).

Throughout life, in general, histone levels decrease but histone modifications have a more complex correlation with age. Each modification has its implication, e.g., in modulating the expression of key genes related to longevity (S. J. Yi & Kim, 2020).

1.2.2 Non-coding RNA

Non-coding RNAs (hereinafter ncRNA) are RNA molecules that do not get translated to form a functional protein. Despite this, they play an important role in

many processes. They are divided into classes and include **tRNAs**, that recognize codons and recruit the amino acids to the mRNA sequence in the correct order, **rRNAs** that form the ribosomes, **snRNAs** involved in splicing events, **snoRNAs** that add chemical modifications to other RNA molecules, and long ncRNAs (**lncRNAs**) that play a crucial role in the regulation of gene expression, X-chromosome inactivation, imprinting and maintenance of nuclear architecture (Hombach & Kretz, 2016).

The relation of ncRNAs with aging still requires better understanding and more extensive studies, since most of the transcriptomic studies in the field of aging focus on protein-coding regions. Some age-related lncRNAs have been identified but the majority of these are specific to only one tissue. There are yet to be identified lncRNAs associated with the aging process in all or most of the tissues (Marttila et al., 2020).

1.2.3 DNA methylation

DNA methylation is the main epigenetic modification found in mammalian cells (Handy et al., 2011). It is the attachment of a methyl group at the C5 position of cytosines and the majority of these alterations occur within CpG dinucleotides (when a cytosine is followed by a guanine in the 5'→3' direction) . This epigenetic alteration is crucial in the silencing of retroviral elements, regulating gene expression in the different types of cells and tissues, genomic imprinting, and X chromosome

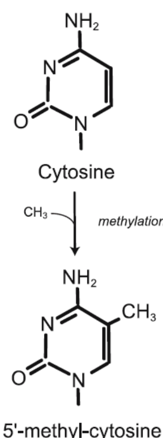

Figure 3: Methylation of cytosines. Adapted from Walsh & Xu, 2006

inactivation. The influence of DNA methylation on gene activity is related to the genomic region where it occurs (Moore et al., 2013).

CpG-rich regions in the human genome, called CpG islands, are very commonly associated with the regulatory or promoter regions of genes. These stretches of DNA are GC rich which is complementary to many transcription factors binding sites. Due to this complementarity, when un-methylated, CpG islands within promoter regions stimulate the binding of the transcription factors and thus promote gene expression. When methylated, CpG islands associated with promotor regions stably repress gene expression and establish imprinting (Moore et al., 2013).

DNA methylation at CpG dinucleotides silences gene expression by suppressing transcription through several mechanisms. One mechanism is the direct blockage of DNA recognition and binding of transcription factors due to the methyl group attached to the CpG dinucleotide. Another mechanism includes the preferential bind of other factors to the methyl group, therefore, blocking the access for transcription factors (Handy et al., 2011).

DNA methylation also occurs outside promotor regions. Tissue-specific methylation is more common and conserved in intragenic regions than in promoter regions. This suggests that methylation at intragenic regions may have a greater functional role in regulating tissue-specific transcription initiation than methylation at promoter regions. It has been shown that greater gene body methylation is correlated with higher gene expression (Maunakea et al., 2010). This seems to be true except for slowly dividing and nondividing cells such as brain cells (Moore et al., 2013).

On the frontal cortex of the mouse, methylation outside CpGs was negatively correlated to gene activity (Xie et al., 2012). DNA methylation of the first intron also showed a clear inverse relation to gene expression and this relation is conserved across vertebrates, genome-wide and tissue-independently (Anastasiadi et al., 2018).

DNA methyltransferases (DNAMTs) are the family of enzymes that catalyze DNA methylation during embryogenesis and development and its maintenance in daughter cells after replication (Handy et al., 2011). The pattern of DNA methylation

changes during development in a predictable manner and is different in the various tissues due to this family of enzymes (Walsh & Xu, 2006).

Many studies have focused on the relationship between DNA methylation and biological aging due to its value in health assessment, disease prevention, and forensic analysis (Hannum et al., 2013).

1.3 Epigenetics and the process of aging

The epigenome, in contrast to the genome, is highly dynamic and is constantly adapting to internal, external and stochastic factors. Throughout development and aging, changes in the epigenome accumulate and can therefore be correlated with age (Heyn et al., 2012). Some studies have focused on finding patterns amongst epigenetic modifications that can be associated with age.

The accumulation of epigenetic modifications can be observed when comparing the epigenome of younger versus older persons, with the latter showing less epigenetic similarities (Fraga et al., 2005; Hannum et al., 2013). Heyn and his colleagues compared the methylomes of T helper cells of the extreme points of human life: newborns and nonagenarians/centenarians. Their study corroborates the previous statement, with newborns having more homogenous methylation patterns amongst them, and also revealed a reduction of methylated CpGs in the older subjects (Heyn et al., 2012).

Monozygotic twins are great subjects to the study of epigenetic modifications since they have practically identical genomes but exhibit different phenotypes which can be explained by epigenetics. When comparing pairs of monozygotic twins, the youngest pairs are epigenetically more identical than the oldest who show clear distinctions. Also, the pairs who spend less time together and/or have a more different natural health/medical history show the greatest epigenetic differences, underlying the significant role of environmental factors in the translation of a genotype to a phenotype (Fraga et al., 2005). However, it should be pointed that, despite being more identical than older pairs of twins, young and healthy monozygotic twins still have different epigenomes (Planterose Jiménez et al., 2021).

Many aspects are associated with epigenetic changes, such as smoking, exposure to toxins, eating habits, and physical activity, and these alterations may or

not be reversible. For example, smoking affects DNA methylation patterns in a time and dose-dependent manner and can be reversed after quitting. It takes 15-19 years of low dose smoking for individuals to display a methylation pattern associated with smoking and less than a year after quitting for this pattern to change to a non-smoker pattern. Regarding heavy-dose smokers, it takes 5-9 years to display a smoker methylation pattern, and less than 2 years after quitting to return to a non-smoker pattern (McCartney et al., 2018). Zong and colleagues (Zong et al., 2019) recently reviewed a series of articles on this topic and outlined the reversible DNA methylation pattern of smokers after quitting for most differentially methylated CpGs and also the irreversible damage of smoking for some genes.

The amount of evidence that correlates age and epigenetic changes is astonishing (e.g. B. C. Christensen et al., 2009; Rakyan et al., 2010) and brought great advances in many fields. For example, in the medical field for the study of the impact of diseases in the aging process (Horvath et al., 2014, 2015, 2016). In the forensic context, this correlation is particularly important since it allows for age predictions and will be further explored in the following chapters.

2. Age estimation

2.1 Introduction

Age can be measured in two different ways: chronological and biological age. Chronological age, by definition, is the amount of time that has elapsed since birth and is the measure of time commonly used. For biological age, there isn't exactly a definition, but the notion that individuals age differently is well known. Biological age tries to explain this notion. It measures the complex biological process of aging that is unique to every person and in a subjective manner (Jackson et al., 2003). In the forensic context, accurately predicting chronological age is preferred over biological age, since what is being searched is a donor external trait. In other contexts, for example, for medical purposes, biological age is preferred since it can give information on the patients' disease risk and mortality (Field et al., 2018), or for the evaluation of the impact of clinical trials on mortality rates (Horvath et al., 2018).

Predicting someone's age from a biological sample can be of great use, both for living persons and human remains, or even for cultured tissues.

Age estimation tools may be used for human identification from remains, aiding in building a biological profile to compare with missing persons (Cunha et al., 2009; Parson, 2018). When an individual lacks valid identification documents, these tools can be used to help in attributing a sentence, since the law is applicable according to age. This is particularly important in migration cases since minors have better care, an increased chance of being granted asylum, and can be accompanied by family members which may lead young adults to pose as minors to benefit from these conditions (Abbott, 2018; Parson, 2018).

