Forensic Science International: Genetics 18 (2015) 33–48

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsig

Forensic DNA Phenotyping: Predicting human appearance from crime scene material for investigative purposes☆

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ARTICLE INFO

Article history:
Received 1 October 2014
Received in revised form 29 January 2015
Accepted 11 February 2015

Keywords:
Appearance
Externally visible traits
Physical traits
Forensic DNA Phenotyping
FDP
DNA intelligence

ABSTRACT

Forensic DNA Phenotyping refers to the prediction of appearance traits of unknown sample donors, or unknown deceased (missing) persons, directly from biological materials found at the scene. "Biological witness" outcomes of Forensic DNA Phenotyping can provide investigative leads to trace unknown persons, who are unidentifiable with current comparative DNA profiling. This intelligence application of DNA marks a substantially different forensic use of genetic material rather than that of current DNA profiling presented in the courtroom. Currently, group-specific pigmentation traits are already predictable from DNA with reasonably high accuracies, while several other externally visible characteristics are under genetic investigation. Until individual-specific appearance becomes accurately predictable from DNA, conventional DNA profiling needs to be performed subsequent to appearance DNA prediction. Notably, and where Forensic DNA Phenotyping shows great promise, this is on a (much) smaller group of potential suspects, who match the appearance characteristics DNA-predicted from the crime scene stain or from the deceased person's remains. Provided sufficient funding being made available, future research to better understand the genetic basis of human appearance will expectedly lead to a substantially more detailed description of an unknown person's appearance from DNA, delivering increased value for police investigations in criminal and missing person cases involving unknowns.

1. Forensic DNA Phenotyping: some general considerations

Forensic DNA analysis, i.e., the identification of persons via short tandem repeat (STR) profile matching of unknown evidence material with reference material from known persons, has been considered the golden standard in forensic sciences [1]. However, one of the major limitations of this comparative approach of DNA identification, likewise applying to STRs and single nucleotide polymorphisms (SNP), is that it typically fails to identify persons whose STR or SNP profile is not already known to the investigators. Persons may be unavailable for comparative DNA profile matching because they have successfully escaped police investigations and thus avoided becoming a known suspect. Although this current approach becomes more effective when forensic DNA (profile) databases are in place [2], cases where the evidence DNA profile does not match that of any known person including all stored in the forensic DNA (profile) database are routinely seen by investigators. In the absence of any other information that provides leads for tracing unknown forensic sample donors, cold cases can wait for various periods of time (sometimes for very long), before the evidence STR profile is matched with a known person subsequently added to the grown forensic DNA database or delivered as suspect by police re-investigation of the given case.

DNA mass screenings can be carried out in cases where no DNA profile match is obtained and no other evidence is available [3]. In such DNA dragnets, larger number of persons (hundreds to thousands), usually those living in the geographic region where the crime occurred, are invited to voluntarily provide a saliva sample for STR profiling. Although the true perpetrator may not participate voluntarily, due to awareness of the provided sample leading to identification, non-participation may raise suspicion and thus directing investigators towards additional leads. If the true perpetrator does not participate but only close relatives do, familial search is able to identify them, which provides investigative leads to find the unknown perpetrator. Using conventional

☆ This article is dedicated to the 10th Anniversary of the Department of Forensic Molecular Biology at Erasmus MC University Medical Center Rotterdam (http://www.erasmusmc.nl/fmb/), and all its current and former members involved in fundamental and applied research on human appearance genetics and DNA prediction, among various other achievements between 2004 and 2014.

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http://dx.doi.org/10.1016/j.fsigen.2015.02.003
1872-4973/

autosomal STR profiling in the DNA dragnet limits the possibilities of familial search to close relatives of the unknown, non-participating perpetrator, which can be overcome by using Y-chromosomal STRs instead (if the evidence DNA originates from a male perpetrator) [4]. Since a Y-STR profile identifies a man together with all his paternal male relatives, close and distant ones, a Y-STR dragnet is more effective than dragnets based on autosomal STRs (or SNPs). For instance, a large Y-STR dragnet involving thousands of local volunteering men finally led to solving a murder case in the Netherlands after 13 years of investigation [5]. Still, in order to be potentially successful, close and/or distant relatives of the true perpetrator (if not the perpetrator himself) have to participate in the DNA mass test. The local presence of relatives may be more likely in rural areas, where relatives are less likely to migrate away, than in urban areas. In general, however, such DNA dragnets without specific cause and evidence to ask volunteers are often seen critically due to ethical concerns, and in some countries are legally forbidden. Furthermore, the economic burden to obtain STR profiles of hundreds or even thousands of individuals in a single case is high. Because of these reasons, DNA dragnets are not applied often [3–5].

These limitations of comparative DNA profiling stimulated a relatively new development within forensic genetics, i.e., Forensic DNA Phenotyping (FDP) [6,7]. FDP aims to infer the unknown stain or sample donor's externally visible characteristics (EVCs) from DNA (or other molecular biomarkers) directly from the biological material left behind at the scene of crime, or obtained from unknown bodies. In essence, FDP outcomes can serve as "biological witness", and may potentially provide even more accurate information than human eyewitnesses do, who are known to be unreliable [8]. As such, FDP is expected to provide investigative leads allowing to trace unknown perpetrators, who are not identifiable via conventional comparative DNA profiling. FDP is also expected to be useful for missing persons identification, i.e., in cases where reference DNA profile from putative ante-mortem samples, or from putative relatives are unavailable. The DNA inference of bio-geographic ancestry (see Philipps in this issue) is sometimes considered part of FDP [7]; however, genetic ancestry does not always portray an externally visible characteristic, particularly in individuals of mixed genetic ancestry.

Appearance prediction from DNA for forensic usage started in the early 2000s and first progressed very slowly. The main reason for the relatively late introduction of forensic appearance prediction from DNA is the (still) limited knowledge about the genetics of most human EVCs. Even though it takes the same technological equipment and statistical methods to identify disease genes as to find EVC genes, our knowledge about inherited diseases is currently more advanced [9] than on how we look. One of the reasons for limited appearance genetic knowledge till today might be related to research funding strategies that typically focus more on disease-related variation than on normal human variation and its genetic exploration. Of all EVCs, those that involve pigmentation i.e., variation in the coloration of the human iris, head hair, and (less so) skin, are the best and currently the only examples of practical FDP (see below). Although all EVCs are considered complex traits, where several to many genes are contributing to the phenotype together with environmental factors, human pigmentation traits in general seem the least genetically complex of all EVCs, with a few handful of genes providing most of the phenotypic information, at least on a broad categorical level. Therefore, understanding the genetic basis of pigmentation traits is currently more advanced than for any other EVC, and thus is DNA-based pigmentation prediction. All other EVCs are, based on current knowledge or expectations developed from current knowledge, genetically much more complex with dozens to expectedly thousands of genes contributing, which complicates the identification of responsible genes and predictive DNA markers.

The problem with highly complex genetic traits, as realized for many common diseases, is that every individual gene contributes only a small proportion of the phenotypic variance, and only the combination of a large number of genetic factors may explain the overall inherited component [10]. Moreover, the larger the environmental component, the less can be explained by DNA, and – of course – any non-genetic contribution can never be explained by a DNA test. According to anecdotal knowledge, and based on previous findings from twin heritability studies [11], human EVCs typically carry a large genetic component, but environmental impacts also exist, for some EVCs more so than others. If however a gene only has a small individual effect on the phenotypic trait, it is difficult to be identified with the current toolbox used by genetic epidemiologists, because the measurable statistical signal is minutely small. Therefore it requires the use of large sets of individuals to identify such small genetic effects with the needed level of statistical significance. Since the genomic tools used for finding genes, such as SNP microarrays, are still expensive (i.e., approx. 250 EUR per individual sample, and exome or whole genome sequencing are by magnitudes more expensive), carrying out genome-wide association studies (GWASs) on large numbers of individuals (i.e., tens of thousands) with large numbers of single nucleotide polymorphisms (SNPs) (i.e., hundreds of thousands and more) quickly becomes unaffordable for the average single laboratory. The formation of large international consortia, has demonstrated to be highly successful in finding complex trait genes, mostly common complex diseases, by combining impressively large numbers of samples (up to hundreds of thousands) [9]. Consequently, given the complex genetic nature of EVCs, only large collaborative efforts will allow unveiling their genetic basis as a prerequisite for developing predictive DNA markers and tools for practical FDP.

2. DNA phenotyping of pigmentation traits: the first FDP success story

In the following three sub-chapters I summarize the current knowledge on DNA-based prediction of eye, hair, and skin color, respectively. Due to space constrains, and because it is the predictive value of a SNP that is relevant for FDP purposes, I mostly leave out association and linkage studies on human pigmentation traits. Table 1 lists all SNPs previously applied for eye and/or hair and/or skin color prediction from DNA.

