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Recent progress, methods and perspectives in forensic epigenetics

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ABSTRACT

Forensic epigenetics, i.e., investigating epigenetics variation to resolve forensically relevant questions unanswerable with standard forensic DNA profiling has been gaining substantial ground over the last few years. Differential DNA methylation among tissues and individuals has been proposed as useful resource for three forensic applications i) determining the tissue type of a human biological trace, ii) estimating the age of an unknown trace donor, and iii) differentiating between monozygotic twins. Thus far, forensic epigenetic investigations have used a wide range of methods for CpG marker discovery, prediction modelling and targeted DNA methylation analysis, all coming with advantages and disadvantages when it comes to forensic trace analysis. In this review, we summarize the most recent literature on these three main topics of current forensic epigenetic investigations and discuss limitations and practical considerations in experimental design and data interpretation, such as technical and biological biases. Moreover, we provide future perspectives with regard to new research questions, new epigenetic markers and recent technological advances that – as we envision – will move the field towards forensic epigenomics in the near future.

1. Introduction

Epigenetics refers to the 'heritable' alterations in gene expression and cellular phenotype that are triggered by molecular mechanisms other than DNA sequence changes, including DNA base modifications (such as cytosine/5′-CpG-3′ methylation) and post-translational histone modifications (such as histone H3 methylation or acetylation) [1]. Epigenetic processes play a significant role in gene expression as a response to various short- or long-term environmental influences [2,3]. Epigenetics regulation of gene expression works under the 'rule' - with many exceptions - that a methylated gene promoter becomes compact and non-accessible to transcription factors leading to the inactivation of the gene [4] (Fig. 1A). Therefore, epigenetics has been extensively studied, where epigenetic mis-programming and aberrant DNA methylation of key regulatory genes is observed and impacts human diseases such as cancer [5,6]. However, the lifelong molecular responses to the 'dynamic' environment via adjusting DNA methylation levels across the genome, resulting in individual epigenomic variation [7–9], also referred to as epigenetic fingerprint, is also relevant in the forensic field (Fig. 1B–D) [10].

Detecting DNA sequence variation in the form of short tandem repeats (STRs) or single nucleotide polymorphisms (SNPs) has been a powerful resource in forensic genetics for identifying individuals, like victims and perpetrators of crime, from smaller and smaller human biological evidence [11]. Although less established thus far, SNPs can also be used in DNA-based forensic intelligence to predict unknown persons' appearance traits and biogeographical ancestry, which can help finding unknown perpetrators of crime who, in principle, cannot be identified with standard forensic DNA profiling [12,13]. Together with exploring genetic variation, the additional investigation of epigenetic variation - mainly DNA methylation differences between CpG sites - has gained substantial ground in the forensic field over the last few years [14–16]. Since its first forensic introduction for sex determination in 1993 [17], differential DNA methylation patterns have been mainly studied for three forensically relevant reasons: i) to identify the tissue/cell-type source of DNA evidence [18], ii) to estimate an individual's age [19], and iii) to differentiate between monozygotic twins [20]. Generally, next to the availability of suitable DNA markers, suitable technology for multiplex analysis of a large number of markers from low-quality and -quantity DNA recovered from crime scene traces is key and currently the limiting factor in the progress of forensic genetics, which also applies to forensic epigenetics. However, advances in targeted next-generation sequencing (NGS) technologies, often referred to as massively parallel sequencing (MPS), are deemed promising [21,22].

In this review, we summarize the most recent literature on DNA methylation profiling in the three currently investigated forensic applications, and present an in-depth overview of the methodology used for marker discovery, statistical modelling and targeted DNA

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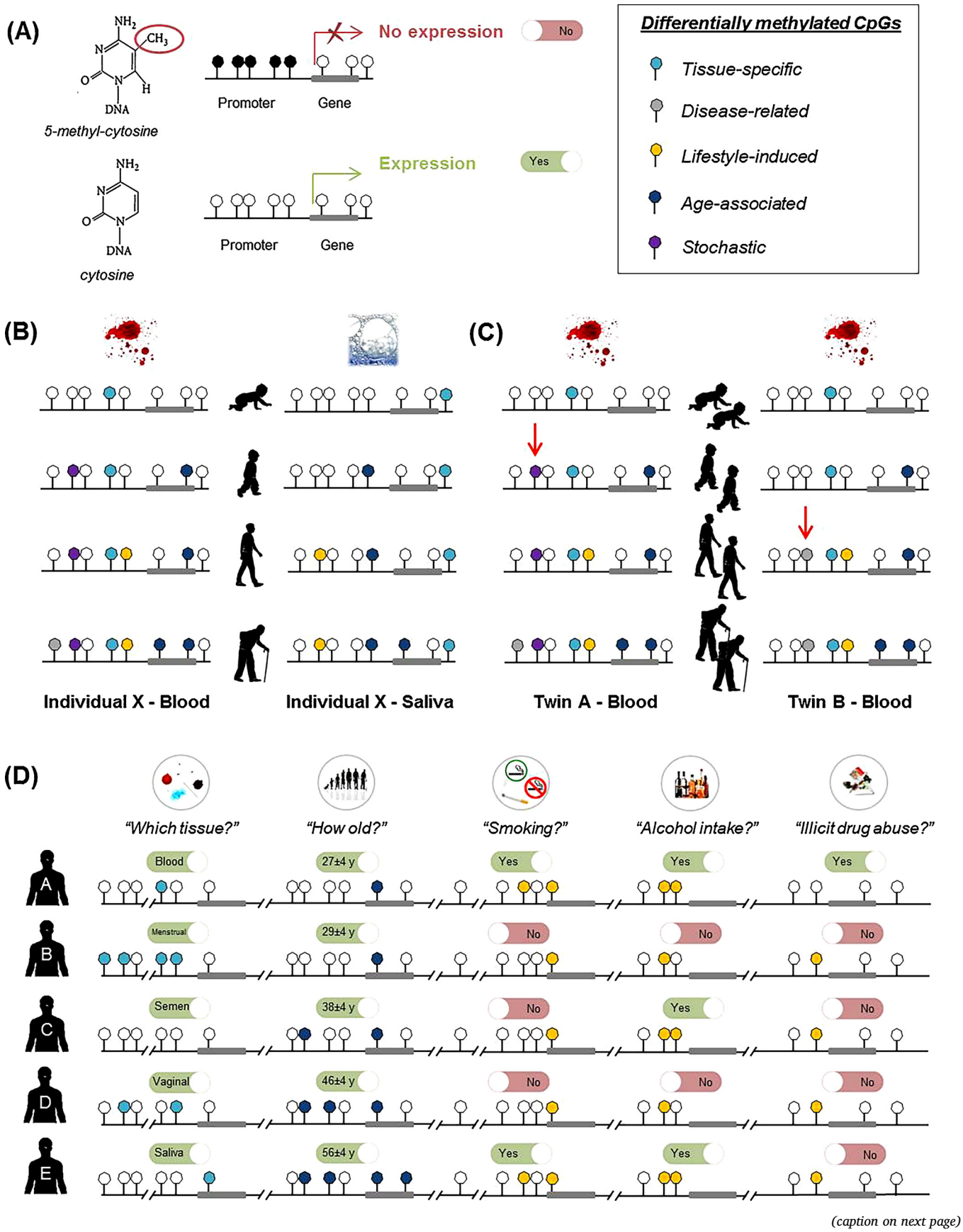

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Fig. 1. (A) Gene expression regulation via DNA methylation 'switches', where a methylated gene promoter (black lollipops) usually leads to gene inactivation by 'blocking' the transcription mechanism, (B) DNA methylation variation within different tissues (blood and saliva) of the same individual, where DNA methylation differences occur either in a tissue-specific or tissue-shared manner, in various locations associated with a gene, (C) DNA methylation variation between individuals of the same monozygotic twin pair, which can be stochastic, disease-related (red arrows), or due to lifestyle choice differences, and (D) future concept of epigenetic fingerprinting, where differential DNA methylation profiling can lead to the simultaneous prediction of a stain's tissue source and an individual's age and lifestyle choices useful for crime scene investigation and investigative intelligence (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

methylation detection including their limitations. Moreover, we present future possibilities that arise as a result of the identification of novel epigenetic modifications and the development of advanced epigenetic technologies. We believe that these will allow for addressing additional forensic questions, which will transform forensic epigenetics into forensic epigenomics in the future.

2. Recent advances in forensic epigenetic applications

To date, a wide range of > 60 papers on forensic epigenetic investigations have been published. Since the first review on the potential opportunities and challenges of forensic DNA methylation profiling published in this Journal in 2013 [14], other reviews on this topic have appeared in other journals in 2013 [23], 2015 [15] and most recently in 2016 [16], reflecting the fast development of this field. The scope of this section is to summarize the most recent forensic epigenetic literature on the above-mentioned three main topics of forensic epigenetic studies i.e., papers published since 2016 when the last epigenetic review paper appeared [16].