If the perpetrator is a minor, he or she may not fully understand the consequences of their actions and must be judged accordingly. Age estimation tools may help in attributing imputability or imputableness to an individual. Determining someone's age may also be useful to assure that an individual is not trying to pose as older to benefit from a pension.

Age estimation can also be required by a court in cases of adoption when a minor's age is not ascertained (Cunha et al., 2009), for eligibility and group assignment in sports competitions (Shi et al., 2018) and cases of child labor or minor's sexual exploitation (Balla et al., 2019)

Age estimation tools can also aid in research studies, e.g., for studying anti-aging properties of compounds on cultured cells, reducing the need to perform such studies on humans (Horvath et al., 2018).

Another great use of age estimation tools is in cases where the Short Tandem Repeat (STR) profile of a sample found on a crime scene doesn't match the profiles of the suspects or other profiles found in DNA databases; this means that there are no leads on whom the perpetrator is and estimating his or her age reduces the pool of possible suspects to a certain age group which is very useful in the course the investigation (Cunha et al., 2009; Parson, 2018).

Several methods for age estimations of living persons have been approved, such as the *Study Group on Forensic Age Diagnostics*' method that consists of a physical exam and X-ray examination of the left hand, dentition, and clavicle (Schmeling et al., 2008). For juvenile age estimation, limitations to these methods

have been found due to some differences in skeletal growth patterns (Zhang et al., 2009) and wisdom tooth mineralization in children belonging to different ethnical groups (Olze et al., 2007).

For age estimation in human remains, various dental and skeletal methods have been proposed. Skeletal age estimation relies on the morphological quantification made by the anthropologist which introduces error and the results are often a wide age interval that is accurate but lacks precision. For example, in a murder investigation, after several methods were applied, the estimated age of the victim was reported to be between 23 to 35 years of age and it was later found that the individual was 26 years old (A. M. Christensen et al., 2014). Bone development is susceptible to physical loading, nutrition, and health status and is variable from individual to individual. For living individuals or intact bodies, the skeletal assessment is accomplished using radiography which may present a different appearance when compared to the actual bone examination (A. M. Christensen et al., 2014).

Dental methods for age estimation require the initial placement of the individual into one of two categories. The juvenile category includes all stages from embryonic development until adolescence and the adult category includes every stage after adolescence. Attributing a category may not be easy, particularly when the individual is a young adult. The sex and ancestry of the individual (A. M. Christensen et al., 2014; Olze et al., 2007) are factors that influence age estimation and are often unknown which may pose limitations to the dental methods.

Histological age estimating methods have also been proposed, with utility especially in fragmented human bones from remains, however, the majority of histological methods rely on determining the osteon population density (OPD). There is a maximum value that can be reached using this technique and after the maximum is reached, it is only possible to assert that the individual is older than a certain age. Also, the maximum ODP happens at different times in different bones and is affected by age, sex, ancestry, physical activity, nutrition, and health. Finally, histological methods are destructive and should only be considered after less destructive methods (A. M. Christensen et al., 2014).

All these methods have their limitations, lack standardization and consensus concerning which method should be used (Cunha et al., 2009), present ambiguous results (Parson, 2018), perform better when certain aspects of the individual are known (such as gender, ethnicity or pathological conditions), and require a body or fragments of a body. Some methods can only indicate if the individual was young or old (A. M. Christensen et al., 2014)

The use of molecular genetic methods for age estimation brings the advantage that they can be applied to any tissue containing DNA and can be used for living persons and human remains (Parson, 2018).

Molecular methods for age prediction include leukocyte telomere length, analysis of age-dependent deletions of mitochondrial DNA, T-Cells DNA rearrangements, and protein alterations, but all these biomarkers have low precision and practical limitations.

Telomeres are repetitive sequences found at the end of chromosomes that shorten with each DNA replication event. Age is correlated to the relative size of telomers and is proposed as an age estimation tool (Márquez-Ruiz et al., 2018). The methods based on telomere shortening estimate biological age with an error between estimated and actual age of 7 to 22 years. A recent study used quantitative polymerase chain reaction (qPCR) for determining telomere length estimated age with an error of 13,8 years (Márquez-Ruiz et al., 2018). This method requires small amounts of DNA, is not very time-consuming, and has high throughput, simplicity, robustness, and reproducibility. Despite these advantages, methods based on telomere shortening are influenced by the environmental factors to which the sample has been exposed and the cause of death, making this technique unsuitable in the forensic field (Zapico et al., 2019).

During the process of aging, mitochondrial DNA accumulates mutations and this damage can be measured to estimate age. With the use of qPCR, mtDNA damage methods for age estimation quantify the damage (mutations) in mtDNA and correlate it with age. (Zapico & Ubelaker, 2016). Mitochondrial DNA methods showed a good correlation with age, can be applied to multiple tissues, and require low amounts of tissue. In comparison to other anthropological approaches for age estimation, it is more accurate, simple, and affordable (Zapico & Ubelaker, 2016). Despite being

one of the first molecular markers studied for age estimation purposes, there is still a shortage of studies that strengthens the correlation between mtDNA damage and age (Parson, 2018). This method also presents populational differences in the amount of damage found in mtDNA (Zapico & Ubelaker, 2016).

Another method currently being studied for age estimation relates the decline of signal joint T-cell receptor rearrangement excision circles (sjTRECs) to increasing age. This method uses qPCR and can be applied to blood (Ou et al., 2011) and bloodstains (Ou et al., 2012), which are very common types of samples found in the forensic field. This method predicts age with an error between actual and predicted age of approximately 10 years and is not influenced by sex. Although blood is a very common forensic sample, other types of samples are found in this field and only blood can be used to predict age using this method (Ou et al., 2012; Ou et al., 2011).

Covering all available methods for age estimation and their specifications is not the main goal of this work. All the methods here mentioned are just a few examples and can be consulted in a simplified table format in the Supplementary Material.

Epigenetics opened new horizons to age predictions using molecular methods. When comparing the various types of biomarkers used for age predictions (mRNA, DNA methylation, sjTREC, mtDNA, and telomere length), DNA methylation showed the highest correlation to age (Weidner et al., 2014; Zubakov et al., 2016). This technique showed to be an improvement in terms of accuracy to the previously mentioned age estimation methods, with a Mean Absolute Error (MAE) of only 3,4 years between estimated and chronological age (Woźniak et al., 2021).

Despite being advantageous, DNA methylation methods for age predictions have their limitations. The *VISible Attributes through GEnomics* (VISAGE) Consortium aims to overcome these limitations and the overall limitations of the use of Forensic DNA Phenotyping (FDP) in providing information on the appearance, age, and ancestry of a person in the forensic field. Among other objectives, the VISAGE Consortium groups and validates data and prototype tools on the DNA predictors of Externally Visible Characteristics (EVCs) and identifies and analyses the associated legal, ethical, and social aspects of these tools. Of interest to this work, the VISAGE Consortium published scientific works on age estimation tools using DNA methylation markers.

2.2 DNA methylation and age estimation

Epigenetics brought a promising tool for age prediction with DNA methylation markers. There is cumulative evidence that the DNA methylation levels are associated with age since it undergoes dynamic alterations during the lifetime (Parson, 2018; Weidner & Wagner, 2014; S. H. Yi et al., 2014).

Many studies have focused on finding age-related CpGs that can be used for age estimations through their methylation patterns. In 2014, Weidner and colleagues (Weidner et al., 2014) proposed an age estimation model using only 3 CpGs and pyrosequencing technology. This method used blood samples, but it was also demonstrated on other tissues with similar results.