2.1. Eye color

The first two studies that performed DNA-based iris (eye) color prediction were published in 2007. Frudakis et al. [12] used 33 SNPs from the *OCA2* gene, which allowed them to classify 8% of the eye colors observed among >1000 samples. Sulem et al. [13], embedded in the first GWAS on human pigmentation traits, used 9 SNPs from 6 genomic regions (*SLC24A4, KITLG, 6p25.3, TYR, OCA2–HERC2,* and *MC1R*) which they identified with significant eye color association among several thousand Europeans, for categorical eye color prediction. Of the individuals DNA-predicted with <0.2 probability for brown and <0.1 probability for green, about 90% were indeed blue eyed, and of the individuals DNA-predicted to be brown with >0.5 probability, about 60% were indeed brown eyed. In 2008, three parallel studies [14–16]

reported the *HERC2* gene as the most important eye color gene. Sturm et al. [14] and Eiberg et al. [15] highlighted *HERC2* rs12913832 as a major eye color predictor, while Kayser et al. [16], due to the SNP content of the microarrays used in their GWAS, highlighted several other *HERC2* SNPs such as rs916977. Sturm et al. [14] reported an R^2 value (see Box 1) of 0.68 for *HERC2* rs12913832 alone. Eiberg et al. [15] noted a particular h-1 haplotype based on 13 SNPs from the *OCA2–HERC2* region, including *HERC2* rs12913832, but also others such as *HERC2* rs916977, being present in 97% of the analysed persons with blue eyes. Kayser et al. [16], who singled-out the *HERC2* gene via GWAS, additionally performed formal DNA-based prediction of eye color using 3 SNPs (*HERC2* rs916977, *OCA2* rs11855019, and *OCA2* rs7495174). The authors obtained prevalence-adjusted average prediction accuracies expressed as area under the receiver characteristic operating curve (AUC, see Box 1) of about 0.8 for brown and blue eye color, respectively (where 0.5 means random prediction and 1.0 means completely accurate prediction); most of the eye color predictive value was provided by the *HERC2* rs916977 alone.

The first comprehensive DNA prediction study on eye color was published in 2009 by Liu et al. [17], where the authors selected from previous publications 37 SNPs from 8 pigmentation genes, and investigated their eye color predictive capacity in a total of >6100 Dutch Europeans. They trained an eye color prediction model based on 24 SNPs from 8 genes in >3800 samples, and validated the model in >2300 independent samples (see Box 1). This model provided AUCs of 0.93 for brown and 0.91 for blue eye color, while for intermediate eye color the AUC was considerably smaller at 0.73. A single SNP, *HERC2* rs12913832 that was emphasized before to carry substantial eye color information [14], expressed most of the predictive effect, solely achieving AUC values of 0.899 for brown and 0.877 for blue. Liu et al. [17] proposed 6 SNPs from 6 pigmentation genes (*HERC2* rs12913832, *OCA2* rs1800407, *SLC24A4* rs12896399, *SLC45A2* rs16891982, *TYR* rs1393350, and *IRF4* rs12203592) as a minimal set of eye color DNA predictors. This 6-SNP set achieved AUC values of 0.93 for brown, 0.91 for blue, and 0.72 for intermediate eye color in the >2300 Dutch Europeans used for model validation [17].

In a parallel study published in 2010, Valenzuela et al. [18] tested 75 SNPs from 24 pigmentation candidate genes for their predictive effect on pigmentation variation in eye, hair, and skin using >780 Europeans and non-Europeans. Three SNPs from 3 pigmentation genes, *HERC2* rs12913832, *SLC45A2* rs16891982, and *SLC24A5* rs1426654 (the former two overlap with the best six from Liu et al. [17]), provided an R^2 from multiple linear regression modelling (see Box 1) of categorical eye color of 76.45%; of this, the vast majority (74.8%) was achieved by *HERC2* rs12913832 alone [18]. However, mixing Europeans and non-Europeans in the ascertainment of eye color predictive SNPs poses a challenge in separating ancestry from eye color effects, so that the achieved prediction outcomes are difficult to interpret. Indeed, additional data [19,20] suggested that *SLC24A5* rs1426654 is unlikely to be directly involved in eye color (see further explanation below for hair color). In 2010 too, Mengel-From et al. [21] confirmed in almost 400 Danish Europeans the eye color predictive value of *HERC2* rs12913832, which together with two other *HERC2* SNPs rs1129038 and rs11636232 in strong linkage disequilibrium (LD) with rs12913832 in Europeans, and *OCA2* rs1800407 provided likelihood ratios (see Box 1) from 4-SNP diplotypes of up to 29.3 for dark and up to 10.7 for light eye color (rs12913832 and rs1800407 overlap with the best 6 SNPs from Liu et al. [17]).

Based on previous findings together with the SNP prediction rankings observed by Liu et al. [17], the first DNA-based eye color prediction system for forensic usage was developed by Walsh et al. [22] and published in 2010/2011. This IrisPlex system includes a sensitive assay for multiplex genotyping of the six most eye color predicting SNPs from Liu et al. [17] (*HERC2* rs12913832, *OCA2* rs1800407, *SLC24A4* rs12896399, *SLC45A2* rs16891982, *TYR* rs1393350, and *IRF4* rs12203592) and implemented the prediction model from Liu et al. [17] into an interactive and easy-to-use Excel sheet that provides categorical eye color probabilities from user input SNP genotypes. The forensic developmental validation of the IrisPlex assay was published in the same year, demonstrating that the assay is fully compatible with all SWGDAM guidelines [23]. The IrisPlex assay is highly sensitive, delivering complete 6-SNP profiles down to about 30 pg input DNA [22,23]. The IrisPlex system was further tested to predict eye color in 940 worldwide DNA samples from HGDP-CEPH [22]. Although exact eye color phenotypes were unavailable for these samples, different eye colors were only predicted in European samples and less so in those from neighboring regions, while in samples from distant regions such as East Asia, Africa, Oceania and from Native Americans, only brown eyes were predicted (except a single Native American who was unpredicted) [22]. Therefore, the distribution of the IrisPlex-predicted eye color is highly concordant with the known global distribution of eye color categories, which strongly suggests that IrisPlex performs well, regardless to the bio-geographic origin of the sample under testing [22].

Although the initially introduced IrisPlex eye color prediction model was based on thousands of Europeans [17], they were all from one population (Dutch); therefore, a subsequent validation of the prediction model on >3800 Europeans from seven countries was performed [24]. The model based on thousands of samples from across Europe performed nearly identical to the initial model based on thousands of Dutch Europeans, demonstrating the robustness of the IrisPlex model [24]. The AUC values achieved in this pan-European study were even higher than established before [22] with the IrisPlex markers (0.96 for blue and brown, respectively). The authors attributed the gain in accuracy to the use of more accurate eye color phenotype data they obtained from high-resolution digital images. In 2014, the enhanced IrisPlex prediction model for eye color was introduced based on >9100 individuals from eight parts of Europe, which achieved AUCs of 0.95 for brown, 0.94 for blue, and 0.74 for intermediate eye color [25]. When applied to an independent set of about 120 Polish individuals, not included in model building or validation, the enhanced IrisPlex model delivered on average eye color prediction accuracies of 84%; or 93% when only brown and blue prediction was assessed and the intermediate category was not included [25].

Recently, the IrisPlex assay was tested by the European DNA Profiling Group (EDNAP) of the International Society for Forensic Genetics (ISFG) in a multi-center exercise involving >20 laboratories with various levels of specific experience (including novices), and was found to be easy to implement and highly reliable [26].

All 6 IrisPlex SNPs were included in a commercial tool, the Identitas V1 Forensic Chip, allowing to infer biogeographic ancestry, appearance, relatedness, and sex from genome-wide SNPs, which had been tested in a large number of DNA samples [27]. This tool (http://identitascorp.com/) provides among other forensically relevant information (including hair color) eye color prediction using the IrisPlex model [27]. However, as with all SNP microarrays, the underlying hybridization technology provides challenges to low quantity and/or low quality input DNA [27].

Subsequent to the initial publication of IrisPlex, other SNP sets with partially overlapping markers have been proposed for eye

color prediction. In 2011, Spichenok et al. [28] introduced a 6-SNP set including *HERC2* rs12193832, *IRF4* rs12203592, *SLC45A2* rs16891982, *OCA2* rs1545397, *ASIP* rs6119471, and *MC1R* rs885479, of which the former three overlap with IrisPlex [22]. These SNPs were ascertained from, and prediction was performed in >550 European and non-European samples. As mentioned before, the across-ethnicity study design for selecting predictive SNPs applied to a regional trait such as European eye (and hair) color variation is, however, unable to differentiate between true eye color effects and ancestry effects. Most of the previous studies could not show an effect of *MC1R* on eye color variation, although this gene is well-known for having a strong effect on light skin color, freckles, and red hair as seen in Europeans.

Pneuman et al. [29] attempted to directly compare the outcomes of the 6-SNP set from Spichenok et al. [28] with the 6-SNP IrisPlex set [22]. The authors reported that the IrisPlex set and prediction approach (error rate 31% plus 26% inconclusive outcomes) predicted eye color much less accurately than the Spichenok set and prediction approach (2.8% error rate without inconclusive outcome). Such a large discrepancy appears unexpected given the overlap in the SNPs used, particularly the top ranked eye color predictor *HERC2* rs12193832 which provides most of the predictive value, and may be explained by multiple factors. However, it should be noted that for the predicted outcomes there is a definitional difference between the two approaches used. The prediction outcomes from IrisPlex are specific in that they are probabilities for having blue, brown, or intermediate eye color. On the other hand, the outcomes of the Spichenok prediction approach [28] include "non-blue" and "non-brown" predictions, which are expected at higher accuracies. Such a "non-color" prediction approach is conceptually prone to argument, and, in the very least, makes a direct comparison with a color prediction approach difficult. Furthermore, IrisPlex eye color prediction [22,23] is based on a statistical model using underlying phenotypic and genotypic data (see Box 1), while the Spichenok approach [28] is based on an ad hoc classification procedure not using statistics. Of the 135 errors reported for IrisPlex by Pneuman et al. [29] 130 (96.3%) were seen with individuals of intermediate eye color. Notably, however, the IrisPlex test was introduced for accurate blue and brown eye color prediction, while its value for predicting intermediate eye colors has always been highlighted as its greatest limitation [22–25]. Recently, the Spichenok 6-SNP set for eye color prediction was updated to a 5-SNP set by excluding the *MC1R* and the *OCA2* SNPs and including an additional IrisPlex SNP (*SLC24A4* rs12896399) [30], so that both sets now overlap in 4 SNPs.