2.1. Determination of body fluids and tissues

Knowing the cell or tissue type origin of a DNA sample used for standard forensic DNA profiling can be a useful information for crime scene investigations, not only for reconstructing the events at the crime scene, but also for associating tissues with donors in mixed samples. While other approaches, such as tissue-specific mRNA or miRNA profiling [18], have been successfully proposed and thoroughly investigated, they lack the direct 'same-molecule' link between a DNA profile and its (RNA-based) tissue information. Recently, DNA copy number variation was proposed as a promising tissue-specific DNA marker, however initial results only allow for the detection of blood and semen [24]. Since the first investigation of DNA methylation-based tissue identification in 2011 [25], suitable tissue-specific CpG markers have been identified and validated as well as multiplex test assays have been developed based on various enzymatic- or bisulfite conversion-based technologies, including in studies published since 2016.

Based on Illumina HumanMethylation450 Beadchip microarray data, Forat et al. identified 150 candidate tissue-specific markers for the identification of blood, saliva, semen, vaginal fluid and menstrual blood [26]. Using a range of targeted techniques, the authors validated a set of nine DNA methylation markers and also showed how disease status can affect reliable identification, for example in vaginal samples from patients suffering from cervix carcinoma. Similarly, Lin et al. identified a set of eight tissue-specific CpG markers from available Illumina 450 K data, which together with two control markers formed a 10-plex assay based on methylation-specific restriction enzyme (MSRE)-PCR system [27]. Not only was this system sensitive and reproducible, but was combined with forensic STR profiling in a single reaction. Moreover, using methylation data from methylated DNA immunoprecipitation (MeDIP), Vidaki et al. proposed novel semen-specific CpG markers (cg04382920 and cg11768416) and developed singleplex pyrosequencing assays that were highly sensitive, able to analyze stains stored for up to 16 years [28]. Using promising data from the literature, the same group also tested a short genomic location of the *EFS* gene (10 CpGs) by means of bisulfite pyrosequencing, and confirmed that it is highly blood-specific with a low inter-individual variability in 65 whole blood samples and robust methylation differences compared to menstrual blood [29]. On this topic, in a more comprehensive approach employing methylation SNaPshot®, Holtkötter et al. evaluated a total of 11 previously reported CpG sites for their potential to differentiate between whole and menstrual blood, and proposed BLU2, initially identified by [28], as the most suitable [30]. Based on Illumina 450 K microarray data, 15 more suitable menstrual blood-specific CpG sites were identified by Lee et al., the two best of which were validated in a large dataset (n = 461) of body fluid samples using bisulfite sequencing [31]. The authors employed these two markers by updating their existing multiplex methylation SNaPshot® system [32].

Based on three previously identified epigenetic markers [33,34], Silva et al. further investigating tissue-specific findings for semen, saliva and blood using bisulfite pyrosequencing by performing an initial developmental validation including sensitivity (down to 0.1 ng), stability, mixtures and forensic-type samples [35]. While the results of this study were promising, showing that the tested markers are robust and reliable, 10 ng was recommended as the minimum DNA input for two of them to avoid PCR bias. Furthermore, using the semen-specific hypomethylated marker of this study (*ZC3H12D*), which demonstrated a clear 'on-off' methylation pattern compared to non-semen tissues, the same group developed a simple high-resolution melt-curve (HRM) analysis assay, able to detect semen with as low as 1 ng of genomic DNA [36]. The ability of quantitative (q)PCR for simple, quick and sensitive single-tissue DNA methylation tools have also been demonstrated by Watanabe et al. [37,38]. Duplex qPCR methods with two probes for the non-methylated and methylated alleles were developed to detect blood and semen using previously identified CpG sites. While the methods were successfully applied in 29-year-old stains, the authors indicated issues with interpreting moderately methylated sequences [37]. Lastly, as it was recently demonstrated [39], independent validation of reported tissue-specific markers is crucial, as it allows for testing a larger pool of samples and selecting the best markers demonstrating 'on-off' methylation, particularly useful for the development of multiplex methylation SNaPshot® assays.

2.2. Estimation of a person's age

Using differential DNA methylation profiling for the purpose of estimating an unknown stain donor's age is by far the most popular and fast-expanding forensic epigenetic application, with > 20 papers published since 2016. This is due to not only the well-established role of DNA methylation in ageing, surpassing other molecular-based approaches [40], but also the high relevance of developing molecular tools for predicting a person's age from biological traces. Establishing the age of an unknown trace DNA is forensically relevant on its own, as age characterizes a person useful for finding unknown perpetrators. Moreover, it is relevant in combination with appearance trait prediction from DNA in Forensic DNA Phenotyping (FDP) since some appearance traits (such as hair loss, hair greying etc.) are highly age-dependent. Since this sub-field has been recently comprehensively reviewed [19,41,42], we will focus only in the newly published papers (since 2016) that aim to validate existing markers and/or expand age prediction in different tissues, ethnic groups, younger individuals and diseased populations.

While most forensic age predictors have been previously built in whole blood, recent studies have also investigated other tissues, such as

saliva or different tissues from postmortem samples. Based on the Illumina 450 K microarray platform, Hong et al. identified six age-associated CpGs in saliva, despite the small sample size used (n = 54) [43]. Combining these with a cell type-specific marker for both blood and buccal cells, they constructed a novel 7-plex methylation SNaPshot® system and tested it in an independent set of 226 saliva samples, providing age prediction with a mean absolute deviation (MAD) of 3.2 years [43]. Using bisulfite pyrosequencing to examine three previously reported genetic loci (*SCGN, DLX5* and KLF14) in saliva, Alghanim et al. proposed single- and dual-locus age models, resulting in MAD = 8 years and MAD = 7.1 years, respectively; the larger error is likely a consequence of the sample size and markers used [44]. In a more simplistic approach, Hamano et al. developed a methylation-sensitive HRM method based on the promoters of two previously reported age-associated genes (*ELOVL2* and *EDARADD*) and tested almost 250 saliva samples [45]. The authors achieved age prediction in their validation test samples with a MAD of 6.25 years in saliva samples and 7.65 years in cigarette butt samples, where the error difference likely reflects cell type differences and hence, cell type-specific epigenetic effects. Using the same experimental approach, this group also tested the promoter regions of *ELOVL2* and *FHL2* in postmortem blood samples, with similar results obtained from blood of living individuals [46]. Similarly, Naue et al. developed an age prediction tool in blood based on MPS technology using 13 CpG sites and a random forest machine learning algorithm achieving MAD = 3.16 years [47]. In a most recent follow-up study, some of these markers (7 out of 13) were found to have multi-tissue information when testing bone, brain, buccal swabs and muscle samples of 29 deceased individuals; however, the observed age-dependent methylation effects differed between the tissues analyzed [48].

Similarly to tissue-specific epigenetic markers, age predictors also need to undergo stringent validation prior to future implementation to forensic casework, including ethnicity-specific effects. Towards this direction, Fleckhaus et al. tested two age predictors developed in buccal swabs [49] and blood [50] in three populations groups from the Middle East, West Africa and Central Europe (N = ~40–50 each), where the results showed significantly lower dispersions of methylation and thus, prediction errors in the Middle East individuals [51]. Cho et al. validated one of these age predictive tools with CpGs in *ELOVL2, C1orf132, TRIM59, KLF14* and *FHL2* originally trained in Polish individuals (MAD = 3.9 years) [50] in Korean blood samples (MAD = 4.18 years), revealing that some of them showed different age-associated methylation effects [52]. These authors also suggested that the inclusion of additional age biomarkers, such as signal-joint T-cell receptor excision circles (sjTRECs) [53], has the potential to decrease the 'typically' higher error in the eldest group reported in the relevant literature. The same age predictor has also been validated in individuals from three age-related disease groups, where particular markers have significantly different age prediction capacities in Alzheimer's and Grave's diseased patients [54]. Moreover, targeting young individuals, Shi et al. investigated age-associated DNA methylation signatures in 48 Chinese children (6–15 years old) using the novel approach of digital PCR, together with skeletal and dental age information via X-ray examination [55]. Using a combination of these types of age markers, the authors achieved high accuracy – mean absolute error (MAE) = 0.47 years for boys and MAE = 0.33 years for girls, highlighting the potential of this approach in immigration cases where confirming the age of a child is necessary. Even when using DNA methylation alone including six novel pre-adult age markers, in a most recent study, Freire-Aradas et al. report highly accurate age prediction (median absolute error, MAE = 0.94 years) in children (N = 180, 2–18 years old) using quantile regression and EpiTYPER [56]. The obtained smaller errors in these studies reflect the smaller age range used for prediction modelling. However, errors still represent ~5–6% of the applied age range, regardless if in adults or children.