A great advantage of age estimation tools based on methylation profiles compared to other molecular methods is that it has been shown that the methylation profiles are not significantly altered by different storage conditions (Li et al., 2018; Zbieć-Piekarska, Spólnicka, Kupiec, Makowska, et al., 2015) and in a post-mortem interval of under 24 hours (Sukawutthiya et al., 2021), the latter being of critical importance in the forensic field. Another study has also suggested that the methylation status of samples collected from human remains within 10 days of the time of death has no statistical difference from samples collected on living persons (Hamano et al., 2016).

Age estimations may also play a role in complementing the prediction of other EVCs, such as hair color, skin pigmentation, or baldness pattern in males since these characteristics are influenced by age. Complementing age with other EVC predictions can aid in building a sketch of the sample's donor (Zbieć-Piekarska, Spólnicka, Kupiec, Parys-Proszek, et al., 2015) .

2.3 Challenges in building an age predicting model

The first challenge in building an age predicting model is "where?". DNA is filled with information and researchers from many fields work in translating that information into something useful. To build an age-predicting model, researchers first search the DNA to find the markers that can provide information about a

person’s age. A genome-wide study performed on pairs of monozygotic twins, with ages between 21 and 55 years, quantified the methylation status of 27 578 CpGs in saliva samples and found a strong correlation between the methylation status of 88 CpGs and age (Bocklandt et al., 2011). It is important to note that these CpGs lay near genes involved in cardiovascular, neurological, and genetic diseases. It makes sense that CpGs that can be used for age estimation lay near or within genes involved in aging-related conditions and several models use CpGs following this logic. For example, Hannum and colleagues proposed an age predicting model with several markers involved in Alzheimer’s disease, obesity, and metabolism (Hannum et al., 2013).

In 2012, the ELOVL2 gene was proposed as an epigenetic marker of age (Garagnani et al., 2012). This study reported that CpG islands of the ELOVL2 gene range from being almost entirely unmethylated in the early stages of life, to being almost completely methylated in the latter stages. To express this in numbers, the study had subjects with ages comprised between 9 and 99 years, and the percentage of methylation ranged from 7 to 91% in a linear age-related manner (Garagnani et al., 2012). A single CpG site within this gene explains 83% of age variance (Zbieć-Piekarska, Spólnicka, Kupiec, Parys-Proszek, et al., 2015).

Apart from the ELOVL2 gene, various other genes have CpGs that are highly correlated with age and by combining them, very accurate models can be achieved. For example, a model proposed by Zbieć-Piekarska and her colleagues includes CpGs within the genes ELOVL2, MIR29B2C (previously denominated C1orf132), TRIM59, KLF14 e FHL2 and explains 95.3% of age variance with an MAE of 4 years (Zbieć-Piekarska, Spólnicka, Kupiec, Parys-Proszek, et al., 2015).

The VISAGE Consortium evaluated a basic prototype tool for age estimation that targets 32 CpG sites located at five genes, ELOVL2, MIR29B2C, FHL2, TRIM59, and KLF14 (Heidegger et al., 2020) based on other studies that found these markers to be strong age predictors. Later, they developed an enhanced tool for age prediction that uses bisulfite multiplex PCR and Massively Parallel Sequencing. They also proposed three new statistical models to predict age in blood, buccal cells, and bones. This tool targets 44 CpGs from 8 DNA methylation markers and by using different combinations of these markers in the appropriate

statistical model it is possible to predict age with MAE of 3,2 years for blood, 3,7 for buccal cells, and 3,4 for bone (Woźniak et al., 2021).

A wide variety of CpGs have been related to age and have been used in age predicting models. Using all available markers may seem attractive, but there are some factors to be considered.

A good question to answer about the candidate markers is: are they good predictors in the different tissues and cell types? Most age predicting models are built based on blood samples, but a forensically relevant sample can be of any type of human biological material. Cell or tissue-type methylation specificity has been shown for some CpGs and it is of great relevance in the context of forensic practice to find CpGs that can be good predictors of age across different tissues.

Among others, a relevant sample in the forensic context could be of unknown or mixed origin, which means that a model based and validated for a single tissue cannot be properly introduced in the routine of a forensic lab. Some studies have addressed this problem, focussing their attention on building models with markers that can be used to predict age in a variety of tissues and cell types (Horvath, 2013; Koch & Wagner, 2011; Woźniak et al., 2021). Particularly, the VISAGE Consortium's enhanced tool for age estimation predicted age in blood, buccal cells, and bone with great accuracy (MAE of 3,2 – 3,7 years). However, it has been previously shown that models that accurately predict age in various tissues don't have a predictive value in semen (H. Y. Lee et al., 2015), which is a relevant type of forensic sample.

Some markers used in the VISAGE enhanced tool for age predictions (Woźniak et al., 2021) were found to have altered DNA methylation patterns that could influence age prediction in alcohol abusers (Piniewska-Róg et al., 2021). When analyzing a forensic sample of unknown origin, it is important to make sure that the results can't be affected by factors unknown to the investigation (such as alcohol consumption of the sample donor).

Another problem is imposed now. Is the model technically feasible in the forensic context?

As mentioned before, using all available markers could seem to lead to a more accurate model. However, the more CpGs a model uses, the greater the amount of biological material required as input. There are some characteristics of forensic

samples (see section 2.4) that need to be considered, namely the amount of sample that is found on the crime scene and degradation of the sample.

2.4 What influences age estimations?

The complexity of the genetics of age

Aging is a complex multifactorial process that varies among individuals and even among the different body tissues. Genetic and epigenetic factors are involved in the aging process and are influenced by genes, environmental and stochastic factors. The contribution of each factor and the combination of factors is still to be determined (Rodríguez-Rodero et al., 2011). Epigenetic markers are being used for age estimations and the identification and validation of the markers that better correlate with age is an ongoing process.

Sex

There isn't a consensus on the influence of sex on epigenetic age predictions.

Some studies demonstrated that sex contributes to the aging rate, with men's methylome aging faster than woman's and leading to men having a higher predicted age (Hannum et al., 2013; Simpkin et al., 2016; Weidner et al., 2014). This trend strengthens over the life course but the difference was found even in 7 years-old children (Simpkin et al., 2016). In many studies, however, sex did not significantly influence age prediction (Bekaert et al., 2015; Correia Dias et al., 2020; Freire-aradas et al., 2016; Koch & Wagner, 2011; Zbieć-Piekarska, Spólnicka, Kupiec, Parys-Proszek, et al., 2015). This discrepancy may be related to the method and markers used; when using a broader approach such as methylome studies, sex does appear to affect age prediction, but when using a smaller set of markers, such as a group of age-informative CpGs, the results of age estimations don't seem to vary significantly between sexes.

Lifestyle choices

The methylome is dynamic and changes over time in response to the surrounding environment. Everybody makes different choices and is exposed to

different environments throughout life, and all these factors will influence the methylome thus affecting age estimations.

Epigenetic age in children is influenced by the mother's lifestyle before and during pregnancy. Maternal alcohol consumption (Simpkin et al., 2016) and smoking (Richmond et al., 2015) during pregnancy and the mother's weight (obesity and underweight) (Sharp et al., 2015) were found to influence the epigenome of the offspring during development.

Alcohol abuse alters DNA methylation patterns at some markers used for age prediction (Piniewska-Róg et al., 2021; Weidner & Wagner, 2014). This means that alcohol consumption may interfere with age estimations and exacerbates the impact that environmental factors can have on specific markers (Piniewska-Róg et al., 2021).