Allwood and Harbison [31] tested 19 SNPs from 10 pigmentation genes in a small sample of 101 New Zealanders of European and non-European origin, and proposed a set of 4 SNPs i.e., *SLC24A4* rs12896399, *OCA2* rs1800407, *TYR* rs1393350, and *HERC2* rs1129038 for eye color prediction, of which the former three overlap with IrisPlex and the latter one is in strong linkage disequilibrium (LD) with *HERC2* rs12913832 ($R^2 > 0.99$ in Europeans) included in both sets. These 4 SNPs achieved prediction accuracies of 89% for blue and 94% for brown eye color using a classification tree model approach (see Box 1).

Ruiz et al. [32] described an eye color prediction test based on 23 SNPs out of 37 that were tested via two multiplex genotyping assays in >410 Europeans, and obtained eye color phenotypes from SNP genotypes via an Bayesian classifier (i.e., Snipper) (see Box 1). The authors particularly emphasized that adding *HERC2* and *OCA2* SNPs that are in LD (partly in strong LD) with *HERC2* and *OCA2* SNPs included in the IrisPlex, leads to increased prediction accuracies, particularly for intermediate eye color [32]. For a subset of 13 SNPs i.e., the 6 IrisPlex SNPs plus 4 *HERC2* SNPs (rs1129038, rs11636232, rs7183877, and rs1667394), and 3 *OCA2* SNPs (rs4778241, rs4778232, and rs8024968) they reported AUC values of 0.999 for blue, 0.990 for brown, and 0.816 for intermediate eyes [32]. This is compared to 0.986, 0.978, and 0.756, respectively, obtained by the authors with the 6 IrisPlex SNPs in the same samples. Previous studies [16,17,21,33] had already noted beneficial predictive values when considering LD SNPs, including those from *HERC2* and *OCA2*, but the observed effects were smaller than those reported by Ruiz et al. [32]. Possible explanations are the use of more accurate phenotypes and/or accuracy over-estimations, for instance due to sample size limitations (see Box 1). Freire-Aradas et al. [34] recently showed that the inclusion of *HERC2* rs1129038, as advocated by Ruiz et al. [32] to improve intermediate (green-hazel) eye color prediction, revealed higher than expected green-hazel predictions in people from the Americas, Middle East, and West Asia for which IrisPlex predicted brown eyes as is expected in such regions (no eye color phenotypes were available in these samples). As emphasized by Ruiz et al. [32], additional studies are needed to better understand the additive values of SNPs in LD with each other, such as those from the *HERC2–OCA2* region, to the prediction of eye color.

Recently, Yun et al. [35] compared two eye color prediction algorithms, the IrisPlex model they implemented in FROG-kb (http://frog.med.yale.edu/FrogKB/) and *Snipper* (http://mathgene.usc.es/snipper/) as used by Ruiz et al. [32], in data from the 6 IrisPlex SNPs obtained in >900 samples from 12 Eurasian populations. Out of >700 individuals with complete IrisPlex profiles, 22% inconsistent predictions were observed between both approaches, demonstrating that the differences in the underlying logic and supporting data of both approaches can yield different prediction outcomes [35]. Overall, the authors reported fewer blue eye predictions and more inconclusive results obtained with *Snipper* versus with the IrisPlex prediction model, whereas IrisPlex revealed no intermediate eye color predictions while *Snipper* did in 29 instances [35]. Due to the lack of eye color phenotypes in the samples used in this study, it cannot be said, which prediction approach was more accurate. Both methods, however, performed similarly well in predicting brown eye color in all individuals from the 4 East Asian populations used [35], one of the geographic region where only brown eye color is expected to exist.

Lately, non-European populations that have experienced European admixture in their population history, such as those from the Americas, are starting to be explored for DNA-based eye color prediction [34,36]. For instance, Dembinski et al. [36] analysed the 6 IrisPlex SNPs in 200 U.S. Americans; using the initial IrisPlex model, they obtained high rates of correct predictions for blue eye color (95% using an 0.7 and 0.5 probability threshold), while for brown eyes less correct predictions where obtained (76% and 88% with the 0.7 and 0.5 threshold, respectively), and no correct intermediate predictions were found. The authors reported more inconclusive results than seen in the initial IrisPlex data, which they explained by the greater number of intermediate eye color individuals used as a result of the hypothesized admixture effect in U.S. Americans studied [36]. Notably, most incorrect and inconclusive eye color predictions were found in individuals that were heterozygote for *HERC2* rs12931382 (which were 30% of the samples they analysed). Clearly, more data on European-admixed populations are needed to better understand the relationship between genetic admixture, eye color, and DNA-based eye color prediction accuracy using existing or newly developed SNP sets.

Although some groups e.g., Ref. [32] favor the increase of SNP predictors for eye color over the 6-SNPs used with IrisPlex, Pietroni

et al. [37] recently suggested the opposite strategy. Referring to the tradition of "conservative statistical weight calculation" in the field of forensic genetics, and given the by far strongest eye color prediction effect known for *HERC2* rs12913832 relative to all other current known SNP predictors for eye color, the authors suggested to limit DNA-based eye color prediction solely to *HERC2* rs12913832 [37]. Without any statistical help (see Box 1), such ad hoc approach concludes blue eyes when the homozygote GG(CC) genotype is observed, brown eyes when the homozygote TT(AA) genotype is found, while all heterozygote individuals remain inconclusive [37]. Although this approach was already applied earlier in the bone DNA identification of Nicolaus Copernicus and his *HERC2*-predicted blue eye color [38], further discussion in the field will show, if this rather simplistic view on DNA-based eye color prediction will be followed more widely.

A different issue with DNA-based eye color prediction that was controversially discussed recently is whether or not gender provides a considerable influence on the prediction accuracies. In 2013, Martinez-Cadenas et al. [39] claimed that gender is a major factor explaining discrepancies in eye color prediction based on *HERC2/OCA2* genotypes and the IrisPlex model. Initially, their conclusion was based on blue eye prediction for which considerably lower prediction sensitivity was observed in males versus females, and intermediate eye color prediction where females displayed a considerably lower sensitivity than males. However, in the Spanish sample used, the sample size for blue ($N = 55$) and intermediate ($N = 69$) eye color, for which they observed a gender effect, was markedly lower than for brown eyes ($N = 369$), for which no gender effect was noted. In a reply letter, Liu et al. [40] suggested that these findings may rather be explained by stochastic effects due to the small sample size used; their much larger pan-European EUREYE and Dutch European datasets did not show such effect. However, Martinez-Cardenas et al. [41], in their reply to Liu et al. [40], presented a second and enlarged dataset ($N = 1170$ Spanish melanoma cases and controls) for which they showed a strong and statistically significant difference in blue eye color frequency among males (22%) and females (13%). The authors re-emphasized that in their samples females tended to present darker eye colors than predicted by IrisPlex in a significantly higher proportion than males [41]. In a subsequent study, Pietroni et al. [37] found gender to be significantly associated with quantitative eye color measurements in an Italian population sample, but not in a Danish and a Swedish sample. Notably, a gender effect on quantitative eye color was observed before by Liu et al. [33], but in this study it only explained 0.04% of hue and 0.09% of saturation in the Dutch population studied, while in the Italian population analysed by Pietrone et al. [37], gender explained 4.9% of the PIR-score that is based on saturation. Pietrone et al. [37] concluded that the gender effect on eye color may be a population specific phenomenon.

However, there is currently no evidence for the existence of a X-chromosomal gene contributing to human eye color variation, which – if existing – could in principle explain gender differences in eye color, and – when used for DNA-based eye color prediction – in prediction accuracies. Moreover, it is completely unclear how the eye color effect of such a hypothetical X-chromosomal gene – if existing – would be population dependent. If population-specificity is indeed involved, this would consequently mean that the sexual selection, which is assumed to have shaped human eye color phenotypic and genotypic variation in and across Europe [42] towards the frequencies we observe today, would have acted differently on men and women in different parts of Europe, for which there currently is no evidence. Clearly, more and reasonably large datasets from different parts of Europe are needed to further explore this issue and its relevance for practical FDP. Liu et al. [40] showed in two large datasets that taking gender into account in the IrisPlex prediction modeling did not improve eye color prediction accuracy to any noticeable degree. If however, future work will indeed demonstrate that including gender information in DNA-based eye color prediction significantly improves the prediction accuracy, the will typically not oppose a problem to practical forensic work. Typically FDP such as eye color DNA prediction is performed after conventional DNA profiling (without obtaining a match), which includes the AMELY/AMELX system for sex determination, so that DNA-derived gender information would be available (or could be established from additional DNA typing) for consideration in the model-based eye color prediction, if indeed proven to be beneficial.

An area in human pigmentation (or other appearance) genetics that has started to be explored, but needs further attention, are epistasis effects and how they contribute to pigmentation (or other appearance) phenotypes and their DNA-based prediction. Such interaction between SNPs from the same and/or different pigmentation genes has been found already, particularly for eye color [32,33,43,44]. Recently, Pospiech et al. [45] in a systematic study using >1000 Polish Europeans, identified new and previously noted SNP–SNP interactions contributing to pigmentation traits. Some of these interactions increased eye color prediction accuracies, albeit not to a large degree. For instance, considering interaction between *HERC2* rs12913832 and *OCA2* rs1800407 as well as between *TYRP1* rs1408799 raised the AUC for green eyes from 0.667 to 0.697 [45].