2.3. Discrimination of monozygotic twins

Differentiating between monozygotic twins (MZ) remains one of the unsolved challenges in forensic DNA identification. Identical twins share almost identical DNA sequences and hence typically the same standard forensic DNA profiles. In very rare cases twin individual-specific somatic mutations in a forensic STR were observed [57]. Moreover, by means of ultra-deep whole genome sequencing twin individual-specific somatic mutations in SNPs can be observed and used to differentiate twins [58]. However, this approach involves high costs, careful validation of the identified SNPs in trace DNA that can be complicated by tissue differences and is not guaranteed to reveal SNP differences in a given twin pair [59], which also depends on which materials are available as reference and trace samples [58]. These circumstances have attracted forensic researchers to use epigenetic variation for investigating monozygotic twin differentiation. Additionally, epigenetic differences have also been shown between both healthy and disease-discordant MZ twins [60,61], explained by stochastic, environmental and phenotype-related molecular events, proposing that such approach might also be suitable to answer the forensic question. Hence, forensically-motivated researchers have initiated genome-wide methylation pattern comparison in limited number of twins [62,63], and also in longitudinal studies over a limited period of time (9 months) [64], but the observed differences are also very rare and with small size effects. A recent study showed that CpG sites showing methylation difference among 12 MZ twin pairs exist in to a small extent [65]; however, larger investigations using hundreds of MZ twin pairs are still needed to shed light on the role and function of those methylation 'hotspots'.

Furthermore, targeted investigations analyzing both reference- and trace-type evidence from the same twin individuals are needed to simulate the forensic case scenario, but such studies are scarce thus far. Vidaki et al. investigated sets of reference-type whole blood samples and saliva samples from monozygotic twin pairs by genome-wide methylation analysis to identify twin-differentiating markers and followed them up by forensically suitable qPCR analysis in trace-type bloodstains, saliva stains and cigarette butts [20,66]. These two studies revealed a range of critical technical and biological factors that impact on the ability to differentiate monozygotic twins via DNA methylation profiling, including method-to-method differences, cell type composition-associated differences in DNA methylation detection. Moreover, the typical time differences between collection of reference and trace samples leading to potential time-wise changes in DNA methylation levels may represent an important biological factor, which could not be investigated in these studies given their sampling design. The technical issues deriving from the situation that the available genome-wide screening technologies are not suitable for low-quality and -quantity DNA found in trace samples requires a switch to a currently unavailable technology to avoid the method-to-method variation observed in these studies. Developing a method appropriate and sensitive enough for the analysis of both reference- and trace-type material and including a cell type counting/sorting step may solve these problems in the future.

3. Current methodologies for forensic epigenetic profiling

Here, given that no forensic epigenetic technology-focused review exists thus far, we aim to review the literature of all forensic epigenetic papers since 2011. We discuss these in terms of techniques used for CpG marker discovery and targeted analysis, method requirements and performances, statistical power and prediction errors of proposed models, as well as implemented developmental/validating strategies.

3.1. CpG marker discovery

Concerning candidate CpG markers potentially useful for forensic applications, forensic researchers highly rely on existing studies in the

Table 1
Summary of forensically motivated studies investigating genome-wide DNA methylation with the Illumina HumanMethylation27/450 platforms as categorized by research question.

Topic	Platform	Data	Tissue	Samples	Sample info	DNA input (μg)	Bisulfite conversion kit	Normalization method	Study
Tissue ID	450 K	New	WB, SA, VA	16	Korean	0.5	EZ DNA Methylation	Background subtraction	Park et al. [34]
		New	WB, SA, SE, VA, MB	42	20-59 years old	1–2	EZ DNA Methylation	ComBat, surrogate variable analysis	Lee et al. [32]
		Public/New	BL, SA, SE, VA, MB, SK	70		1–2	EZ DNA Methylation	ComBat, surrogate variable analysis	Lee et al. [31]
	27 K 450 K	New	WB, SA, SE, VA, MB, EM, SK, PM	14	Pooled samples	0.5	EpiTect Bisulfite	Not performed	Forat et al. [26]
	450 K	Public	WB, SA, SE, VA	61			Various	Background subtraction	Lin et al. [27]
Age	450 K	New	WB	16	21-32 years old	1	EZ DNA Methylation	Not provided	Xu et al. [72]
		New	SE	12	20-59 years old	1–2	EZ DNA Methylation	ComBat	Lee et al. [32]
		Public	WB	3,702	19-101 years old		Various	Functional, subset-quantile, quantile	Freire-Aradas et al. [73]
		Public	WB	2,173	Chinese,African, European		Various	Not provided/Not performed	Park et al. [74]
		Public	SA	54	18-73 years old	1–2	EZ DNA Methylation	ComBat, surrogate variable analysis	Hong et al. [43]
		Public	BL	1,156	2-90 years old		Various	Quantile	Vidaki et al. [75]
		Public	BL	1,246	0-75 years old	1.3–1	Various	Functional	Naue et al. [47]
		Public	BL	1,121	3-94 years old	0.5–2	Various	Not provided	Freire-Aradas et al. [56]
Twins	27 K	New	WB	44	17-74 years old, Chinese	1–2	EZ DNA methylation Gold	Background subtraction	Li et al. [62]
	450 K	New	WB	22	Chinese	1	EZ DNA methylation Gold	Quantile, beta-mixture quantile	Zhang et al. [64]
		New	WB	20	52–62 years old, European	0.75	EZ DNA Methylation	Functional, subset-quantile	Vidaki et al. [20]
		New	BU	2	53 years old	0.75	EZ DNA Methylation	Functional, subset-quantile	Vidaki et al. [66]
		New	WB	48	Korean	0.5	EZ DNA methylation-Gold	Not performed	Park et al. [65]

Abbreviations - WB: whole blood, SA: saliva, VA: vaginal fluid, MB: menstrual blood, SE: semen, SK: skin, EM: endometrium, PM: penis mucosa, BU: buccal cells.

medical and other fields; particularly in the case of age-associated CpG markers. Firstly, CpGs are selected via candidate marker approaches, such as in the case of the age-dependent *ELOLV2* [67] and whole blood-specific *EFS* gene [29]; nevertheless, these need to be further validated in large pools of other forensically relevant tissues. More often, however, forensic epigenetic markers have been identified via DNA methylation microarray screening that allow for the analysis of hundreds of thousands of CpG sites simultaneously (Illumina Infinium 27 K/450 K) (Table 1). These microarrays are the best compromise thus far in terms of coverage, sample throughput, analysis time and reagent costs, and have been the gold standard in epigenetic biomarker research [68]. Prior to analysis, careful data normalization to account for various technical and biological variation and biases is also required [69], especially when pooling large number of samples. While they provide single-CpG resolution, probes are mainly associated with disease-related gene regions and often contain multiple CpGs that can affect probe binding, hence accurate methylation quantification. Moreover, such hybridization-based technology requires good-quality and -quantity (several hundreds of ng input) DNA for successful performance [70], which limits their forensic application to reference-type samples but does not allow their use for trace-type samples.

There is a wide range of publicly available DNA methylation microarray datasets, e.g. in the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/), that can be useful as data resource for forensic studies. However, limiting factors include knowledge availability regarding phenotypes of forensic interest and availability of data from the particular tissues of forensic interest as well as missing details on normalization procedures. Moreover, some of these medically-motivated studies are performed in patients, where it is typically unknown how the disease status impacts on the forensic question of interest. Regarding the three main forensic epigenetic applications mentioned thus far, age prediction research using such data is the most popular. Age information is often provided together with the microarray data because age is an important co-factor in medical epigenomic studies. On the other hand, especially for forensically relevant tissues such as semen, vaginal fluid or menstrual blood, public data resources are less abundant, since these tissues are rarely included in medical studies. This situation has led forensic researchers to generate microarray data for such tissues [26,31,32,34,76]; however, typically in limited numbers due to the high per-sample costs involved. This is also the case for forensic epigenetic studies involving healthy MZ twins, as most available datasets include disease-discordant MZ twins.