Diseases

Some diseases have been shown to significantly impact age estimations, particularly diseases that have been associated with accelerated aging or reduced lifespan. These diseases include obesity (Horvath et al., 2014), osteoarthritis (Vidal et al., 2016), Huntington's disease (Horvath et al., 2016), Down Syndrome (Horvath et al., 2015).

Forensic samples

Forensic samples are unique. They can be found on any type of substrate and belong to any type of biological material, are exposed to different environmental conditions, and may come in very little quantities. Also, they are mixed with PCR inhibitors, prone to degradation, and can be found within a few hours, days, or years from the moment it was left on the crime scene (S. B. Lee & Shewale, 2017).

To test the forensic utility of samples obtained from bloodstains and kept at room conditions for 5, 10, and 15 years old, Zbieć-Piekarska and her colleagues (Zbieć-Piekarska, Spólnicka, Kupiec, Makowska, et al., 2015) proceeded to estimate the age of the individuals to whom those samples belong and whose actual age was known. Not all 10- and 15- years old samples could be amplified by PCR due to DNA degradation. Despite this, the samples with positive PCR amplification had a

percentage of correct predictions similar to those collected and processed immediately.

DNA methylation patterns are different between tissues, so when a forensic sample is in hands, finding the tissue of origin may be crucial to the age estimation and other tests. A recent study performed on blood, buccal cells, and bone used a smaller set of CpGs that are informative across different tissues. This seems to be the solution for applying DNA methylation age estimation tools in routine forensic practice (Woźniak et al., 2021).

Most studies published so far in the forensic field focused on somatic cells, mainly blood, but other types of cells and tissues are found in crime scenes or other forensic contexts. Semen samples are frequently used in forensic practices, particularly in sexual assault cases. The VISAGE Consortium recently published a study on the prediction of age in semen samples and found this to be a more complex method compared to age prediction in other tissues (Pisarek et al., 2021). Differentially methylated sites in semen don't have a powerful individual predictive power. The markers used for age prediction in blood can explain between 95,2% to 98,2% of the variation in age (Woźniak et al., 2021) whilst the markers used for semen only explain 35% to 60% (Pisarek et al., 2021). Since individually each marker used for age prediction in semen is only moderately correlated with age, using more markers it is possible to build an accurate model. Jenkins et al. (Jenkins et al., 2018) built a model that uses 51 CpGs and predicts age with MAE of only 2 years whilst the VISAGE model for age prediction in semen only uses 6 CpGs and predicts age with MAE of 5 years (Pisarek et al., 2021). However, using more CpGs in a model requires higher DNA quantity and quality which, in the forensic field, is difficult to control.

A major problem found in the forensic field is the quantity of sample available. Relevant samples are frequently a drop of blood or a few hair strands where the amount of DNA that can be extracted is very limited. Bisulfite conversion is a standard tool for methylation analyses, but it leads to inferior results when the DNA input is smaller due to DNA loss during this process. This means that erroneous methylation quantification, and therefore, less accurate age prediction may be a result of the low number of DNA molecules used for this purpose (Heidegger et al.,

2020; Vidaki & Kayser, 2018). In conclusion, samples usually found in the forensic context may not have the necessary quantity and quality to perform the methods used for age estimation.

Funding limitations

The study of the genetics of diseases uses the same technologies and statistical methods as the study of Externally Visible Characteristics (EVC), including age estimations, but is far more advanced. This is in part due to research funding that is, typically, more focused on disease-related genetic variations. To find the markers that correlate with age (and other ECVs) it is required to study a large number of individuals and the genomic tools to carry this mission, such as SNPs microarrays or Genome-Wide Association Studies are still very expensive (Kayser, 2015).

Biogeographic ancestry

It has been suggested that the methylation status of some markers may be population-specific. In a recent study, Dias and her colleagues replicated a study performed on individuals from a Korean population (Jung et al., 2018) in samples from Portuguese people. By applying the Korean model on Portuguese individuals, differences in specific markers between the two populations were found, e.g., the CpG in the gene ELOVL2 showed the strongest correlation in Portuguese individuals, but for the Koreans, the strongest correlation was found on the CpG at the FHL2 gene. The CpG with the lowest correlation was found at the KLF14 gene for the Portuguese and at the C1orf132 gene for the Koreans. The statistical model used for age prediction of the Portuguese individuals suffered some alterations from the original model which allowed for an accurate age prediction (MAD=4.25) but, when the methylation data from the Portuguese individuals was applied to the Korean algorithm, the results were strongly less accurate (MAD=15.26) (Dias, Cordeiro, et al., 2020).

Not many age estimation studies have focused on the population differences in methylation patterns since most only use a sample set from one populational group. As seen in the previously mentioned study (Dias, Cordeiro, et al., 2020), these differences may influence the accuracy of the predicted age.

Finally, most studies on age estimations up to now have been performed on European and West Eurasian populations (Parson, 2018) and since different populations experience different environmental conditions, health care, and lifestyles, future studies should include populations from different geographic backgrounds.

Chronological age

As mentioned before, the methylome, as opposed to the genome, is not static and accumulates changes with time. This means that older individuals tend to have a greater discrepancy in their methylomes compared to younger individuals, and this is reflected in age-estimation studies.

It is often found that older individuals (over 40 years of age) have a higher Mean Average Error and percentage of incorrect age predictions (Dias, Cordeiro, et al., 2020; Dias, Cunha, et al., 2020; Hamano et al., 2016; Zbieć-Piekarska, Spólnicka, Kupiec, Parys-Proszek, et al., 2015). Overwhelming evidence of this can be found in studies involving monozygotic twins, where younger pairs of twins appear to be epigenetically more similar than older pairs of twins, who show more significant differences in methylation and acetylation patterns (Fraga et al., 2005).

Epigenetic drift

Epigenetic drift is a result of errors in the maintenance of the epigenome and is considered a hallmark of aging. These errors can result in changes in gene expression and lead to age-related phenotypes (Mendelsohn & Larrick, 2017). Using the example of monozygotic twins, who share the same genome and, in early ages, the same epigenome, epigenetic drift can explain the different phenotypes presented throughout life. In the early stages of life, if a pair of MZ twins share the same genotype, epigenotype and grow up exposed to the same external factors, what explains different disease outcomes and other age-related phenotypes of these twins? As errors in maintenance of the epigenome are random and accumulate with time, the epigenomes of MZ twin pairs start to depart from each other (Fraga et al., 2005). Considering this, it is safe to assume that epigenetic drift

may influence the estimation of age through DNA methylation since random errors on the used markers may occur.

3. Methods used for Age Estimations through Methylation Analysis

3.1 DNA purification

There are two types of forensic biological evidence: single-source samples and evidence samples. The first refers to samples collected from a single known donor, for example, for paternity testing, DNA database generation, reference samples in casework, and relationship testing. They contain an abundant quantity of biological material, are collected and stored in relatively controlled environments, are less likely to be degraded, and contain minimal inhibitors of PCR. Evidence samples are collected at the crime scene and can be any type of biological material. They can be deposited on various types of substrates, are often mixed with PCR inhibitors, exposed to variable environmental conditions, and in limited quantities (S. B. Lee & Shewale, 2017).

DNA purification is the method by which the cells in a sample are lysate so the DNA and other nucleic acids can be isolated from contaminants.

Purifying DNA from forensic samples is challenged by the characteristics of the sample mentioned above. Therefore, finding the most suitable method that will lead to a maximum amount of purified DNA extracted from the sample is a step with great impact in many downstream analyses such as STR profiling and FDP. Some commercial kits were validated for a variety of tissues and body fluids, such as the QIAamp DNA Blood Maxi Kit (QIAGEN) used in the VISAGE's age estimation tools (Heidegger et al., 2020; Woźniak et al., 2021). The VISAGE model developed for age estimation in semen uses the Sherlock AX Kits (A&A Biotechnology) for DNA purification (Pisarek et al., 2021), and this kit can also be used for blood and saliva stains, hair, preserved and fresh tissues, and frozen and fresh blood.