Future research activities shall particularly concentrate on solving the limitation of all currently available eye color DNA test systems in being least accurate to predict non-blue, non-brown eye colors. The nature of non-blue, non-brown eyes representing the continuum between the two extremes of blue and brown eyes makes it expectable that DNA prediction of the intermediate eye color category is more difficult than for blue and brown, which all currently available DNA tests show. More studies need to be performed to find out if additional, previously unrecognized DNA variants exist that particularly contribute to these intermediate colors. Or instead, if these intermediate eye colors can be explained by increasing numbers of genetic loci with small individual effects also seen for blue and brown. In the future, it would also be beneficial to expand DNA prediction of eye color, as available on the categorical level with the current tools, to continuous eye color. A quantitative approach to eye color DNA prediction would increase the level of detail eye color information can be achieved. Such an approach, which would finally hand-out to investigators a specific color chart, is expected to minimize final interpretation problems that may exist with current categorical verbal outcomes provided to the investigators. For example, different investigators may have different shades of color in mind (e.g., blue, light blue, light gray) when searching for an unknown suspect after being told by forensic DNA experts that the suspect has blue eyes with a probability of 95%. Although to date accurate DNA prediction of continuous eye (as well as hair and skin) color is far from reality, because the necessary DNA markers are not yet available, achieving this goal may eventually be possible as indicated by the first studies that identified DNA predictors of quantitative eye color. Liu et al. [33] exemplified that a GWAS on continuous eye color obtained from high-resolution digital eye images in thousands of Europeans allows the identification of additional genes and predictive SNPs that remained elusive in previous gene search studies using categorical eye color phenotypes. Some other studies too have been conducted to investigate quantitative eye color [37,46].

Table 1
SNPs previously applied for DNA prediction of human pigmentation traits.[a]

Gene	SNP-ID	Chr.	Predicted phenotype			References
			Eye color	Hair color	Skin color	
LYST	rs3768056G	1	✓			[33]
SLC45A2	rs13289	5			✓	[57]
SLC45A2	rs16891982	5	✓	✓	✓	[17–19,22–36,45,50–52,57]
SLC45A2	rs26722	5	✓			[17,31,32,34]
SLC45A2	rs28777	5		✓	✓	[19,25,27,45,50–52]
EXOC2	rs4959270	6		✓		[19,25,50,52]
IRF4	rs12203592	6	✓	✓	✓	[17,19,22–36,45,50–52]
IRF4-EXOC2	rs1540771	6	✓	✓		[13]
TYRP1	rs1325127	9	✓			[33]
TYRP1	rs1408799	9	✓		✓	[17,32,34,45,57]
TYRP1	rs2733832	9	✓			[31]
TYRP1	rs683	9	✓	✓		[17,19,25,27,32,34,50–52]
TPCN2	rs35264875	11		✓		[45]
TPCN2	rs3829241	11			✓	[57]
TYR	rs1042602	11	✓	✓	✓	[13,19,25,27,31,45,50–52]
TYR	rs1393350	11	✓	✓	✓	[13,17,22–27,29,31–36,45,50–52]
KITLG	rs10777129	12			✓	[57]
KITLG	rs12821256	12	✓	✓		[13,19,25,27,45,50–52]
SLC24A4	rs12896399	14	✓	✓		[13,17,22–27,29–36,45,50–52]
SLC24A4	rs2402130	14		✓	✓	[19,25,27,45,50,52,57]
HERC2	rs1129038	15	✓			[21,31–34]
HERC2	rs1667394	15	✓	✓		[13,17,32,34]
HERC2	rs12592730	15	✓			[17,32,34]
HERC2	rs12913832	15	✓	✓	✓	[17–19,21–38,45,50–52]
HERC2	rs3935591	15	✓			[17]
HERC2	rs7183877	15	✓			[17,32,34]
HERC2	rs916977	15	✓			[16,31,32,34]
HERC2	rs11636232	15	✓			[21,32,34]
MYO5A	rs1724630	15	✓			[31]
OCA2	rs1004611	15	✓			[12]
OCA2	rs1037208	15	✓			[12]
OCA2	rs10852218	15	✓			[12]
OCA2	rs11855019	15	✓			[12,16]
OCA2	rs123439067	15	✓			[12]
OCA2	rs1375170	15	✓			[12]
OCA2	rs1375164	15	✓			[32,34]
OCA2	rs1448484	15			✓	[57]
OCA2	rs1448485	15	✓			[17]
OCA2	rs1448490	15	✓			[12]
OCA2	rs1498519	15	✓			[12]
OCA2	rs1545397	15	✓		✓	[12,28–30]
OCA2	rs1597196	15	✓			[17]
OCA2	rs1800401	15	✓			[31]
OCA2	rs1800404	15	✓			[12]
OCA2	rs1800407	15	✓	✓	✓	[12,17,19,21–27,29,31–36,45,50–52]
OCA2	rs180041	15	✓			[12]
OCA2	rs1874835	15	✓			[12]
OCA2	rs1900758	15	✓			[12]
OCA2	rs2036213	15	✓			[12]
OCA2	rs2305252	15	✓			[12]
OCA2	rs2311470	15	✓			[12]
OCA2	rs2594935	15	✓			[17]
OCA2	rs2871886	15	✓			[12]
OCA2	rs3099645	15	✓			[12]
OCA2	rs3794606	15	✓			[12]
OCA2	rs4778137	15	✓			[12]
OCA2	rs4778138	15	✓			[17,31,32,34]
OCA2	rs4778177	15	✓			[12]
OCA2	rs4778190	15	✓			[12]
OCA2	rs4778232	15	✓			[17,32,34]
OCA2	rs4778241	15	✓			[17,32,34]
OCA2	rs6497268	15	✓			[12]
OCA2	rs7179994	15	✓			[17]
OCA2	rs728405	15	✓			[17,33]
OCA2	rs735066	15	✓			[12]
OCA2	rs7495174	15	✓	✓	✓	[13,16,17,31,32,34]
OCA2	rs749846	15	✓			[12]
OCA2	rs8023340	15	✓			[12]
OCA2	rs8024968	15	✓			[17,32,34]
OCA2	rs895828	15	✓			[12]
OCA2	rs895829	15	✓			[12]
OCA2	rs924312	15	✓			[12]
OCA2	rs924314	15	✓			[12]
OCA2	rs977588	15	✓			[12]

Table 1 (*Continued*)

Gene	SNP-ID	Chr.	Predicted phenotype			References
			Eye color	Hair color	Skin color	
OCA2	rs977589	15	✓			[12]
SLC24A5	rs1426654	15	✓	✓	✓	[18,28,30,31,57]
MC1R	179insC	16		✓		[47]
MC1R	N29insA	16		✓	✓	[19,25,45,47,50–52]
MC1R	rs1110400	16		✓	✓	[19,25,27,45,47,50–52]
MC1R	rs11547464	16		✓	✓	[19,25,27,45,47,48,50–52]
MC1R	rs1805005	16		✓	✓	[19,25,27,45,47,50–52]
MC1R	rs1805006	16		✓	✓	[19,25,27,45,47,48,50–52]
MC1R	rs1805007	16	✓	✓	✓	[13,18,19,25,31,45,47,48,50–52]
MC1R	rs1805008	16	✓	✓	✓	[13,19,25,27,31,45,47,48,50–52]
MC1R	rs1805009	16		✓	✓	[19,25,45,47,48,50–52]
MC1R	rs2228479	16	✓	✓	✓	[19,25,27,31,45,47,50–52]
MC1R	rs885479	16	✓	✓	✓	[19,25,27–30,45,47,50–52]
MC1R	Y152OCH	16		✓	✓	[19,25,45,47,50–52]
NPLOC4	rs9894429	17	✓			[33]
ASIP	rs1015362	20	✓	✓		[32,34,45]
ASIP	rs2424984	20			✓	[18]
ASIP	rs4911414	20		✓	✓	[45]
ASIP	rs6058017	20	✓		✓	[17,32,34,57]
ASIP	rs6119471	20	✓		✓	[28–30,57]
ASIP/PIGU	rs2378249	20		✓		[19,25,50–52]
DSCR9	rs7277820	21	✓			[33]

[a] Genetic association studies on human pigmentation traits serving as prerequisite for subsequent developments of pigmentation predictive DNA markers are not cited here, but can be found in the reference lists of the cited articles.

2.2. Hair color

The first DNA test allowing to predict hair color was restricted to red hair and published in 2001 by Grimes et al. [47]. The authors demonstrated in a small sample that by using their DNA test based on 12 *MC1R* DNA variants developed from previous knowledge of *MC1R* determining red hair color, 96% of individuals identified with two red hair causing mutations indeed had red hair [47]. The two non-red haired individuals in the study (one blond haired, the other light brown haired) who had two red hair causing mutations described themselves as red haired in youth. In 2007, Branicki et al. [48] sequenced the entire *MC1R* gene in >180 individuals of various hair colors including 40 with red hair and additional 36 with blond-red hair, and developed a DNA test for red hair color prediction based on 5 *MC1R* DNA variants. The first DNA prediction attempt for all categorical hair colors was published in 2007 as part of the Sulem et al. pigmentation GWAS [13]. Using 2 *MC1R* SNPs, rs1805008 and rs1805007, the authors first predicted red hair. Of the individuals whose hair color was predicted red with a >0.5 probability, about 70% indeed had red hair [13]. In a subsequent approach excluding red hair individuals, they predicted other hair colors based on 9 associated SNPs from 6 genes/regions; however, the prediction of the non-red categorical hair colors were much less accurate [13]. In 2010, Valenzuela et al. [18] reported 3 SNPs i.e., *SLC45A2* rs16891982, *SLC24A5* rs1426654, and *HERC2* rs12913832 to achieve an R^2 for total hair melanin of 76.3%. In 2011, Branicki et al. [19] in their systematic hair color prediction study used 46 SNPs from 13 genes previously associated with hair color and tested them for their predictive value in 385 Europeans from Poland. The authors presented a model including 22 SNPs from 11 genes that achieved AUC values of 0.93 for red hair, 0.87 for black, 0.82 for brown, and 0.81 for blond [19]. They could not confirm a hair color prediction effect for *SLC24A5* rs1426654 as previously reported by Valenzuela et al. [18] as 98.7% of the Polish samples tested were homozygote for the derived allele and the 5 heterozygote carriers had different hair colors [19]. Together with previous data [20,49], it appears that the hair (and eye) color effect reported by Valenzuela et al. [18] for *SLC24A5* rs1426654 is likely a European ancestry effect picked-up due to the multi-ethnic study design applied [19], and because of its involvement in skin color variation (see below).