3.2. Prediction modelling and statistical analysis

Once suitable CpG markers have been identified, such as via microarray screening described above, at best by analyzing large datasets, appropriate subsequent data analysis is then required such as for classifying a tissue type, estimating an individual's age, or differentiating between MZ twins. There are various statistical approaches to do so, which differ within and between the research questions. In the case of forensic tissue identification, no interpretation guidelines using the developed multiplex tissue ID systems have yet been proposed, highlighting the need for additional developmental validation and reaching a consensus opinion. Simply observing distinct (often 'on-off') tissue-specific DNA methylation patterns is the first step, but not sufficient. Natural intra- and inter-individual variation as well as age-, lifestyle- and disease-associated effects need to be carefully investigated before a candidate tissue-specific CpG can be concluded as practically useful marker for forensic tissue identification. Once such studies reveal that the tissue effects is largely independent from such factors, statistical frameworks based on likelihood ratios can be developed. Similar requirements are also involved in the case of MZ twin differentiation, where methylation data will eventually be used for individual identification and presented in court. The data analysis here is not trivial and require additional investigations from what has already been

mentioned, such as establishing CpG methylation frequencies and testing the *in vivo* and *in vitro* time wise stability of the markers. Moreover, the crucial question on how many twin-differentiating CpGs are required to conclude individual identification in the context of a given twin pair with STR profile evidence pointing to one or the other individual remains to be answered. Future research is expected to shed light in these questions, which is required as additional prerequisite for practical casework applications.

For age prediction modeling using DNA methylation markers, forensic researchers have used various statistical approaches, including *regression modelling* – multivariate linear [74,77–79], univariate linear [43,76], multivariate nonlinear [72], multivariate quantile [56,80,81], quadratic [82], backward stepwise [79], weighted-least-squares [81], ordinary-least-squares [81], support vector [72] – and *neural networks* – generalized regression [75], random forest regression [47], and back propagation [72]. The method-of-choice should be determined by the (non-)normal distribution of CpG methylation, (non-)linear relationships, collinearity, non-constant variance or heteroscedasticity in our dataset. For example, when comparing multiple statistical methods using the same dataset, a quantile regression seemed to perform better when dealing with non-constant, non-normally distributed variance [81]. Similarly, support vector regression was identified as the most robust model with the least mean absolute deviation from the true chronological age and a less cross-validated error, when compared to linear regression [72]. Recently, artificial neural networks seem to outperform 'standard' multiple linear regression analysis, significantly improving the age prediction error [75]. However, thus far the underlying reason is unclear and shall be understood before such artificial intelligence methods replace classical methods in data analysis and interpretation. Systematic feature selection was recently shown to play a critical role in determining the performance of the final age prediction model [79], suggesting an influence of optimal marker selection strategies.

3.3. Targeted DNA methylation detection

In this section, we summarize the most commonly used targeted methods for detecting differential DNA methylation and discuss their performance and forensic suitability. Given that the methylation status of a CpG site turns into a 'C/T 'SNP variation following bisulfite conversion, with C corresponding to the methylated and T to the unmethylated alleles, many techniques successfully applied in forensic SNP typing have also been used for analyzing CpG sites. These include the methylation-sensitive genotyping version of restriction enzyme-based fragment analysis, single-based extension (SBE) systems like SNaPshot®, pyrosequencing, qPCR, NGS and mass spectrometry-based genotyping (Tables 2 and 3, Supplementary Table 1 containing additional information on experimental design).

3.3.1. Methylation-sensitive restriction enzyme (MSRE)-based fragment analysis

MSREs have been widely used in epigenetics research, due to their highly-specific ability to recognize and cut methylated DNA. MSRE-PCR has been described in the past as a fast and reliable way to detect DNA methylation patterns in multiple fragments simultaneously, in a sensitive and reproducible way [101]. The basic steps of the MSRE-based fragment analysis include extensive digestion of genomic DNA with the MSRE-of-choice, multiplex PCR amplification of the genes-of-interest with gene-specific primers, and detection of the resulting PCR fragments via capillary electrophoresis using genetic analyzers. This has been one of the most frequently applied methods in forensic epigenetic tissue identification papers, likely because genetic analyzers are the most common instruments in forensic laboratories. The reported assays are all based on the HhaI restriction enzyme (5′-GCGˆC-3′), and can simultaneously analyze up to 15 CpG sites (Table 2). The most recent paper using this technique is by Lin et al. describing a 10-plex reaction optimized to be co-amplified in a single reaction together with an STR kit, which thus provides both tissue and individual identification, highlighting the advantage of this method [102]. To account for technical issues that can rise from incomplete digestion and suboptimal amplification, digest and positive controls were incorporated as reference markers in this 10-plex. The combined assay was highly sensitive (down to 250 pg of template DNA) and promising in analyzing imbalanced mixtures (10:90). A general advantage of enzymatic methods over other epigenetic analysis methods is that they do not require prior bisulfite conversion, which introduces serious problems to forensic epigenetic trace analysis (discussed in section 4). A general disadvantage lies in the necessary availability of a suitable methylation-sensitive restriction enzyme, especially for those epigenetic applications that rely on a restricted number of CpGs.

3.3.2. Bisulfite methylation-sensitive single-nucleotide primer extension (Ms-SNuPE)

SBE systems have been widely applied for SNP genotyping in a wide range of forensic applications, from analyzing highly degraded DNA to estimating the biogeographical ancestry and physical appearance of an unknown trace donor [103]. Over the last few years, the MS-SNuPE technology (in other words, methylation SNaPshot® [104]) has been also been applied for DNA methylation profiling, mainly for the age estimation and tissue identification. This technique was described in 2007 [105] and works similarly to standard SNaPshot®, but instead is performed on bisulfite-converted DNA. An advantage is that it is run on the widely available genetic analyzer platform, while a disadvantage lies in its multiplexing ability being restricted to ~10 CpG sites. SBE primer design is crucial to sufficiently separate the peaks from each other, while poly-T tails, commonly used in standard SNaPshot® should be avoided to prevent interactions with A-rich, bisulfite-converted DNA templates. As an example, Lee et al. successfully used a multiplex methylation SNaPshot® system to simultaneously analyze nine CpGs for the identification on five different body fluids [31]. The success relies on the necessary large methylation differences between the target and non-target tissues, where at best one signal/peak is present in all relevant non-target tissues. A detection threshold of 100rfu has been suggested for the interpretation of the multiplex results with reduced peak heights [31]. Hong et al. developed a 7-plex methylation SNaPshot® method for estimating age in saliva [43], where intermediate methylation levels are expected, but successfully detected down to 4 ng of input DNA. While this technology seems very promising for targeted epigenetic analysis in forensics, appropriate developmental validation testing is still missing to properly assess its reproducibility and accuracy capabilities. A general disadvantage of this mostly qualitative technology is the often quantitative nature of DNA methylation variation, meaning that lower-level DNA methylation differences may not be accurately detected with this method.

3.3.3. Bisulfite Sanger sequencing

Sanger sequencing is a well-established method based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during *in vitro* DNA replication. It is particularly useful in forensics for screening long contiguous DNA sequences (> 500bp) [106], for validating SNP typing results [58], and for establishing mitochondrial DNA haplotypes [107]. Within the forensic epigenetics community, Sanger sequencing of bisulfite-converted PCR products has been mainly performed for validating CpG markers obtained from microarray data, prior to the construction of targeted multiplex epigenetic assays using other technologies [26,32,91,92]. Overall, bisulfite sequencing using the Sanger technology can be useful for this purpose as it can also give information about adjacent CpG sites in the sequence, but methylation detection is only qualitative (suitable for large differences) and time-consuming.

Table 2
Summary of targeted forensic studies based on methylation-sensitive restriction enzymes (MSRE) and categorized per detection technology used.

Technology		Topic	Marker selection	Tissue/sample types	Samples	Assays	CpGs	Enzyme	Study
MSRE-based	Capillary Electrophoresis	TissueID	Literature	WB, SA, SE, SK	50	1	15	HhaI restriction enzyme	Frumkin et al. [25]
			Literature	WB, SA, SE, VA, MB, UR	135	1	8		Wasserstrom et al. [83]
			Sequence-based	WB, SA, BU, SE	64	1	8		LaRue et al. [84]
			Previous study	WB, SA, SE	144	1	4		An et al. [85]
			Previous study	WB, SA, SE, VA, MB	88	1	12		Choi et al. [86]
			Illumina 450 K	WB, SA, SE, VA, MB, NM, SK	110	1	10		Lin et al. [27]
	Quantitative PCR	TissueID	Literature	WB, SE	63	1	4	HhaI restriction enzyme	Bai et al. [87]
		Age	Literature	WB	80	4	4	EpiTect Methyl II DNA restriction Kit	Mawlood et al. [88]
	MPS	TissueID	Literature	WB, SA, SE	16	1	10	HhaI restriction enzyme	Bartling et al. [89]
	Mass spectrometry	TissueID	Representational methylation analysis	WB, SA, SE, VA	60	6	63	HhaI restriction enzyme	Ma et al. [90]

*Abbreviations - WB: whole blood, SA: saliva, SE: semen, SK: skin, VA: vaginal fluid, MB: menstrual blood, UR: urine, BU: buccal cells, NM: nasal mucosa, MPS: massively parallel sequencing.