3.2 Bisulfite Conversion (BC)

Methylation of cytosine occurs at the 5' position of the pyrimidine ring. This is a very common post-replicative modification of DNA, mostly found in CpG dinucleotides, and is involved in the regulation of gene expression (Walsh & Xu, 2006).

Bisulfite conversion is a method used to distinguish cytosines from methylated cytosines, which is needed in methylation studies. The bisulfite deaminates the cytosines causing its chemical conversion to uracil, leaving the methylated cytosines intact. The deamination of the methylated cytosines is much slower and, in the duration of this treatment, doesn't occur. After the treatment, the converted DNA can be used in other molecular biology techniques, such as PCR and sequencing, and the methylated cytosines will be detected since the unmethylated ones were converted to uracil.

A major advantage of this technique is that it provides information on the methylation status of every cytosine examined. Compared to other conversion methods, bisulfite conversion is less time-consuming and reduces the possibility of contamination and human error because it has fewer tube changing steps (Hernández et al., 2013).

Some obstacles with this technique have also been identified; the deamination of methylated cytosines may occur and results in the conversion of the cytosine into thymine, which cannot be distinguished from an unmethylated cytosine that has been converted to uracil (adenine will bind to both). Low deamination efficiency of the unmethylated cytosines may be caused by the oxidation of the bisulfite, incomplete strand separation, protein residues after DNA extraction, or the use of too much DNA in BC (may lead to re-annealing of complementary sequences) (Darst et al., 2010). DNA loss and degradation may be a result of DNA fragmentation during the bisulfite treatment (Hernández et al., 2013).

It is important to use controls for the process of bisulfite conversion to validate the use of the converted DNA in other downstream analyses. These controls are used to evaluate the quality of the converted DNA, the efficiency of conversion, and the quantity of DNA remaining (Hernández et al., 2013).

Several commercially available kits were tested for the basic prototype tool developed by Heidegger et al. (Heidegger et al., 2020). The EZ DNA Methylation-Direct™ Kit (Zymo Research) and the Premium Bisulfite kit (Diagenode) were the kits that yielded better concentrations of DNA even for low DNA input (500 pg) and had great conversion efficacy (Heidegger et al., 2020). In the VISAGE enhanced tool for age estimation (Woźniak et al., 2021), bisulfite conversion is performed using the Premium Bisulfite kit, and in the model developed for age estimation in semen (Pisarek et al., 2021), bisulfite conversion is performed using the EZ DNA Methylation Direct™ Kit.

3.3 Multiplex PCR

Multiplex PCR is a widely used molecular biology technique that allows for the amplification of several targets in a single PCR reaction. Developing a multiplex technique capable of analyzing a large set of markers from low quality and quantity of DNA input is a goal for the progress of forensic genetics and epigenetics.

The use of multiplex PCR for bisulfite-converted samples is limited by the quality and quantity of DNA obtained after the treatment. Since DNA fragmentation and lower complexity sequences are common results of the conversion, non-specific primer binding and formation of dimers occur during the multiplex PCR. At the moment, DNA input of 20 ng for BC samples is the lowest quantity that allows a robust quantification of DNA methylation of the sample (Heidegger et al., 2020).

The Multiplex PCR Kit (QIAGEN) is used in the VISAGE basic tool (Heidegger et al., 2020) and enhanced tool (Woźniak et al., 2021) in combination with primers designed in other studies for age estimations (Weidner et al., 2014; Zbieć-Piekarska, Spólnicka, Kupiec, Makowska, et al., 2015).

3.4 Pyrosequencing

Pyrosequencing is a sequencing-by-synthesis method that consists of the release of a Pyrophosphate (PP_i) molecule whenever a nucleotide is incorporated by a DNA polymerase in the template DNA strand. Nucleotides are released in the

reaction one at a time, so that, if the nucleotide is incorporated, the PP_i molecule is released and quantitatively converted into a bioluminometric signal.

Shortly, pyrosequencing can be divided into 7 steps: **1) assay design**, a crucial step for a successful and accurate quantitative analysis, and includes the design of PCR and pyrosequencing primers; **2) template preparation,** consisting of DNA extraction, determination of DNA concentration and the bisulfite conversion; **3) PCR amplification** of the target region; **4) pyrosequencing template preparation,** where PCR products undergo streptavidin-coated sepharose beads and alkali treatment to obtain single-strand DNA molecules. This step also removes salts that may influence subsequent enzymatic reactions; **5) annealing of the pyrosequencing primer** several bases upstream of the site of interest; **6) pyrosequencing** and finally **7) data analyses.**

This technique uses PCR products amplified from bisulfite-treated DNA and combines the ability of direct quantitative sequencing, reproducibility, speed, and ease-of-use. It can analyze the DNA methylation in CpG rich and poor regions and is a reference method used for the validation of newly developed methods in this field. Several epigenetic age estimation clocks use pyrosequencing (Bekaert et al., 2015; Cho et al., 2017; Weidner & Wagner, 2014; Zbieć-Piekarska, Spólnicka, Kupiec, Makowska, et al., 2015; Zbieć-Piekarska, Spólnicka, Kupiec, Parys-Proszek, et al., 2015).

3.5 Single-base extension reaction

Traditional methylation quantification methods involve cloning and sequencing of a high number of sequences per individual after bisulfite conversion and PCR. These approaches can be very arduous and time-consuming. Methylation-sensitive single nucleotide primer extension is a more efficient method developed to quickly and accurately quantify DNA methylation levels of bisulfite-treated samples.

This method is based on the repeated annealing of primers exactly one base-pair upstream of the target CpGs and the extension of the primer consists of the incorporation of a single fluorescent dideoxynucleotide. The proportion of fluorescent cytosines and thymines incorporated, representing methylated and non-methylated CpGs respectively can then be quantified. Different platforms use

different methods for the quantification of methylated cytosines, e.g., capillary electrophoresis, radioactively labeled dNTPs, or mass spectrometry (Kaminsky & Petronis, 2009).

This technology is used in a model for age estimation in blood, saliva, and buccal swabs (Jung et al., 2018) that was later replicated on a different population (Dias, Cordeiro, et al., 2020).

3.6 Massively Parallel Sequencing (MPS)

After the introduction of Sanger sequencing (Sanger et al., 1977), improvements to this method have led to increased accuracy and efficiency in more recent sequencing methods. Massively parallel sequencing allows for the analysis of all types of nucleic acids sequences, and the distinction between methylated and unmethylated cytosines after BC conversion. MPS is a Next-Generation Sequencing method used worldwide and in a wide variety of research areas. It uses reversible terminator chemistry and fluorescently labeled modified dNTPs in reaction with the sequence of interest (Bentley et al., 2008).

This technology is composed of 4 steps (Illumina Inc., 2017): **1) sample preparation,** where the DNA from the sample is randomly fragmentized and bonded to adaptor molecules**; 2) cluster generation**, which starts with loading the library prepared on the first step into a flow cell with surface-bound oligos. These oligos are complementary to the adapter molecules bonded to the DNA in the first step and capture the DNA fragments. The DNA fragments are amplified and finally ready for **3) sequencing;** this step begins with the extension of the primer. dNTPs are added to the reaction and bind to the template DNA in a stepwise manner, first for all forward strands and latter for all reverse strands; **4) data analysis** consist of the clustering of the millions of reads obtained in the previous steps, both in the reverse and forward sense. Then, the obtained sequences are aligned to the reference genome to find variants.