Based on previous findings together with the SNP prediction rankings observed by Branicki et al. [19], the first DNA test system for predicting all categorical hair colors in combination with categorical eye color prediction, was developed and published in 2013 [50]. This HIrisPlex system [50] includes a single multiplex genotyping assay for 24 eye and hair color predicting SNPs, including all 6 from IrisPlex, as well as two prediction models, one for hair color and the previous IrisPlex model for eye color. Of the 24 SNPs included, 11 were from *MC1R* including one indel, Y152OCH, N29insA, rs1805006, rs11547464, rs1805007, rs1805008, rs1805009, rs1805005, rs2228479, rs1110400, and rs885479; two from *SLC45A2*, rs28777 and rs16891982; one from *KITLG*, rs12821256; one from *EXOC2*, rs4959270; one from *IRF4*, rs12203592; two from *TYR*, rs1042602 and rs1393350; one from *OCA2*, rs1800407; two from *SLC24A4*, rs2402130 and rs12896399; one from *HERC2*, rs12913832; one from *ASIP/PIGU*, rs2378249; and one from *TYRP1*, rs683. All but 2 SNPs (*TYR* rs1393350 and *SLC24A4* rs12896399) were used for model-based hair color prediction, and the 6 IrisPlex SNP included were used for model-based eye color prediction. Tested in >1500 individuals from three parts of Europe, 80% of the samples ($n = 1243$) were used for hair color model building and 20% ($N = 308$) for model validation. Using a hair color prediction guide based on probability thresholds, correct predictions were obtained in 80% of red, 87.5% black, 78.5% brown, and 69.5% blond hair individuals [50]. In 2014, the forensic developmental validation study of the HIrisPlex assay was published [25], demonstrating that it is fully compatible with all SWGDAM guidelines. The HIrisPlex assay delivered complete 24-SNP profiles down to about 60 pg input DNA [25]. Also included in this publication was the enhanced hair color prediction model based on >1600 individuals. With this model, AUC values of 0.92 were obtained for red, 0.85 for black, 0.81 for blond, and 0.75 for brown hair color [25]. When applied to an independent set of about 120 Polish individuals not used for model building or validation, the enhanced HIrisPlex hair color prediction model delivered on an average hair color prediction accuracy of 73%, mostly caused by inaccurate predictions of several blonds and browns [25].

Furthermore in this paper, a freely accessible online prediction tool was introduced and is online available at http://www.erasmusmc.nl/fmb/resources/Irisplex_HIrisPlex/ which allows eye and hair color probabilities to be estimated from complete and partial HIrisPlex profiles [25]. Fig. 1 shows outcomes from HIrisPlex based eye and hair color DNA prediction in 12 individuals with varying eye and hair colors together with their eye and hair images for visual phenotype inspection. The prediction probabilities in these example individuals were obtained from complete HIrisPlex SNP profiles via the enhanced IrisPlex eye color model and the enhanced HIrisPlex hair color prediction model [25].

Moreover, by performing HIrisPlex hair color prediction in the worldwide HGDP-CEPH samples it was demonstrated that HIrisPlex performs independent of bio-geographic ancestry in predicting hair color [50]. Although hair color phenotypes are unavailable in the HGDP samples tested, different hair colors were only predicted in samples from Europe and less so neighboring regions, while in samples from East Asia, Africa, Oceania and from Native Americans i.e., regions where only black hair color is expected, only black hair was predicted [50]. The HIrisPlex system was also applied to DNA samples extracted from old and ancient bones and teeth, demonstrating its suitability in degraded DNA analysis [51]. Of the 26 DNA extracts from bones and teeth between 1 and about 800 years of post-mortem age, 23 yielded complete 24-SNP HIrisPlex profiles [51]. More recently, the HIrisPlex DNA markers (not the genotyping assay) were successfully used to obtain eye and hair color information of King Richard III of England (1452–1485) from skeleton remains that were DNA-identified to be those of King Richard III via mitochondrial DNA matching with living relatives (among other means of evidence) [52]. By using the IrisPlex and HIrisPlex models the authors revealed for the skeleton a 96% probability of having blue eyes together with a 77% probability of having blond hair. The DNA-predicted hair and eye colors are consistent with Richard's appearance in an early portrait [52].

Kenny et al. found that R93C, a functional SNP in *TYRP1,* is highly associated with blond hair in Oceanians accounting for 46.4% of

Fig. 1. Individual examples of HIrisPlex-based eye and hair color DNA prediction. Probability outcomes are provided for eye and hair color categories as obtained from complete HIrisPlex SNP profiles [50] using the enhanced IrisPlex eye color and the enhance HIrisPlex hair color prediction models [25] (http://www.erasmusmc.nl/fmb/resources/Irisplex_HIrisPlex/) for 12 individuals chosen with varying eye and hair colors. Eye and hair photographs are provided to allow visual phenotype inspection and comparison with DNA predicted conclusions. Those probabilities that led to the eye and hair color conclusions are highlighted in grey based on the highest probability rule for eye color and by using the HIrisiPlex hair color prediction guide described elsewhere [25,50]. Individual numbering is 1–6 on the left side and 7–12 on the right side. DNA-based prediction conclusions are as follows 1: black hair and brown eyes, 2: dark brown/black hair and brown eyes, 3: dark brown/black hair and blue eyes, 4: brown/dark brown hair and blue eyes, 5: brown/medium brown hair and brown eyes, 6: brown hair and brown eyes (likely with non-brown parts), 7: blond/dark blond hair and blue eyes, 8: blond hair and blue eyes, 9: blond/dark blond hair and blue eyes, 10: red hair and blue eyes, 11: red hair and brown eyes (likely with non-brown parts), and 12: red hair and blue eyes.

blond hair color [53]. However, overall, blond hair color is rare in this part of the world. R93C, marks an independent mutation in the *TYRP1* gene that is not involved in European blond hair, however, the gene is with a different SNP justifying the inclusion of rs683 but not R93C in the HIrisPlex system.

The likely reason for the least accurate predictability of blond and brown hair with the HIrisPlex system (and likely any other system developed based on currently known DNA markers) is grounded in those individuals that were blond as children but turned brown (rarely darker) during adolescence (note, that all current hair color genetic studies used adult individuals). This is supported by hair color prediction data obtained from individuals for which information on age-depended hair color change was collected via interview and non-dyed, non-greyed hair images were available [50]. Of the 157 Irish individuals used in this study, 8 were classified as blond from the images and all of them were correctly predicted as being blond with HIrisPlex [50]. For 14 individuals classified as light brown to black, the HIrisPlex model revealed a high ($p > 0.7$) probability for blond, eight of them noted that they had been blond during childhood [50]. The molecular mechanism of age-dependent hair darkening, and why it occurs in some but not all blonds, is currently unknown. Time-dependent changes in the expression of hair color genes during childhood until around puberty, when typically adult hair color is reached, are expected to be involved, but data evidence is lacking thus far. Also why this effect is hair color dependent and also shows individual variation remains to be identified. Obviously, this mechanism needs to be understood first before it may eventually be usable to improve blond/brown hair color prediction accuracies. Another age-dependent hair color feature currently not considered in the HIrisPlex model, or any other current models, is hair graying or whitening i.e., the loss of hair color with advanced age. This hair color effect also needs to be understood on the molecular level before predictive biomarkers for hair graying or whitening may be developed in the future (i.e., at best in combination with DNA prediction of chronological age, see below).

Eighteen of the 22 SNPs used in the HIrisPlex system for hair color prediction were included in the commercial Identitas V1 Forensic Chip tested in a large number of samples [27]. This tool (http://identitascorp.com/) provides among other forensically relevant information (including eye color) hair color prediction using an adjusted HIrisPlex model excluding the 4 *MC1R* SNPs (N29insA, Y152OCH, rs1805007, and rs1805009) that are not present on this chip due to technical reasons. Consequently, the hair color prediction accuracies obtainable from DNA by using this tool, particularly (but not only) for red hair, are lowered relative to the complete HIrisPlex profile and prediction model. As with all SNP microarrays, the underlying hybridization technology provides challenges to low quantity and/or low quality input DNA [27].

As already emphasized before for eye color prediction, in the future, categorical hair color DNA prediction shall also move towards quantitative hair color prediction to increase the level of detail hair color can be predicted from DNA, and to avoid putative interpretation issues of the prediction outcomes during police investigation. However, genetic studies on quantitative hair color are yet scarce [18,54].