3.3.4. Bisulfite pyrosequencing

Bisulfite pyrosequencing is by far the most popular targeted method for forensic epigenetic analysis, not only due to its simplicity, ease, and availability to forensic laboratories, but also due to its single CpG-resolution, highly quantitative nature and sensitivity [108]. The main steps of this technique include the bisulfite conversion of the DNA template, the amplification of the target gene region using bisulfite-converted gene-specific primers, template preparation and real-time sequencing-by-synthesis technology based on the detection of the release of inorganic pyrophosphate during nucleotide incorporation [109]. Multiplex pyrosequencing is possible but can be very complex [110], so that currently it is mainly used for singleplex analysis, which reflects a forensic disadvantage for cases where the evidence DNA is highly limited. This method has been successfully applied for the analysis of small genomic regions (50-100bps) that can contain multiple adjacent CpG sites [28,67]. Initial developmental validation studies have shown that singleplex bisulfite pyrosequencing assays are very sensitive (down to 50–100 pg of DNA input, depending on the locus), applicable to old stains and simulated case samples [28,29,35]. Nevertheless, due to the recommended high number of PCR cycles used during amplification (45 cycles), the reported standard deviation of methylation quantification can be as high as 10%, depending again on the locus/assay, but can be compensated by using higher bisulfite DNA input into the PCR.

3.3.5. Quantitative (q)PCR-based analysis

Real-time qPCR assays are commonly used in forensics due to their high sensitivity, for example when it comes to human-specific assessment of genomic DNA quantity and quality [111]. Moreover, qPCR is fully quantitative, which is especially suitable for analyzing DNA methylation variation, which is of quantitative nature. Forensic epigenetic qPCR assays come in various different forms, either MSRE- [87] or bisulfite-based [20], as well as either SYBR™-Green- [88] or TaqMan™ probe-based [37,38,66], and they can also include a HRM analysis at the end of the amplification process [36,45,46]. These are the key factors that determine the assay's sensitivity, specificity, multiplex capabilities and CpG-resolution. For example, SYBR™-Green-based qPCR assays come only as singleplex reactions targeting one or a few CpG sites in the primer binding region, but are cheaper than TaqMan™ probe-based ones. Using a nested-PCR approach or higher PCR cycles could increase their sensitivity, but that would also affect their accuracy [20]. On the other hand, TaqMan™ probe-based assays are more sensitive with lower standard deviation of methylation detection (< 5%) and can be multiplexed as well using different fluorescent dyes [66]. HRM analysis can be useful to study the methylation level of entire PCR fragments that may contain several CpGs, especially when large/distinct regional methylation differences are expected, but as a result, their CpG resolution is lower than the other qPCR assays mentioned above, which highly depends on the CpG density of the DNA sequence itself.

3.3.6. Massively parallel sequencing (MPS)

MPS, or alternatively known as NGS, generally refers to a high-throughput approach for the simultaneous analysis of millions of short reads from multiple (up to hundreds or thousands) amplicons being sequenced simultaneously. MPS applied in a targeted way (i.e. to sequence selected marker regions instead of the entire DNA sequence in an aliquot) has gained significant ground in the forensic field research, sometimes deemed as future technology for forensic STR/SNP analysis [112]. Targeted MPS was also proposed for DNA methylation profiling in the biomedical field [113], and it was recently introduced to the forensic epigenetic field [22]. Currently, there are two main MPS platforms used in forensics, the MiSeq system offered by Illumina based on sequencing-by-synthesis chemistry, and the PGM/S5 systems offered by Thermo Fisher Scientific based on semiconductor sequencing. Thus far, only studies using the MiSeq for DNA methylation profiling were published [26,47,75,89], showing small 9–16 multiplex capability, which is, however, related to other factors than the technology itself. While Bartling et al. has developed an HhaI restriction enzyme-based method [89], most epigenetic MPS protocols are bisulfite-based.

Advantages of this technology include fully quantitative analysis (read counts), high read depth leading to high methylation resolution (< 5%) and its ability to multiplex both epigenetic and potentially genetic markers in the future, which would be highly relevant for forensic casework analysis. Potentially high sensitivity still needs to be established. Disadvantages are the required specialized equipment not yet available in most forensic laboratories (in contrast to genetic analyzers and PCR machines required for all other epigenetic methods used in forensics), complex bioinformatics and high, but constantly decreasing, costs. Thus far, published protocols are based on various library preparation protocols that slightly differ from each other in terms of reagents used, library DNA input, library amplification cycles, and minimum read coverage, that can all influence its performance and accuracy. Future developmental validation of MPS-based DNA

methylation assays will unravel the full potential of MPS technologies, in general and in particular regarding sensitivity and multiplex ability. Next to targeted MPS used in forensic epigenetic analysis, bisulfite sequencing using MPS technology can also be applied in a non-targeted epigenome-wide way, sequencing all converted DNA sequences in an aliquot. Thus far, non-targeted bisulfite sequencing has not been applied in forensically-motivated studies likely because of the high costs involved but also because of the current forensic interest in a selected set of CpGs rather than epigenome-wide DNA methylation variation.

3.3.7. Mass spectrometry

The Agena Bioscience EpiTYPER® technology is a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry-based method for region-specific bisulfite sequencing in a quantitative, single-CpG-resolution and high-throughput manner [114]. While it is not a typical genetic method used in forensic laboratories, it has gained popularity among forensic epigenetic protocols [40,56,77,80,100]. Using the EpiDesigner® software tool, EpiTYPER® assays are easily designed and DNA methylation levels are calculated by comparing the mass signal intensity between the methylated and non-methylated DNA (a mass difference of 16 Daltons, Da per CpG position). Since DNA fragments with different mass are required, the technology cannot separate ones with identical size or detect ones outside a mass window of 1,000–7,000 Da [80]. Since EpiTYPER® reports methylation levels as CpG clusters, containing one or more CpGs (usually 2–4 CpGs) in the same fragment, the ones closely positioned to each other they will be detected as a set, lowering the technology's resolution.

3.3.8. Targeted method-of-choice depending on the purpose

Screening or validating longer candidate genes: Screening or validating gene promoters and other genomic regions (100-500bp) can be useful in small-scale marker discovery and validation of genome-wide methylation data. Targeted bisulfite Sanger sequencing and mass spectrometry-based methods are the most suitable for this purpose. The EpiTYPER® technology provides more accurate quantitative values of DNA methylation levels compared to methods base on capillary electrophoresis, and is suitable for large-scare efforts due to its high-throughput fashion. However, most forensic laboratories have access to capillary electrophoresis platforms due to their use in standard STR profiling.

Investigating short genomic regions: Short DNA regions (< 100bp) can be analyzed to provide information on the methylation levels of a small set of adjacent CpGs. For short region DNA sequencing, pyrosequencing has been by far the preferred choice, due to both its quantitative nature (~5% standard error) and easy use with a variety of pre-designed assays available, for example the EpiTect®/PyroMark® options from QIAGEN. Nevertheless, bisulfite pyrosequencing is used mainly in its singleplex form, therefore more time-, costs- and sample volume-consuming. When single-CpG resolution is not required, HRM analysis of entire PCR fragments can also be useful in its singleplex form. Additionally, when using qPCR-based approaches, researchers can investigate single or multiple CpGs present in the DNA sequence of primers or probes (~20–30bp).

Co-analyzing CpGs in multiple genomic regions: MSRE-based fragment analysis and Ms-SNuPE are the most suitable methods for simultaneously analyzing single CpGs present in multiple DNA fragments, however the maximum capacity is 10–15 CpGs in one reaction. As they are both based on capillary electrophoresis, they offer high-throughput capacity, however caution is needed during data interpretation, which tend to be more on the qualitative side. Moreover, the rise of MPS in forensic DNA and DNA methylation profiling, cannot be overlooked, and is envisioned as the tool of choice when highly multiplex assays (> 25–50 short fragments [115]) are required for multiple samples, provided that suitable multiplex PCR systems can be designed.

4. Challenges and practical considerations in forensic epigenetic analysis

Here, we outline potential sources of error and biases commonly introduced throughout the epigenetic analysis methods used. These affect not only the experimental design but also the resulting DNA methylation detection. We also discuss various practical considerations and limitations that arise from these challenges, which we suggest to be taken into consideration when developing, validating and implementing epigenetic tools, both statistical and laboratory ones, particularly for forensic purposes.