MPS has brought great advances to sequencing, with high throughput and accurate results at lower costs than previous methods. Some advantages of MPS are the ability to combine age estimation with other markers used in the forensic context (Vidaki et al., 2017) and the universality of this platform, which can also be

used for SNP variation analysis and STR profiling (Gross et al., 2021; Woźniak et al., 2021).

A recent study performed by the VISAGE Consortium (Gross et al., 2021) surveyed European laboratories that work with criminal cases, regarding the use of MPS. They concluded that over 70% of the inquired labs already owned an MPS platform, or will own one within the next 2 years. However, owning an MPS platform is not the only requirement to implementing MPS for the prediction of age and other externally visible characteristics. These technologies require specialized personnel and bioinformatics software for the interpretation of millions of reads obtained from each sample. This study demonstrated that European laboratories are on a good path to implementing MPS platforms for FDP and that there is a high interest in implementing these technologies (Gross et al., 2021).

4. Ethical issues and current legal landscape

The history of DNA in forensic science has a few landmarks, starting with Professor Sir Alec Jeffreys' discovery of DNA fingerprinting in 1984. In 1985, the first immigration, paternity, and identification of identical twins cases were solved using DNA fingerprinting.

Shortly after, DNA fingerprinting started being used in criminal investigations. For example, in 1986 when trying to solve the rape and murder of two girls. In this case, a suspect had confessed to committing one of the murders, however, DNA evidence collected from both crimes revealed that both murders had been committed by the same person and this person was not the confessing suspect.

In the years that followed, the value of DNA evidence to solve crimes started to be recognized but also raised questions in some courts of law on whether or not to admit evidence and convict a suspect based on DNA evidence.

Fast-forwarding to the present, DNA evidence can be used, not only to confirm if a sample found on a crime scene belongs or not to a known suspect but to infer on personal traits of the person who left the sample in the crime scene when there are no suspects.

The VISAGE Consortium describes Forensic DNA Phenotyping (FDP) as "a tool which seeks to probabilistically infer the age, appearance and biogeographical ancestry (BGA) (…) of an individual from their anonymous DNA sample". Being a probabilistic tool, age estimation and other FDP tools infer a phenotype to a certain degree of probability (Samuel & Prainsack, 2018).

Epigenetic Age Estimation is included in FDP tools, and with the advance of these techniques, concerns on ethical and legal aspects around it have risen.

Here, we analyze the use of Forensic DNA Phenotyping in unknown donor samples found on crime victims or at the crime scene in terms of ethical and societal issues and current legislation.

4.1 Ethical and societal issues

Discrimination

Discrimination is one of the major problems of FDP since these technologies can more easily discriminate against minorities. However, this is more applicable to BGA than age predictions and therefore, won't be further discussed here.

The "CSI effect"

Another problem is the "CSI effect" which arose with the growing popularity of television shows about forensic investigations (Chin & Workewych, 2016). These fictional programs create a perception of DNA technologies as infallible. This leads to over-interpretation of the findings obtained by FDP, which are always probabilistic and shouldn't be considered absolute truths. Despite the probability of the suspect having a determinate phenotype, other phenotypes shouldn't be ruled out. Police officers aren't typically instructed on the scientific and probabilistic nature of FDP findings which can lead to over-interpretation of FDP findings (Samuel & Prainsack, 2019).

The "Right not to know"

When the trait being tested is an EVC, including eye and hair color or age, it is mainly considered that FDP doesn't violate privacy rights since these pieces of information are known by the person, everybody who has ever seen the person, and

the police through personal identification documents (Kayser, 2015; Samuel & Prainsack, 2019). The same doesn't apply to other traits that may be unknown to the sample donor, such as information on health conditions. Putting in perspective, when a violent crime is being investigated, how deep should the investigation go to solve it? Some diseases already have a cure or have a low impact on the person. In these cases, solving the crime may outweigh the "right not to know" (de Cerqueira et al., 2016). Since age is a trait known to the person, at least approximately, investigating this trait shouldn't be considered as a violation of the "Right not to know".

Suspect populations

Reports of FDP findings introduce to the investigation the probability of the potential suspect displaying certain characteristics. This creates a suspect population, a group of all the persons who resemble the suspect, and introduces people to the investigation that would otherwise not be involved. When someone is involved in a criminal investigation, both their privacy, family, and professional lives are negatively impacted (Samuel & Prainsack, 2019).

The concept of suspect populations didn't emerge with FDP but, if the number of innocent people involved in criminal investigations was to be inflated by these technologies, not only would it affect more lives, but could even be negative to the investigation.

Communication and interpretation of FDP results

Another great issue being addressed is the communication of FDP results to the general public. Opinions are divided on this subject, with some defending that there shouldn't be a public communication of FDP results. First, it may lead to discrimination of people whose phenotype is similar to the described phenotype. Second, results may indicate a different phenotype from that of the perpetrator, either due to false results or because the sample may belong to an innocent person who passed by the crime scene. Erroneous results may in term lead the public to

diminish their trust in these tools or in the police that has spent time following a false lead.

Police and law enforcement agents should be taught to understand the basis and limitations of FDP to properly use the results given by these tools. Some police officers refuse to use these results since they don't understand them or just don't believe in them. Educating these agents on FDP would also improve the interpretation of the results and minimize the CSI effect.

Artificially altered appearance

Most EVCs can be artificially altered. Eye color can be hidden by contact lenses, hair color can be altered by hair dye, and can be straightened or curled by esthetic produce and techniques. Baldness patterns may be changed by hair transplantations and plastic surgeries may remove freckles or influence apparent age.

All these alterations can lead investigators in the opposite direction of the perpetrator and, in these cases, FDP can be disadvantageous. However, ID Cards, driver's licenses, and passports usually have portrait images and descriptions of the individual regarding their height, eye color, and age (Kayser, 2015). The perpetrator could successfully hide from the investigators at plain sight, but would probably be traced through their documentation, especially through age, which is present in every personal ID document.

Eyewitness

Many analogies can be drawn between eyewitnesses and FDP or "biological witnesses". When someone witnesses a crime and reports what they saw to the police, the information will most likely be the sex, approximate height, and age, hair color, structure, and race of the perpetrator. These include some of the EVCs currently determined by FDP.

One advantage of an eyewitness over a biological witness is that the description given is the description of the perpetrator and a DNA sample used for FDP may belong to someone who just passed through the crime scene and isn't related to the

crime. Even if the DNA belongs to the perpetrator, the results of FDP are probabilistic and may not correctly describe the perpetrator.

However, eyewitnesses of a crime are usually surprised by the event and many variables could affect their ability to correctly identify the culprit. Some of the main variables include viewing conditions (e.g. lightning), distracting stimuli (e.g. weapons), and the internal state of the witness during the crime (e.g. surprised) (Albright, 2017).

The main point in this analogy is not to argue in favor of biological witnesses over eyewitnesses. It is instead to compare both in terms of ethic and societal issues. If eyewitnesses can describe the external traits of a perpetrator without violating any rights, then the same can be extrapolated to FDP findings.

Misuse of FDP technologies

As with any technology, FDP doesn't come without the risk of misuse. For example, an insurance company has been licensed to use epigenetic age predicting tools, the same used for criminal investigations, to differentially attribute life insurance according to the epigenetically determined age (Dupras et al., 2018).

Even within the context of criminal justice, the debate on this topic is either it is acceptable that this technology can be used in any case or not. We can divide the opinions into 3 categories: FDP should be used independently of the severity of the crime; FDP should not be used at all or FDP should be used with limitation to the severity of the crime.