2.3. Skin color

Compared to eye and hair color prediction, much less genetic knowledge is currently available for skin color variation. Our knowledge about the genes that determine skin color variation is far less complete than it is for eye and hair color. This mostly is because of the global distribution of skin color variation versus the mostly European distribution of eye and hair color variation, which allows the use of a rather homogenous European population for eye and hair color gene mapping, while for skin color a heterogeneous global population would need to be considered. However, classical approaches for gene mapping, such as GWAS, are unsuitable when using genetically heterogeneous study populations. Therefore, previous GWAS on skin color were performed within Europeans [13,55] or within Asians [56], but the considered skin color variation within continental groups is limited, because most skin color variation is expressed between continental groups, limiting the success of such studies in completing the list of skin color genes. This is illustrated for instance by the Valenzuela et al. [18] pigmentation prediction study. Using the same multi-ethnic study group achieved R^2 values of 76.4% for eye color and 76.3% for hair color (see above), while only 45.7% for skin reflectance based on 3 SNPs (*SLC45A2* rs16891982, *SLC24A5* rs1426654, and *ASIP* rs2424984) [18].

Spichenok et al. [28] used a 7-SNP set (the 6 SNPs described above for eye color plus *SLC24A5* rs1426654), including 2 SNPs previously described for skin color prediction by Valenzuela et al. [18], to predict not-white and not-dark skin color (see problem with a no-color prediction approach as mentioned above) and reported three errors among 398 predictions; for the remaining 28% of the tested samples no prediction was obtained due to inconclusive test outcomes. Pneuman et al. [29] verified this 7-SNP set in >250 independent samples, reported 1% error and 19% inconclusive outcomes. Hart et al. [30] used 6 SNPs (the Spichenok et al. [28] 7-SNP set without *IRF4* rs12203592), and reported no error in an >200 additional test set, while 38% were reported inconclusive. However, the extremely small error rates reported by these three studies [28–30], which all used the same ad hoc prediction approach and the same no-color prediction outcomes, must be considered with care considering the approach and outcomes used, as well as the lack of specific detail on the Europeans tested, who were all defined as white thus ignoring skin color variation existing among Europeans.

In 2014, Maronas et al. [57] published the first comprehensive skin color prediction study investigating 59 SNPs previously associated with skin, eye, and hair color (including the SNPs used by Ruiz et al. [32] for eye color prediction) in a small set of ~280 samples from European and non-European individuals, for which questionnaire-based skin color information was collected and skin reflectance was measured. The authors identified a subset of 29 SNPs that were most correlated with skin color variation in their samples. These 29 SNPs provided a separation of most white skin colored individuals from most intermediate/black skin colored individuals in a principal component analysis (PCA), while intermediate and black overlapped considerable [57]. The authors suggested 6 SNPs, *SLC45A2* rs16891982, *SLC24A5* rs1426654, *KITLG* rs10777129, *ASIP* rs6058017, *TYRP1* rs1408799, and *OCA2* rs1448484 (the former two suggested for skin color prediction by previous studies [18,28,30]), for which they reported classification success in iterative naïve Bayes analysis (see Box 1) of 98.3% for white, 92.7% for black, and 83.7% for intermediate skin color versus rest in comparison. An enlarged 10-SNP set, additionally including *SLC45A2* rs13289, *SLC24A4* rs2402130, *TPCN2* rs3829241, and *ASIP* rs6119471 (the latter one previously suggested for skin color prediction [28,30]) was also emphasized, for which AUC values of 0.999 for white, 0.966 for black and 0.803 for intermediate skin color were reported from a small validation set of 118 individuals [57]. Given the relatively small sample size used in this study (see Box 1), additional data from many more individuals are needed to better judge how accurate these and other SNPs can predict skin color categories. As emphasized before for eye and hair color, also skin color DNA prediction should eventually be moved towards quantitative prediction in the future; the first genes involved in quantitative skin color variation have already been identified [58].

Box 1. How to get from genotypes to phenotypes

A systematic (model-based) prediction analysis typically consists of three steps. The most critical concern about the validity of a prediction model is that the observations of each step, i.e., the samples used in each step, must be independent. Discovery step: the identification of the genotype–phenotype relationship is often achieved through GWAS, where a large number of SNPs are iteratively tested in a large number of individuals for their phenotype relationship. Typically, SNPs identified with genome-wide significant association ($p < 5 \times 10^{-8}$) are considered as candidate markers for model-building. Model-building step: a functional form (for example linear) is hypothesized for the genotype–phenotype relationship, and the parameters of the function are estimated as to optimize the fit of the function. Selecting a prediction model is a matter of choice, typically within two fields: statistics (for example regression models) or machine-learning techniques (for example classification tree or naive Bayes classifiers). Model-validation step: explanatory variable values are input to the parameterized function to generate predictions for the outcome. The predicted outcome values are then compared with the truly observed values to estimate the prediction accuracy via different parameters. For categorical prediction outcomes, the most frequently used accuracy parameters are the area under the receiver-operating-characteristic curves (AUC), sensitivity, and specificity; for quantitative prediction outcomes (mostly Gaussian), prediction accuracy is often expressed as correlation (R or R^2), and mean squared error (MSE).

Multinomial logistic regression (MLR)

MLR is a statistical classification method that generalizes logistic regression (LR) to multiclass problems, i.e., an outcome consisting of more than two possible discrete categories. The model can provide predicted probabilities, inside the probability space (sum equal to 1.0), for all categories of the response variable without additional assumptions. MLR was for instance used for DNA prediction of eye and hair color [17,19].

Gaussian naïve Bayes (GNB) classifier

In machine learning, naïve Bayes classifiers are a family of simple probabilistic classifiers based on Bayes' rule assuming conditional independence between all predictors. Since the form implied by the assumptions of a GNB classifier is precisely the parametric form used by LR, GNB classifier is a closely related alternative to MLR. In fact, if the GNB assumptions of independence fully hold, then asymptotically the GNB and LR converge toward identical classifiers. Indeed, a naïve Bayes classifier for eye color prediction (such as available with *Snipper*) could provide similar accuracy estimates as MLR when the same set of SNP predictors are used [32]. However, when the GNB modeling assumptions of independence are violated, the asymptotic classification accuracy for LR is often better than that of GNB. On the other hand, GNB converges toward its' asymptotic accuracy at a much faster rate than LR; consequently, GNB may outperform LR when the sample size is highly limited. Naïve Bayes classifier i.e., *Snipper* was for instance used for DNA prediction of eye and skin color [32,57].

AUC

A receiver operating characteristic (ROC) curve is a visual presentation of the performance of a binary classifier by plotting the true positive prediction rate against the false positive prediction rate at all possible thresholds. The area under the ROC curve (AUC) is the integral of the ROC curve, ranging from 0.5 (random prediction) to 1.0 (perfect prediction). An AUC value can be interpreted as the probability that the prediction model will assign a higher score to a randomly chosen positive instance (e.g., blue eye color) than a randomly chosen negative one (e.g., non-blue eye color). Thus, it represents an overall but highly condensed measure of the accuracy from a binary classifier. AUC was for instance used for DNA prediction of eye/hair color, age, and body height [17,19,62,68].

Likelihood ratio (LR)

The accuracy of a binary classifier can also be expressed as positive likelihood ratio (LR+), which can be derived from sensitivity and specificity values: LR+ = sensitivity/(1 − specificity). For interpretation, an obtained LR+ value of 10 for e.g., blue eyes means that the likelihood of a blue eye DNA prediction for a blue eye person is 10× higher than the likelihood of a blue eye DNA prediction for a non-blue eye person. LR was for instance used for DNA prediction of eye color [21].

Correlation coefficient (R or R^2)

When predicting quantitative (not categorical) traits, the prediction accuracy can be expressed as the correlation (R) between the predicted and the truly observed outcome values. R^2 is simply the square of the correlation coefficient R, and can be interpreted as the fraction of trait variance explained by the prediction model. Both R and R^2 range from 0 (random prediction) to 1 (perfect prediction). R^2 was for instance used for DNA prediction of pigmentation traits and age [18,68,75].

Cross-validation

If the sample-size is limited, cross-validation can be used to guard the required independency. A cross-validation involves splitting the whole sample set into complementary subsets, building the prediction model in some subsets, and evaluating the model performance in the remaining sets. Typically, multiple rounds of cross-validation are performed using different partitions, and the averaged accuracy and 95% quantile values are reported. If carried out properly, cross-validation is nearly unbiased; however, additional validation studies are always necessary for generalization of the model parameters in different population samples. Cross-validation was used for instance for hair color DNA prediction [19].

3. Forensic DNA Phenotyping: current progress and future perspectives

Besides pigmentation traits, no molecular prediction tests are currently available for any other EVCs (perhaps with the exception of age if considered as an EVC, see below) due to limited knowledge on genes and predictive DNA markers. Below, a brief knowledge summary of EVCs for which the first genetic data are available appearing promising for near and distant future FDP developments, is provided.

3.1. Body height/stature

The by far, largest genetic dataset of any EVC is available for body height with several large genome-wide association studies published over the last few years. Body height was used as a model for complex traits from the beginning of the GWAS era. This was possible because height is one of the few traits measured in many cohort studies for which genome-wide SNP data were obtained for disease genetic purposes, allowing the combination of large datasets for GWAS on height. The success however, in explaining

the inherited height variation with DNA, is still limited, illustrating the problem in identifying the genetic make-up of complex common traits in general, and body height in particular. A previous large height GWAS carried out by the international Genetics of Anthropomorphic Traits (GIANT) consortium including >183,000 individuals, identified hundreds of SNPs at 180 genetic loci with genome-wide significant height association, including more than 100 that were never identified before [59]. However, these 180 significantly associated SNPs only explain 10% of the height variation of the study population [59], while the heritability of body height was estimated from twin studies to be about 80%. The most recent height GWAS published by the GIANT consortium carried out on >250,000 individuals identified 697 SNPs with genome-wide significant association, which together explain 16% of height variance in their study population [60]. The authors also showed that increasing the number of SNPs below the genome-wide significant threshold allows explaining more height variance, as may generally be expected, with ~2000, ~3700 and ~9500 SNPs explaining ~21%, ~24% and ~29% height variance, respectively [60]. Notably, using large numbers of SNPs for FDP purposes likely provides no technical challenges to practical forensic DNA analysis in the near future due to massive parallel sequencing technologies (see below).