4.1. Cell type-specific DNA methylation effects

In SNP typing of single-source DNA samples it is expected that there are three versions of genotypes possible – for example, for a C/T SNP the possible genotypes are: C/C, C/T or T/T. Forensic researchers are, therefore, used to develop methods that are sufficient in their qualitative form, without the need of exact quantitative analysis, which might however be useful in mixture analysis. While this is also true for DNA methylation, meaning that each cell can also have three possible 'epigenotypes': $_{m}$C/$_{m}$C, $_{m}$C/C (hemi-methylation [116,117] or C/C, these profiles are not only tissue-specific [118] but also cell (type)-specific [119]. This implies that within a cell population contained in a forensic casework stain, the epigenome of each cell type will differ which may cause the forensic DNA methylation profile between cell types to differ. Nevertheless, what is typically detected with both epigenome-wide and targeted epigenetic techniques is the collective epigenotype of a DNA sample, meaning that we have a continuous spectrum of epigenotypes, ranging from complete non-methylation (all DNA strands of all cells being non-methylated) to complete methylation (all DNA strands of all cells being methylated). Therefore, cell type composition of a forensic sample can influence the detected DNA methylation levels. At the moment it is difficult to know the relevance of epigenetic cell-type differences for the considered forensic applications as this effect is typically ignored as of yet in forensic studies. However, in non-forensic investigations of epigenetic variation, such as in epigenome-wide association studies (EWAS) to investigate the link between epigenetic variation and phenotype trait variation (particularly disease traits), epidemiologists are aware of this effect and typically adjust for cell type composition [120]. In the future, cell-type DNA methylation effects should be taken into account also in forensically-motivated studies, both in experimental design and in data interpretation. This is highly relevant in complex tissues with multiple cell types present, such as whole blood (mixture of different types of leukocytes), saliva (mixture of buccal cells and leukocytes) and menstrual blood (mixture of vaginal cells and leukocytes). For instance, it is often seen that researchers assume that buccal swabs can be 'safely' used instead of oral fluid (saliva), which should not be the case due to saliva's complex cell composition [28,121]. Additionally, cell type composition-related DNA methylation can also be one of the factors contributing to the observed, perhaps higher than expected, inter- and intra-individual variation, especially when analyzing highly heterogeneous tissues. One approach to account for these effects and to 'correct' the obtained methylation data is to use CpGs with established profiles for each cell types - for example, via cell type-specific or single-cell epigenomic profiling. The forensic use of such approach was recently proposed [49]. We also envision that in the future combining tissue/cell type identification with other forensic epigenetic applications, such as age prediction, can be very advantageous.

4.2. Epigenome-wide data normalization

Large-scale, epigenome-wide data, such as the ones obtained with DNA methylation microarrays, need to undergo various pre-processing steps and data normalization procedures to account for a wide range of

unwanted technical and biological variation [122]. Technical variation can be caused by the different chemistries/probes involved (e.g. Infinium I and II), colour intensity differences between the non-methylated and methylated measurements, background signals, systematic errors and batch effects, introduced by different runs, instruments or operators. Biological variation can be caused by differences in cell type composition, multiple-binding probes, probes containing a SNP site and co-founding factors like age that highly affects the epigenome as a whole [123]. Various methods and pipelines have been developed by epidemiologists for preprocessing and normalizing DNA methylation microarray data, such as peak-based correction, functional, all-sample mean, subset-quantile within array, subset quantile and β-mixture quantile normalization [69,123,124]. The method, or combination of methods, to be used should be determined depending on the study and research question. When using publicly-available DNA methylation data, the normalization strategies used should be reported and accounted for (but unfortunately not always are), since differences can affect outcomes in marker discovery and prediction modelling. Furthermore, especially when pooling together different datasets, which is a common strategy also in forensic epigenetics, researchers should be aware of potential technical and biological differences between the different datasets and find ways to account for them in harmonizing the pooled datasets using available methods, such as ComBat [125] and surrogate variable analysis [126]. Nevertheless, publicly available data are not always available in the right format (for example,. idat files), which can restrict researchers from performing such analysis.

4.3. Bisulfite conversion affecting DNA quantity/quality

Treatment with sodium bisulfite ($NaHSO_3$) is a chemical modification of the cytosine residues in the DNA of a sample, during which non-methylated cytosines are converted to uracils via hydrolytic deamination, while methylated cytosines remain unchanged [127]. Subsequently, during PCR amplification and due to DNA polymerase's inability to recognize and incorporate uracil, uracils are replaced by thymines, creating a C/T variation to be detected for each CpG site. While this process revolutionized epigenetic profiling in early 90 s, and is mostly used in forensic applications until today (see above), it causes extensive DNA fragmentation and DNA loss as a result of harsh chemical processes during bisulfite conversion [128]. Currently, there are various kits offered by various manufacturers, which are based on the same principle including DNA naturation, incubation with sodium bisulfite, binding of bisulfite converted DNA on a silica-based column, removal of bisulfite by desalting, desulfonation, washing and elution of bisulfite-converted DNA. These kits can differ slightly in terms of incubation duration and minimum DNA input requirements [129,130], but usually promise ~80% recovery during elution. Furthermore, most kits require high levels of DNA amount for optimal conversion (usually 200–500 ng) [129], which is not always available from forensic-type stains. Some of the kits promise good performance with input DNA amounts as low as 50–100 pg, however the achievement of their suggested 99% conversion efficiency is questionable. The minimum requirement of starting DNA material should be established to avoid incomplete conversion, therefore overestimation of DNA methylation levels.

While there have been some efforts to develop methods to assess bisulfite DNA quality [131] and bisulfite conversion rates [132], there is no method developed thus far that can provide accurate measurement of both bisulfite DNA quantity, quality and conversion efficiency in a simultaneous manner. Hence, current experimental workflows do not include bisulfite DNA assessment prior to downstream analysis, reflecting a clear limitation that shall be overcome in future studies. In fact, different bisulfite conversion kits perform differently, which will impact downstream analysis also in forensic applications. A bias in the conversion will automatically lead to a bias in the DNA methylation detection. Using standard techniques - qPCR, UV, clone sequencing, HPLC, and agarose gel electrophoresis – Holmes et al. compared nine bisulfite kits in terms of DNA yield, DNA degradation, DNA purity, conversion efficiency, stability and handling [129]. The results indicated that the performance varied significantly between the kits, for example conversion efficiency ranged from 98.7% to 99.9% and wrongful (methylated cytosine being converted) conversion ranged from 0.9% to 2.7%. Similarly, using a more accurate digital PCR approach, Kint et al. evaluated twelve bisulfite kits, showing a wide difference in terms of DNA fragmentation (half kits resulted in qPCR Cq values > 35 when amplifying a 476bp fragment) and DNA recovery (ranging from ~30% to ~90%) [130]. Thus far, amplification of longer fragments (> 500bp) of bisulfite-converted DNA has not been reported in the literature, so this should be taken into account in primer design. Lastly, manufacturers recommend a short-term storage of bisulfite-treated DNA, but experiments are still needed to establish the *in vitro* stability of bisulfite converted DNA.

4.4. Reduced bisulfite-converted DNA complexity

Due to the majority of cytosines being converted into thymines, like non-methylated non-CpG cytosines, bisulfite-converted DNA mainly consists of three nucleotides. This reduces its complexity, making its amplification in targeted analysis particularly challenging. More specifically, designing locus-specific primers is therefore more difficult than for genetic analyses, which can be partly overcome by increasing their length, and hence, their annealing temperature. Primer specificity should be checked against the bisulfite version of the human reference genome, which is usually not included in most primer design software, except BiSearch [133]. It is important that primers also contain several non-CpG cytosines to make them bisulfite-specific, and avoid amplification of non-converted DNA strands. Moreover, the amplification of bisulfite-converted DNA is also considered more challenging, often requiring further optimized buffer conditions (e.g. increased $MgCl_2$ concentration) and DNA polymerases that handle such sequences. Secondary structures in particular can be formed, especially at lower (room) temperatures applied in pyrosequencing [109]. For the same reason, poly-T stretches should be avoided when possible, from PCR primers, adapter, sequencing primers and probes.

4.5. Amplification bias affecting methylation detection accuracy

During the PCR amplification of genomic DNA to detect short sequence variation, such as in STR or SNP typing, alleles contain mostly identical DNA sequences, except a few base pairs (i.e. repeat and SNP allele differences). Therefore, all DNA strands are being largely amplified similarly in a single source sample, with same efficiencies despite their (minor) nucleotide differences. In bisulfite-converted DNA, however, especially in fragments with high CpG-density like CpG islands and intermediate methylation levels, DNA strands are amplified differently depending on their methylation status [134]. Differences in C/T contain lead to differences in the number of hydrogen bonds connecting the two strands together, further resulting in different optimal denaturation and primer annealing temperatures. Practically, when alleles are amplified with different rates, bias are created in the detected collective DNA methylation status. Non-methylated CpGs might be amplified faster with higher efficiency due to their T-rich sequence, but the opposite is also possible depending on the sequences itself and PCR conditions. Bias can be further increased by primers containing one or more CpG site(s) with unknown methylation levels, which should be completely avoided, or controlled with dual-primers when not possible or unknown. Due to the exponential effects, PCR cycles (also during library amplification) should also be kept as low as possible, Even taking all these factors into account, biases might still be occurred, resulting in skirted 'linearity' graphs [135]. DNA controls with known methylation levels can be used for this purpose for linearity testing and data normalization. Their preparation should be made with caution by

Table 3
Summary of targeted forensic studies based on bisulfite conversion and categorized per detection technology used.