The main opinion is that FDP should only be used for serious crimes, such as murders and rapes, and only when no other leads are available (Samuel & Prainsack, 2019).

Data protection issues

Genetic material collected from biological samples contains personal information of the person to whom it belongs. As so, it is important to treat this information concerning the ethical values of collecting, processing, and/or storing personal data.

As stated in the General Data Protection Regulation (GDPR), personal data: should be processed “lawfully, fairly and transparently”; should strictly be collected with a specified, explicit and legitimate purpose and the information obtained should be limited to this purpose; should always be updated and, if not, must be erased or rectified; should only be kept for as long as necessary and, if stored longer than this period, can only be accessed if a new purpose is stated; finally, should be ensured to be protected against unauthorized processing and its loss, destruction or damage.

4.2 Legal aspects

The legality of DNA phenotyping varies between countries or even between states of the same country. As reported by the VISAGE Consortium (Samuel & Prainsack, 2018), at the time, FDP was practiced in Belgium, the Czech Republic, Hungary, Italy, Slovakia, France, Poland, Spain, Sweden, The Netherlands, and United Kingdom and in some states of the United States. Other countries, despite not practicing FDP, have used these technologies in sporadic cases (e.g. Australia).

Some countries have laws implicitly forbidding FDP (e.g. Austria, Germany), others have laws to explicitly regulate the use of FDP, such as The Netherlands where inferring biogeographical ancestry, hair, and eye color is permitted, and Slovakia, where the law allows for the use of FDP on samples related to severe crimes and for the identification of a corpse or body parts. This law also states that FDP should only be used when DNA profiles can’t provide the person’s identification (Samuel & Prainsack, 2018). In the U.S. state of Texas, FDP can be used as long as it is for investigating an offense or suspect or for the prosecution of a case (Kayser, 2015).

In France, the current legislation prohibits FDP since it only permits DNA phenotyping with the person’s consent. However, the Cour de Cassation, which has jurisdiction over civil and criminal matters, allows the examination of morphological characteristics by FDP. In conclusion, the French laws forbid FDP unless it is requested by a court order.

The legislation and regulation of FDP in the European Union (EU) falls within 3 documents: The Charter of Fundamental Rights of the European Union (CRF), the GDPR, and the Police Directive (Directive 2016/680).

The Police Directive is of special relevance to this topic since its basis is the protection of people in what regards their personal data processing by law enforcement authorities. Here, "genetic data" is defined as all data resulting from the analysis of a biological sample and that relates to inherited or acquired genetic characteristics.

Some countries have laws that restrict the use of DNA for forensic purposes to non-coding regions of the DNA (Kayser, 2015). This doesn't exclude FDP as long as the markers used are in non-coding regions. However, it seems that these laws refer to non-coding as a synonym to non-informative (Samuel & Prainsack, 2018). This is an outdated view since it is now well-established that the non-coding part of DNA contains coding information (Samuel & Prainsack, 2019).

In some countries, laws only state that genetic data should only be stored from non-coding regions of the DNA (e.g. Poland). This means that, in these countries, FDP can be practiced as long as the data obtained from coding regions aren't stored.

Most countries covered by Samuel and Prainsack's report (Samuel & Prainsack, 2018) don't have laws dedicated to FDP, or the use of DNA in forensics. Since no laws are saying otherwise, FDP is practiced in some of these countries.

There is a need to regulate the use of FDP in all its aspects, especially in those countries practicing these technologies. By practicing FDP without proper legislation, misuse is a strong possibility. Some examples of misuse are in cases when other possibilities for the subject's identification are still available or in minor offenses. The use of FDP to identify diseases or disease risks should also be legislated considering the "Right not to know", the privacy rights, and the impact that this discovery might have on the person. Finally, legislation relating to laboratories that can practice such techniques should be taken into account.

5. Discussion

Our genome is constant and contains all the information that makes us who we are. However, we are not constant, quite the opposite, we are constantly changing. This is possible due to Epigenetics. Epigenetics is responsible for the regulation of gene expression through mechanisms that make genes accessible or not to transcription.

A very important type of epigenetic alteration is DNA methylation, which consists of the addition of a methyl group to a cytosine. This cytosine is usually paired with guanine, composing a CpG site and CpG sites are often in clusters (CpG islands). The influence of DNA methylation on gene expression is related to the genomic region that is methylated. CpG islands are very common within promotor regions and, if methylated, gene expression is repressed (Moore et al., 2013). Differentiated cells have unique patterns of methylation that are dynamic during development and in adaptation to internal, external, and stochastic factors. These factors can be diseases, exposure to toxins, lifestyle, eating habits, among others. Over time, the changes in epigenome accumulate and can be correlated with age (Heyn et al., 2012). This correlation brought great advances, particularly in the forensic field for age predictions.

One of the main applications of age estimation tools is when a sample found on a crime scene doesn't have a match in national DNA databases. In these cases, the sample's DNA can be used to provide leads on whom the sample belongs to through the prediction of traits such as eye and hair color, biogeographic ancestry, and age (Cunha et al., 2009; Parson, 2018). The VISAGE Consortium aims to overcome the limitations associated with epigenetic age estimation tools and the overall limitations of the use of Forensic DNA Phenotyping (FDP), from a technical, ethical and legal point of view.

Age estimation studies try to find CpGs that can be related to age due to their methylation patterns (Bocklandt et al., 2011; Hannum et al., 2013; Zbieć-Piekarska, Spólnicka, Kupiec, Makowska, et al., 2015). To build an age-predicting model, several age-related CpGs are combined, but like in any good recipe, this combination has to be perfectly calibrated. One of the challenges in building a model is: how CpGs can be useful for the model. Typically, the greater the amount of CpGs

used, the more accurate the model is, but also requires more sample (Pisarek et al., 2021). Forensic samples are usually found in rough conditions and small amounts. To build a forensically useful age estimation model the amount of CpGs used should be balanced with the characteristics of forensic samples. Another challenge is to build an age prediction model that can be used in a variety of tissues (Horvath, 2013; Koch & Wagner, 2011). Forensic samples can be of any tissue or cell type or even a mixture of biological materials. CpGs can have different predictive power in different biological materials whereby a model that uses CpGs with strong predictive power in the wider possible variety of tissues and cell types is of great use in the forensic context. Semen seems to be a more complex type of biological material (Pisarek et al., 2021) and, even with advances both in knowledge and in the technologies surrounding age estimations, a separate model or a model with with a larger number of CpGs for this type of sample will possibly be maintained in the future.

Several factors seem to influence age estimations. On top of these factors is the intrinsic complexity of the aging process (Rodríguez-Rodero et al., 2011) which is still a matter of study. Whether or not sex influences age estimation doesn't gather consensus. One characteristic of the epigenome is that it changes due to lifestyle choices (Simpkin et al., 2016; Weidner & Wagner, 2014) and diseases (Horvath et al., 2014, 2015, 2016; Vidal et al., 2016) and these can have an impact on age estimation. Another factor that strongly affects age estimation is the group of characteristics of forensic samples, such as the presence of PCR inhibitors, degradation, and time it may take to find a sample (S. B. Lee & Shewale, 2017). There is a shortage of studies that apply age estimation tools across different populations but it has been shown that a model built based on a specific population isn't equally accurate on other populations (Dias, Cordeiro, et al., 2020). In most age estimation models, the actual chronological age affects the prediction in older subjects, typically over 40 years of age (Dias, Cordeiro, et al., 2020; Dias, Cunha, et al., 2020; Hamano et al., 2016; Zbieć-Piekarska, Spólnicka, Kupiec, Makowska, et al., 2015). Finally, epigenetic drift, which is a result of random errors in the maintenance of the epigenome and can explain the gradually larger discrepancy in

monozygotic twins over time, can have an impact on age estimations (Mendelsohn & Larrick, 2017).