The first formal DNA prediction study on body height was published in 2009 by Aulchenko et al. [61] and achieved an AUC of 0.65 in predicting the tallest top 5% of individuals from thousands of Dutch Europeans used via all 54 height-associated SNPs known from the GWAS at the time. Thus, with 54 SNPs, height was predicted only slightly better than at random, or the toss of a coin (equalling an AUC of 0.5). Liu et al. [62] investigated the power of the 180 genetic loci identified by GIANT in 2010 with significant association to normal height variation [59], to predict extremely tall stature. By examining 770 extremely tall and >9500 Dutch Europeans of normal height, the authors reported an AUC for predicting tall stature of 0.75 based on these 180 SNPs [62], marking a considerable improvement of prediction accuracy compared with the previously used 54 SNPs [61]. It will be interesting to see how much the DNA prediction accuracy for extremely tall individuals can be further increased when using the 697 (or larger sets) identified by the 2014 GIANT GWAS [60] for formal prediction, which may be done soon.

The currently available genetic knowledge on body height clearly illustrates on one hand just how much genetic information on height is still missing, and on the other hand that accurate DNA prediction of normal height is not around the corner, and if ever possible will likely involve many thousands of SNPs (see below).

3.2. Hair loss/baldness

Currently, there are 12 genes and genomic regions known with genome-wide significant association with early-onset androgenetic alopecia (male pattern baldness, AGA), the most common form of hair loss in humans: *AR/EDA2R, TARDBP, HDAC9, AUTS2, SETBP1, PAX1/FOXA2*,*WNT10A*, 17q21.31, 3q25, 5q33.3, and 12p12.1 [63–65]. The by far strongest association is seen for SNPs located in the *AR*/*EDA2R* region on the X-chromosome. This explains why baldness is typically a male phenomenon and it means that a man inherits some of his genetic risk for baldness from his maternal grandfather. Based on 8 genetic loci, Li et al. [64] calculated a genetic risk score for AGA and established that individuals belonging to the highest risk quantile of the genotypic risk score had about six-fold increase in risk for early-onset male pattern baldness. Early-onset female pattern hair loss (FPHL), which is much more rare than male pattern baldness, seem to share some genetic basis with the male form, such as the X-chromosomal region containing the *AR* and *EDA2R* genes [66]. However, various other genes associated with AGA do not seem to be involved in FPHL [67], leaving the etiology of female hair loss largely unknown as of yet.

Although the genetics data for male pattern baldness look promising for DNA prediction, it should be kept in mind that all previous studies were performed on early-onset patients. When applied to the general population, including more typical late-onset forms, the noticeable genetic effects may be less pronounced. However, no dedicated genetic prediction study of baldness in general populations has been carried out as of yet, but is expected in the near future.

3.3. Age

Practical FDP of age-dependant EVCs, such as baldness, wrinkles etc., would strongly benefit from DNA prediction of chronological age. Furthermore, age itself may be seen as an EVC because it is visible to a certain extent. Knowing the approximate age of an unknown person certainly can provide investigative leads. In 2010, Zubakov et al. [68], based on former knowledge of the decrease in T-cells and a particular T-cell DNA rearrangement (sjTREC) with increased age, introduced a DNA test for chronological age estimation based on sjTREC DNA quantification as proxy for T-cell number. This normalized quantitative DNA test system achieved AUCs of 0.88–0.97 for age groups separated by 20 years (i.e., the generation time that typically can be concluded from appearance) [68]. Its value to predict point age was more limited, as may be expected, with an achieved R^2 of 0.835 (SE ± 8.9 years), which for a single DNA marker is remarkable high [68]. An independent study reported a slightly lower R^2 of 0.6686 and a highly significant alteration of sjTREC levels among three age groups [69]. Previously proposed genetic methods for human age estimation such as the age-dependent accumulation of particular mtDNA deletions or telomere shortening showed low accuracies and various technical problems, and are therefore regarded as not being suitable for forensic applications [70].

Recent improvements in understanding human variation in DNA methylation (i.e., the field of epigenetics), including its age dependency, has delivered a number of highly promising CpG candidate markers for age prediction. For instance, Bockland et al. [71] highlighted three sites, the promotors of *EDARADD, TOM1L1,* and *NPTX2*; two CpG markers explained 73% of age variance and predicted individual age with an average accuracy of about 5 years. Garagnani et al. [72] focussed on CpG sites in 3 genes *ELOVL2, FHL2,* and *PENK* of which *ELOVL2* appeared most promising as age prediction marker with a Spearman's correlation coefficient of 0.92. Weidner et al. [73], using >100 CpG sites, correlated DNA methylation with chronological age and reported a mean absolute deviation from age of only 3.34 years and an R^2 of 0.98; they also introduced a freely available online calculator for epigenetic aging signature. However, it had been suggested that age-dependent DNA methylation changes are associated more with biological than with chronological age [73], and the chronological and biological age of a person can be quite different, depending on, for instance, the disease status. Therefore, careful validation of DNA methylation age candidate markers, including the understanding of their biological role, will be crucial before they can be used in forensic practice.

Based on the accumulating knowledge, forensic DNA tests for age prediction based on DNA methylation are expected in the near future, and the first studies appeared already. Yi et al. [74] reported a correlation between predicted age from eight loci and observed age of $R = 0.91$ (i.e., $R^2 = 0.828$), albeit on a very small sample set of only 65 individuals, while the marker identification was performed on an even smaller set of 10 young and 10 old individuals and no validation of the markers' involvement in biological age was

performed. Based on better data evidence, Zbiec-Piekarska et al. focussed on the *ELOVL2* gene and reported age prediction using two CpGs of $R^2 = 0.859$ from blood samples of >300 individuals aged 2–75 years [75]. Evaluating their model in 124 additional samples revealed a very similar R^2 of 0.866, and an average 68.5% correct prediction when grouping individual samples into 4 age categories. Notably, R^2 values were higher in the three young and medium age categories (73–85%) and lower in the advanced age category (30%). The reduced age correlation observed in the advanced age group (60–80 years) i.e., age when typically the disease load is increased, may indicate a biological rather than chronological age effect of *ELOVL2* methylation. However, given that most crime perpetrators are not of advanced age, the practical forensic consequence for age prediction of perpetrators (but not necessarily victims) using this marker may not be severe. The authors also provide preliminary validation of their test assay [75]. The strong age correlation of *ELOVL2* methylation has now been reported in several independent studies [72,75–78], suggesting *ELOVL2* as one of the most promising age prediction marker available to date.

3.4. Hair structure

Three genes have been involved in variation of human hair morphology as of yet, two in Asians and one in Europeans. In 2008, Fujimoto et al. [79] published a genome scan on hair morphology in Asians and identified the *EDAR* gene to be associated with Asian hair thickness, which has been confirmed by subsequent studies [80] including functional work in mice [81]. In 2009, Fujimoto et al. [82] provided evidence for another gene, *FGFR2*, to be involved in hair thickness in Asians. However, because hair thickness in Asians appears uniform across people from Asia, genes like *EDAR* and *FGFR2* are not useful for FDP purposes in Asia (or elsewhere). In 2009, Medland et al. [83] published a GWAS on hair morphology in Australian Europeans and found the *TCHH* gene to be significantly associated with straight hair, explaining 6% of phenotypic variance in the study population. The derived minor T allele of one of the 4 most associated SNPs, rs11803731, which represents a coding, non-synonymous variant in exon 3 of *TCHH*, was found to be absent from East Asia, Oceania, Sub-Saharan Africa, and in Native Americans, but widespread across Europe and neighbouring regions such as North Africa, Middle East, and West Asia [83]. With more T-alleles, the proportion of straight hair increased. The authors also investigated the 170 candidate genes suggested by Fujimoto et al. [79], and reported a strong association for *WNT10A*, a gene previously associated with odonto–onycho–dermal dysplasia [83]. Although twin studies estimated a high heritability for hair curliness in Europeans [84], the genes responsible have not been identified as of yet. Given the diversity of hair morphology among Europeans, DNA prediction of straight, wavy, or curly hair would in principle be useful for FDP purposes, once enough predictive DNA markers are available via future studies.

3.5. Face

Clearly, being able to predict individual-specific faces from DNA would be the ultimate goal of FDP and the dream of police men and women. However, direct information about the genes that determine morphology of the human face is very scarce as of yet. In 2012, Liu et al. [85] published a GWAS on facial shapes, which together with a parallel GWAS by Paternoster et al. [86] currently represent the only systematic studies in search for genes involved in human facial shape variation. Liu et al. [85] using almost 10,000 Europeans identified 5 candidate genes *PAX3, PRDM16, TP63, C5orf50,* and *COL17A1* with genome-wide significant association to different facial distances measured from automated facial landmarking of 3D magnetic resonance images (MRI) of the head and of 2D portrait pictures. Three of the 5 genes (*PAX3, PRDM16,* and *TP63*) have been implicated previously in vertebrate craniofacial development and disease. Their finding of *PAX3* influencing the position of the nasion replicated parallel findings by Paternoster et al. [86], who used >3800 children; this was the only gene identified with genome-wide significance by Paternoster et al. Both studies demonstrate that, as expected for complex traits, the identified genetic effects are small and that a large number of DNA variants is likely involved in determining facial morphology. Notably, the largest effect seen by Liu et al. [85] was for *TP63* rs17447439, where in comparison to the wild-type carriers, the heterozygote carriers had a 0.9 mm and the homozygote carriers a 1.8 mm reduced eye-to-eye distance. Further indirect support for the involvement of these 5 genes in facial morphology comes from a subsequent study [87] that explored the genomic regions surrounding the previously identified face-associated SNPs [85,86] for signals of positive selection, and found elevated diversity consistent with frequency-dependent selection. Local adaptation and/or sexual selection are assumed to have shaped facial morphology during recent evolutionary history [88].