Technology		Topic	Marker selection	Tissue/sample types	Samples	Assays	CpGs	Bisulfite conversion kit	Study
Bisulfite conversion-based	Bisulfite Sanger sequencing	TissueID	Literature	WB, SA, SE, VA, MB	54	5	5	Imprint DNA Modification	Lee et al. [91]
			Previous study	WB. SA, SE	11	3	3	Imprint DNA Modification	An et al. [92]
			Previous study	WB, SA, SE, VA, MB	20	64	64	Imprint DNA Modification	Lee et al. [32]
			Illumina 450 K	WB, SA, SE, VA, MB	5	8	8	EpiTect Plus/96 Bisulfite	Forat et al. [26]
	Methylation-sensitive SNuPE	TissueID	Previous study	WB, SA, SE, VA, MB	144	2	4	Imprint DNA Modification	An et al. [92]
		Age	Illumina 450 K	SE	151	24	24	Imprint DNA Modification	Lee et al. [76]
		TissueID	Illumina 450 K	WB, SA, SE, VA, MB	316	1	8	Imprint DNA Modification	Lee et al. [32]
			Illumina 450 K	WB, SA, SE, VA, MB	461	1	9	Imprint DNA Modification	Lee et al. [31]
			Illumina 450 K	WB, SA, SE, VA, MB	484	1	9	EpiTect Plus/96 Bisulfite	Forat et al. [26]
		Age	Illumina 450 K	SA	226	1	7	EpiTect Fast DNA Bisulfite, Imprint DNA Modification	Hong et al. [43]
		TissueID	Literature	WB, SA, SE, MB	96	1	4	EZ DNA Methylation	Holtkötter et al. [39]
			Literature	WB, BU, SE, VA, MB	103	11	11	EZ DNA Methylation	Holtkötter et al. [30]
	Pyrosequencing	TissueID	Literature	WB, BU, SE, SK	42	4	24	EpiTect Bisulfite	Madi et al. [33]
			Illumina 450 K	WB, SA, SE	80	8	8	EZ DNA Methylation Gold	Park et al. [74]
			Literature	WB, SA, BU, SE, VA, MB	67	5	33	EpiTect Bisulfite	Fu et al. [93]
		Age	Literature	WB	95	10	5	EpiTect Bisulfite	Huang et al. [94]
			Literature	WB	427	1	7	EpiTect Bisulfite	Zbiec-Piekarska et al. [67]
			Literature	WB	420	8	5	EpiTect Bisulfite	Zbiec-Piekarska et al. [50]
		Twins	Literature	WB, BU	352	1	3	EZ DNA Methylation Gold	Xu et al. [95]
			Illumina 450 K	WB	8	5	5	EpiTect Bisulfite	Zhang et al. [64]
		TissueID	Literature	WB, SA, BU, SE	54	3	15	EpiTect Fast DNA Bisulfite	Silva et al. [35]
		Age	Illumina 450 K	WB	765	3	3	EZ DNA Methylation Gold	Park et al. [96]
		TissueID	Literature	WB, SA, BU, SE, VA, MB	109	11	11	MethylEdge Bisulfite	Vidaki et al. [28]
			Literature	WB, SA, BU, SE, VA, MB	122	1	10	EZ DNA Methylation Gold	Vidaki et al. [29]
		Age	Literature	WB	100	5	5	EZ DNA Methylation Gold	Cho et al. [52]
Bisulfite conversion-based	Pyrosequencing	Age	Literature	WB	190	5	5	EpiTect 96 Bisulfite	Spólnicka et al. [54]
			Literature	BL, BU	163	3	27	EpiTect Fast DNA Bisulfite	Alghanim et al. [44]
	Melt-curve analysis	Twins	Literature	BU	10	2	19	EpiTect Bisulfite	Stewart et al. [97]
		Age	Literature	WB	104	2	24	EpiTect Fast DNA Bisulfite	Hamano et al. [46]
		TissueID	Literature	WB, BU, SE	26	1	6	Bisulfite Conversion	Antunes et al. [36]
		Age	Literature	SA, CB	254	2	14	EpiTect Fast DNA Bisulfite	Hamano et al. [45]
	Quantitative PCR	Tissue ID	Literature	WB, SA, SE, VA	58	1	3	EpiTect Bisulfite	Watanabe et al. [38]
			Literature	WB, SA, SE, VA	45	1	3	EpiTect Bisulfite	Watanabe et al. [37]
		Twins	Illumina 450 K	WB	40	28	28	EZ DNA Methylation Gold, MethylEdge Bisulfite	Vidaki et al. [20]
			Illumina 450 K	SA, BU, CB	6	22	22	MethylEdge Bisulfite	Vidaki et al. [66]
	MPS	Age	mtDNA	WB	82	1	54	EZ DNA Methylation-Direct	Mawlood et al. [98]
		TissueID	Illumina 450 K	WB, SA, SE, VA, MB	25	1	9	EpiTect Plus/96 Bisulfite	Forat et al. [26]
		Age	Illumina 450 K	WB	46	1	16	MethylEdge Bisulfite	Vidaki et al. [75]
			Illumina 450 K	WB	324	1	13	EZ DNA Methylation Gold	Naue et al. [47]
			Previous study	WB, BU, BR, BO	29	1	13	EZ DNA Methylation Gold	Naue et al. [48]
	Mass spectrometry	Age	Literature	WB	65	3	58	EZ DNA Methylation	Yi et al. [99]
			Illumina 450 K	WB	68	12	11	EZ DNA Methylation	Xu et al. [72]
			Affymetrix	WB	216	3	8	EZ DNA Methylation	Zubakov et al. [40]
			Illumina 450 K	WB	725	7	7	EZ DNA Methylation	Freire-Aradas et al. [80]
			Literature	Teeth	22	3	13	EZ-96 DNA Methylation	Giuliani et al. [100]
			Illumina 450 K	WB	209	6	6	EZ DNA Methylation	Freire-Aradas et al. [56]

*Abbreviations - WB: whole blood, SA: saliva, SE: semen, VA: vaginal fluid, MB: menstrual blood, SK: skin, BU: buccal cells, SnUPE: single nucleotide primer extension, MPS: massively parallel sequencing, CB: cigarette butts, BR: brain, BO: bones.

mixing artificially-made non- and methylated DNA controls.

4.6. Comparing DNA methylation levels between methods

As shown in Tables 2 and 3 forensic researchers use different targeted epigenetic methods to validate findings in the literature, which might have been generated using different methods. For example, prediction models are based on data generated by Illumina 450 K, while forensic tools are developed using targeted methylation techniques. When comparing data generated from two different methods, method-specific bias might become evident. Data normalization, such as Z-score transformation of the datasets [79] and others, can solve this problem, but depending on the magnitude of method-to-method differences. Potential method-specific bias shall be investigated such as by analyzing the same set of samples with the different methods. Reed et al. assessed measured DNA methylation by bisulfite Sanger sequencing and pyrosequencing [136]. While both methods seemed to provide reliable results, bisulfite Sanger sequencing seemed more sensitive than pyrosequencing at detecting DNA hypermethylation (high methylation levels), however it also resulted in higher variability. In another study comparing Ms-SNuPE, bisulfite sequencing and MPS [26], results were promising but method-to-method differences could results in methylation differences of up to 30%, which need to be carefully interpreted. Similar observations were also reported when comparing microarray- and qPCR-generated data from the same blood samples [20].

4.7. Validating candidate CpG markers

DNA methylation patterns are maintained during DNA replication by the addition of methylated groups to the daughter strands by methyltransferases [1]. However, *de-novo* methylation is also possible to introduce novel methylation tags as part of environmental impact and gene regulation [2,137], which makes DNA methylation very dynamic and often unstable. Therefore, when a candidate CpG marker is discovered, it should undergo extensive validation to investigate *in vivo* and *in vitro* stability and potential co-founding effects, like genotypic and phenotypic influences [138]. While longitudinal studies show that the majority of CpG sites are stable *in vivo* for up to 9 months as currently known [64], these effects can be CpG-specific. These include gender-specific, tissue-specific, age-dependent, population-specific, lifestyle-associated and disease-induced methylation. For instance, once a CpG site is established as age-dependent in blood in a set of healthy European males, this association should be tested not only in more European males from the same tissue but also other tissues, individual of different than European bio-geographical ancestry, females, or patients with age-related diseases.