Epigenetic age estimation models use different technologies but usually start by extracting DNA from the sample and bisulfite-convert the DNA. Extracting DNA from a forensic sample is very challenging hence the importance of choosing the right method to do so (S. B. Lee & Shewale, 2017). The goal is to extract as much purified DNA as possible so that all downstream analyses can be performed. Bisulfite conversion is a crucial step in age estimation models. It is this technique that allows for the distinction between methylated and unmethylated cytosines. Despite being fundamental, bisulfite conversion has its disadvantages: the deamination of methylated cytosines, DNA loss, and DNA degradation. The use of controls in the bisulfite conversion process validates the converted DNA to be used in the next steps of the age estimation model. Multiplex PCR is also a crucial step in most molecular biology assays since it allows for the amplification of multiple DNA targets in a single PCR reaction. This is not straightforward in the cases of forensic samples that have been bisulfite-converted due to the usual low quantities of DNA extracted from this type of sample and further degradation caused by the bisulfite conversion. Currently, it is possible to perform a multiplex PCR from as little as 20 ng of bisulfite-converted DNA. After these three steps, different models use different techniques for the analysis of the DNA methylation patterns. One possibility is pyrosequencing (Sukawutthiya et al., 2021; Tost & Gut, 2007), a sequencing-by-synthesis method. Another possibility is the Methylation SNaPshot (Dias, Cordeiro, et al., 2020), which quantifies DNA methylation levels of bisulfite-converted samples at target CpGs. Finally, a very promising method is Massively Parallel Sequencing (Gross et al., 2021; Naue et al., 2017; Pisarek et al., 2021) since it not only allows for the sequencing of age-related markers but also other methylation markers used in FDP.

Since the discovery of DNA profiles, the use of DNA in forensic cases has had major advances. FDP tools have been studied that could aid in criminal investigations. One of these tools is age estimation through DNA methylation profiles. To implement FDP tools in routine forensic practices it is necessary to first discuss the ethical, societal, and legal issues around it (Samuel & Prainsack, 2019). Some of these issues aren't particularly connected to age estimations, such as

discrimination and the "Right not to know" which are more related to FDP of biogeographical ancestry or genetic diseases. Other issues, however, can arise from age estimations such as the "CSI effect", the creation of suspect populations, the communication and interpretation of FDP results to law enforcement agents, the possibility to artificially modify the appearance, and the misuse of the technology. Many analogies are drawn between eyewitnesses and FDP results since both can report the same EVC. Since eyewitnesses can describe externally visible characteristics of a perpetrator without violating any rights, then the same can be extrapolated to FDP tools. Recently, data protection issues have been heavily debated and regulated and, to implement FDP tools, there is an urgent need to make sure that data protection isn't being violated (Samuel & Prainsack, 2019).

Legally, FDP is not on solid grounds (Samuel & Prainsack, 2018). In many countries, the laws regarding the use of DNA in forensic practices were made for STR profiling and can't be applied to FDP. This means that, even though no laws are forbidding FDP in these countries, there are also no laws regulating its use. Other countries have ambiguous laws, e.g. France, where FDP is both permitted and illegal. The Netherlands is a good example for the implementation of FDP since, not only is it practiced for some traits, but it is properly regulated.

6. Conclusions and future perspectives

Forensic DNA Phenotyping could be a great introduction to the field, in particular for age estimations. However, its limitations must be thoroughly addressed. Previous age estimation methods such as skeletal, dental, and histological methods, lack standardization, present ambiguous results, require a body or fragments of the body and are tissue-specific. More recent age estimation tools use DNA methylation markers for the prediction of a person's chronological age from small samples and in a variety of tissues. DNA methylation is an epigenetic alteration that can be affected by a variety of factors (e.g. cigarette smoking) in a reversible or irreversible manner. Biological age reflects the impact that these factors had in the aging process and differs from chronological age, which is the amount of time passed since birth. Both measures of age are relevant in different

contexts but for forensic purposes, chronological age is of greater use than biological age. Age estimation models used for these purposes should favor DNA methylation markers that better explain chronological as opposed to biological age.

Many aspects may influence age estimation, such as gender, lifestyle choices, diseases, biogeographic ancestry, and epigenetic drift. It is impossible to build an age estimation model without limitations, but it is necessary to study the interference of several factors on the models. Most models are calibrated using samples from one population and should be practiced on samples from subjects of different biogeographic ancestry. Once this and other interference factors are properly applied to a model, then it can be validated as a universal tool. Several molecular biology methods can be used for age estimation and most models start by DNA extraction from the sample, bisulfite-conversion, and amplification of the DNA. After these steps, DNA methylation analysis varies between models. In the future, a standardized model should be built to apply this technology in all forensic laboratories, and this model should include all steps from DNA extraction to DNA methylation analysis. The probability that, in the future, only one age estimation model will be used is very small. Not all tissues and cell types have the same age-related markers and even though the same model can be applied to several types of biological material, it will hardly be possible to build a model that can be applied to all tissues and cell types.

Finally, ethical, societal, and legal aspects surrounding FDP are being discussed and should continue to be discussed in the future to properly regulate the use of age estimations in forensic practices, without violating any human rights, data protection issues, or laws.

In conclusion, developing and validating FDP tools, especially age estimation, could have a great positive impact on solving cases that couldn’t be solved otherwise, but should be properly regulated to prevent misuse.

7. References

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Supplementary Material

Table 1- Overview of methods used for age estimations

Method	Overview	Advantages	Disadvantages	References
Physical and X-Ray Exams	Age estimation in living persons. Consists of a physical exam, an X-ray of the left hand, dentition, and clavicle.	• Approved for age estimation in living persons	• Not accurate for juvenile age estimations • Ethnical differences	(Schmeling et al., 2008; Zhang et al., 2009; Olze et al., 2007)
Skeletal examination	Age estimation for human remains; Morphological quantification performed by an anthropologist.	• Accuracy	• Human error • Lacks precision	(Christensen et al., 2014)
Dental examination	Age is estimated according to the developmental stage of all available teeth	• Very useful for juveniles • Very accurate	• Requires prior classification of the individual as juvenile or adult • Influenced by sex and ancestry	(A. M. Christensen et al., 2014; Olze et al., 2007)
Bone histological analysis	Consist on the determination of osteon population density (OPD) from fragments of bones.	• Useful from fragmented bones;	• Can only accurately estimate age until a certain age • Doesn't perform equally for every bone • Affected by sex, ancestry, physical activity, nutrition, and health • Destructive and invasive method • Require specific equipment and training.	(A. M. Christensen et al., 2014)
Telomere shortening	Determination of telomere length	• Estimates age with an error between 7 to 9 years • Requires small amounts of DNA • High throughput, simplicity, robustness, and reproducibility.	• Susceptible to environmental factors to which the sample is exposed • Influenced by cause of death	(Márquez-Ruiz et al., 2018; Zapico et al., 2019)
Mitochondrial DNA damage	Using qPCR, the amount of damage accumulated in mtDNA during aging can be measured	• Apparent good correlation with age • Requires small amounts of sample • Simple and affordable	• Shortage of studies to validate this method • Populational differences	(Parson, 2018; Zapico & Ubelaker, 2016)
Quantification of sjTREC decline	Through the quantification of sjTRECs by qPCR, age can be related to its decline	• Can be used in blood and bloodstains • Can be used in aged samples	• Can only be used for blood samples • May be affected by genetic and environmental factors • Requires further studies	(Ou et al., 2012; Ou et al., 2011)