4.8. Future implementation into forensic casework

Prior to implementing a DNA methylation-based method for forensic casework, scientists need to test its performance, based on which data interpretation guidelines should be developed [181]. Taking into account all challenges and limitations discussed above, an epigenetic method-independent developmental validation plan should include, but not limited to, testing of its: (a) *linearity*, by using DNA methylation controls to account for amplification bias, (b) *sensitivity*, by decreasing the input genomic DNA into the bisulfite conversion step, (c) *reproducibility*, by analyzing > seven replicates of the same sample under optimized conditions, to estimate standard error, (d) *bisulfite conversion* or *enzymatic digestion efficiency*, by including appropriate controls and testing samples of different DNA quality/quantity, (e) *applicability*, by testing commonly found forensic-type samples and stains, to establish potential sample/cell type-specific effects. Observed methylation values should finally be reported with a confidence interval taking into account starting DNA amounts, like it was recently demonstrated [139], and prediction/identification results in an appropriate statistical framework including likelihood ratios. The method-of-choice will determine additional aspects of a developmental validation plan.

5. Future possibilities towards forensic epigenomics

The last part of this review focuses on presenting how current and expected advances in human epigenetic science and technology will impact on the further progress and expansion of forensic epigenetics in expanding towards forensic epigenomics, as we see it. Such developments concern the introduction of new epigenetic markers beyond CpG methylation, the investigation of new forensic investigative questions as well as the application of novel technological advances that have the potential to revolutionize targeted forensic epigenetic analyses.

5.1. Promising new epigenetic marks

Epigenetics is a fast developing field of fundamental life sciences, where technological advances of the last decade now allow for the thorough investigation of the human epigenome, also beyond DNA methylation and histone modifications. Cytosine modifications at CpG sites do not only include 5-methylcytosine (5-mC) - mainly studied in forensics thus far, driven mostly by sensitivity reasons – but also other recently proposed, less abundant modifications risen from its biochemical processing, like 5-hydroxymethylcytosine (5-hmC) [140], 5-formylcytosine (5-fC) [141], and 5-carboxylcytosine (5-caC) [142]. While these cytosine variants have not thoroughly been studies yet, their promising functional role in epigenomic mechanisms in health and disease has already been highlighted, especially for 5-hydroxymethylation [143,144]. Together with these, 5-methylcytosine at non-CpG sites has also been found in the human genome, however the biological role still needs to be determined [145]. Moreover, going beyond cytosine, 6-methyladenine (6-mA) has been recently detected [146], especially as an mRNA modification, potentially adding an extra dynamic level of gene expression regulation (the so-called epitranscriptome) [147,148]. Such modifications have also been detected in mitochondrial genome (mtDNA) [149], with controversial results and extra technical complexities [150,151], which nevertheless can also be useful in the forensic context [98]. We envision that increasing evidence regarding the function of these additional epigenetic modification will determine their potential in answering forensic research questions, and we expect that their future studies will benefit forensic investigations.

5.2. Novel forensic questions

Human epigenomics, the study of (large fractions of) the entire human epigenome via epigenome-wide DNA methylation microarrays or whole genome bisulfite sequencing, has opened new doors in understanding how environmental factors impact our DNA. Amongst others, lifestyle factors [3], such as smoking [152], alcohol intake [153], drug abuse [154], diet [155], physical exercise [156] and educational attainment [157] have been reported to impact our epigenome, some more than others as far as current studies suggest. According to our view and as detailed elsewhere recently [158], forensic epigenomic profiling as an additional investigative intelligence tool to find unknown perpetrators, for predicting the age lifestyle habits, perhaps socioeconomic status or geographic area of residence, of an unknown person from crime scene DNA is the logical way to broaden FDP beyond currently used SNP analysis to predict appearance traits and bio-geographic ancestry [12]. Combining epigenetic profiling on age with epigenomic profiling on lifestyle habits, on top of genomic profiling an appearance and ancestry can guide police investigation by narrowing down the suspect pool further and further. For instance, the strong correlation between illegal drug abuse and criminal behavior is well known [159]. However, ethical, societal and legal aspects together with privacy protection issues should be carefully considered prior to

implementation of such predictive tools in forensic practice [158,160]. As this discussion is currently ongoing regarding forensic DNA prediction of appearance and ancestry in some European countries, while in others it has been legalized already, it can be expected that such discussion on epigenetic prediction of lifestyle habits will follow with advancing scientific and technological developments.

5.3. Technological advances in epigenetics and epigenomics

Scientific developments in discovering and understanding well-studied and novel epigenetic modifications has been largely enabled by advances in epigenetic technology. For example, this includes the development and popular use of DNA methylation microarray technology, which constantly being updated by increasing number of markers and their epigenome-wide coverage [161]. However, the current Illumina 850 K array for instance, still only covers ~4% of the human epigenome and it needs to be seen how many more CpGs will be analyzable with hybridization-based microarrays in the future. Alternative technologies have also been developed for unraveling the DNA methylation status on a epigenome-wide scale such as reduced representation bisulfite sequencing (RRBS) [162], methylated DNA sequencing (MeD-seq) [163] and high-throughput deep sequencing for methylation mapping (Methyl-MAPS) [164]. For now, they require relatively large amounts of starting DNA (in the order of hundreds of ng) limiting forensic applications, which however is expected to go down. Another interesting technological development, also including epigenomic analysis, is single cell 'omics', which if developed further and able to deal with the specific biological material typically found at crime scenes may eventually overcome the current problem of characterizing the average epigenomic features of a sample including all its cells [165,166] and to offer a solution for complex and heterogeneous tissues [167]. High-depth single-cell bisulfite sequencing has not been applied in forensics yet, but in principle can be advantageous especially in DNA mixture analysis.

All currently available DNA methylation microarray platforms are restrictive to CpG methylation only and focus on disease-associated genomic regions. To expand both the epigenetic marks and the research questions in forensic epigenomics, scientists will rely on the use of novel technologies, such as PCR-free, long-read DNA sequencing, currently mainly provided by Oxford Nanopore [168] and PacBio [169]. While the potential of MinION (Oxford Nanopore), the only portable real-time device for DNA/RNA sequencing available thus far, was only recently demonstrated in forensics for 'standard' or random SNP [170,171], and mtDNA [172] analysis, it can also be applied for investigating novel nucleotide modifications as these are differently charged DNA molecules driven electrophoretically through a nanopore [146,173,175]. Another advantage of such technologies lies in the potential combination of genomic and epigenomic information [176]. However their sequencing error rate is higher than other sequencing technologies (5–15%), which can pose a serious problem in forensics, but is improving [177,178].

Lastly, developments also in the targeted epigenetic technology side may become useful for forensic applications, including methods like droplet digital PCR (ddPCR) [179,180]. ddPCR was recently applied in forensics by Shi et al. with successful results [55], and is thus expected to be used more often in the future. The main advantage of this technology is the digitalized form of methylation detection, which can offer high sensitivity and accuracy, extremely useful to detect small methylation effects (< 5%). It currently comes in its singleplex/duplex form, meaning that separate assays need to be developed for different CpGs, which is also the case with technologies like pyrosequencing. Lastly, a targeted, high-accuracy, medium-throughput MiSeq-based method based on PCR-free library preparation was very recently introduced that combined 29 amplicons in 96 samples per run and promises a mean accuracy of methylation detection at < 1% [115], which – if confirmed in future studies - can revolutionize targeted forensic epigenetic profiling.

6. Conclusion

Forensic epigenetics is a relatively new, but fast developing, subfield of forensic genetics, with exciting current developments in mainly three forensically relevant areas i) tissue identification, ii) age estimation and iii) monozygotic twin differentiation, while various future applications to expand towards forensic epigenomics are already foreseen. Forensic researchers have taken advantage of previously reported epigenetic markers and publically available large-scale genome-wide DNA methylation microarray data to identify suitable candidate CpG markers and have analyzed them with different targeted methylation detection techniques suitable for forensic trace DNA analysis. However, each of these methods comes with advantages and disadvantages, which need to be considered when choosing the most suitable method also depending on the magnitude of methylation differences the chosen markers carry. Due their mostly quantitative nature, accurately detecting DNA methylation levels is more challenging than detecting qualitative SNP or STR genotypes, and is additionally troubled by practical considerations, such as bisulfite conversion or cell type-specific effects that should be taken into account both the study design and outcome interpretation. We expect that current and foreseen technological and scientific developments in human epigenomics will allow many more than the current three forensic questions to be addressable, which will move forensic epigenetics towards forensic epigenomics. Final casework applications of forensic epigenomic profiling will also depend on a broad discussion involving various stakeholders on the societal benefits *versus* individual privacy protection.

Conflict of interests

The authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.fsigen.2018.08.008.

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