Claes et al. [89] employed a more complex approach to facial phenotyping, and used SNPs from craniofacial candidate genes with large frequency differences between three populations (US Americans, Brazilians, and Cape Verdeans). With this specific approach, the authors identified 24 SNPs from 20 genes with nominal significant facial association ($p < 0.1$) of which three (*SLC35D1, FGFR1,* and *LRP6*) were particularly highlighted by the authors [89]. In a subsequent paper apparently using the same data, Claes et al. [90] emphasized that sex and genetic ancestry provided most of the DNA-based facial composites, while the effect of the 24 "facial" SNPs was marginal (e.g., 1% accuracy increase). The selection of candidate genes and the statistical genetic approach applied by Claes et al. was criticized recently [91].

As can be seen from the very few currently available studies, we are just at the beginning of understanding which genes determine normal facial variation, and it will likely be a long way (and wait) until enough predictive DNA markers are available for practical FDP of the face. In terms of what lies in the future, if indeed complete facial appearance will ever be predictable from crime scene DNA with a high-enough accuracy to allow individual identification in a non-comparative manner via facial FDP, conventional DNA profiling including forensic DNA (profile) databases will become gratuitous. Likely, this will not happen anytime soon.

4. Burdens of forensic DNA Phenotyping

4.1. Artificially altered appearance

Obviously, EVCs can be altered artificially via cosmetic means e.g., colored contact lenses, dyed hair color, self-tanning skin lotions, artificial hairstyling, or even surgery e.g., hair transplantations, facial and other plastic surgery etc. However, to avoid being traced via police investigations, perpetrators would need to fake their appearance not only during the criminal act but also afterwards. Additionally, perpetrators would need to get their feigned appearance registered in police documentations. ID cards, drivers licenses, passports etc. all have portrait images, passports in some countries have height and eye color records; all this documented appearance information in principle could be used, together with DNA-predicted appearance from evidence material, for investigative purposes. Hence a good deal of activities and planning are required to successfully avoid FDP-guided police investigations via falsified appearance. However, even the very simple act of largely hiding from crime scenes that does not involve much planning, namely wearing gloves, is often not practised by

criminals, so that human identification from physical fingerprints remains effective to this day, even after more than 100 years of use. Thus, although theoretically and practically possible, it seems unlikely that artificially altered appearance will be a burden of practical FDP in many cases.

4.2. Multiplex genotyping issues

A serious technical burden for further expanding FDP, namely missing multiplex genotyping technologies for in-parallel analyses of large numbers of SNPs suitable for low quality and quantity forensic DNA, is currently been lifted by the application of massive parallel sequencing technologies. These so-called next generation sequencing (NGS) or second generation sequencing (SGS) technologies (see Borsting & Morling in this issue), in principle allow the targeted multiplex analysis of large numbers of SNPs, as would likely be needed in the future for a more detailed appearance prediction, once fundamental research into the genetic basis of various appearance traits has successfully provided the necessary DNA markers. Although studies showing the multiplexing analysis of thousands of SNPs via targeted NGS are still pending, it has already been demonstrated that over 500 SNPs can be successfully combined in a single targeted sequencing run combining several individuals using the Ion Torrent Personal Genome Machine (PGM) [92].

4.3. Ethical issues

Ethical burdens of practical FDP have been raised and can be found elsewhere (e.g., Ref. [7] particularly Box 2 and references cited therein). Not being an ethics professional myself, my opinion is that when it comes to EVCs, privacy issues including the right-not-to-know do not apply. This simply is because appearance traits are not only known to the person itself, but to everybody who has ever seen this person, including the police who have portrait photographs in passports, ID cards, driver-licenses etc. Therefore, EVCs in principle cannot be considered private data. Notably, this can be different for bio-geographic ancestry and its inference from DNA (see Philipps in this issue); while non-admixed ancestry on the broad continental level is usually visible externally (hence cannot be considered private information), mixed ancestry such as between continents, may not necessarily be visible depending on the number of generations back when it occurred and the geographic origin of the ancestors involved since. If indeed not externally visible, privacy issues including the right-not-to-know can apply for genetic ancestry testing.

One ethical issue not touched previously (Ref. [7] Box 2) and therefore mention here is disease-linked appearance. The use of disease information obtained from DNA can be viewed not appropriate for forensic purposes with the ethical argument that patient discrimination is to be avoided. Such reasoning likely has influenced the modification of the forensic DNA legislation in the Netherlands in 2003, which generally allows the use of DNA information on EVCs for forensic purposes, while excluding disease-linked appearance. That this reasoning is not always followed is indicated by the forensic DNA legislation of US state of Texas that implicitly allows FDP, even for genetic diseases [7].

However, although it can be the same genes that cause pathological and normal variation of a given trait including EVCs, the mutations that determine disease are usually different from those DNA variants involved in normal trait variation. For instance, the *OCA2* gene determines oculocutaneous albinism type 2 (hence the gene name), a specific form of albinism, and the *OCA2* gene is also involved in normal variation of skin, eye, and hair coloration (see above); however, the particular *OCA2* mutations that cause this disease [93] are different from those SNPs involved in normal pigmentation (see above and Table 1). Therefore, DNA tests developed for predicting normal appearance as applied for FDP purposes are typically not informative for revealing disease-linked appearance information.

Problems may arise for appearance traits where the disease phenotype reflects the extreme of the normal phenotypic variation together with associated non-causal DNA markers are used for DNA prediction (because the causal ones are yet unknown). For instance, SNPs associated with the disease cleft-lips (i.e., non-syndromic cleft lip with or without cleft palate, NSCL/P) were also shown to be associated with normal facial width – albeit less strongly than with cleft-lips [85,94], perhaps because cleft has been hypothesized to be the pathological extreme form of facial width [95]. In such case, the same SNPs are informative for the disease-linked and the normal form of an appearance trait, albeit less so for the normal form; however, the currently known SNPs are far away from allowing the DNA prediction of neither facial width nor NSCL/P. Future research may identify the causal genetic factors of cleft that likely allow differentiating between disease and normal forms of facial width (as indeed these phenotypes are clearly differentiated albeit related).

Furthermore, in the longer run one may commonly conclude (or not) that the benefit for the society and its people i.e., to catch a murderer who otherwise cannot be caught and thus continues murdering, overwrites ethical concern on patient discrimination, which could lead to using general disease information including those obtained from DNA (with and without EVC manifestation) for investigative purposes. Under such hypothetical scenario, FDP would then not be restricted to DNA prediction of externally visible traits, as it is currently understood (see all above), but would also include DNA prediction of disease traits. Besides necessary broad discussions by ethics professionals and other stakeholders on the risks and benefits of forensic use of disease information including from DNA, the medical genetic knowledge provides restrictions at least for now. Currently, our genetic understanding of disease is quite complete for many monogenic diseases (so called Mendelian diseases) i.e., diseases determined by one or very few genes, which therefore allows accurate DNA prediction as used in medical DNA diagnostics. Arguably, monogenic diseases that run in families, are of limited value for forensic investigation, because they are very rare in the general population. In contrast, genetic knowledge is yet largely incomplete for common diseases reflecting complex traits with many genes and environmental factors determining the disease outcome, which therefore does not (yet) allow accurate DNA prediction [96], for the same reasons as discussed above for complex appearance traits.

4.4. Legal issues

Legal burdens on practical FDP exist depending on the country and its legislation, for more information I refer to the specialized literature (e.g., Ref. [7] particularly Box 3 and references cited therein). In brief, for many countries the legislation that regulates the use of human DNA for forensic purposes comes from a time when DNA fingerprinting or DNA profiling were introduced, and therefore typically do not cover FDP without pending modifications. Some countries have already updated their forensic DNA legislation to allow the forensic application of DNA-based appearance (and ancestry) information such as the Netherlands. Some countries, such as the UK, allow FDP without dedicated legislation. However, I take the liberty here to draw direct parallels between FDP i.e., a “biological witness” and a human eye witness. In principle, FDP delivers the very same type of information as human eye witnesses do i.e., what did/does the suspect look like. To my knowledge however, there is no specific law that allows the police to involve human eye witnesses, so in essence, why does one need it for doing the same from DNA?

In several countries (e.g., Germany), the use of DNA for forensic purposes is currently restricted by law to non-coding markers. Strictly speaking however, the allowance of non-coding DNA markers for forensic purposes not necessarily excludes FDP, even though this may violate the intention of such laws. Non-coding DNA markers can carry the very same trait information as coding ones, as long as the linkage disequilibrium between them is high enough (e.g., because of close physical proximity with each other). Indeed, many DNA markers used and suggested for FDP purposes are intronic SNPs [22,50][e.g. 22,50] and thus by definition are non-coding. Their FDP values comes from the EVC association likely because of strong linkage disequilibrium with causal (including coding) yet unknown SNPs. Furthermore, most regulatory DNA markers are either intronic or intergenetic; thus, strictly speaking there are non-coding. Indeed, several DNA markers used for FDP purposes are regulatory SNPs from intronic or inter-genetic regions, such as the most eye color predictive SNP *HERC2* rs12913832 [97] and other SNPs from human pigmentation genes [98–100]. Because of the knowledge on the dynamic of the human genome, including its haplotype block structure and regulatory elements, which has severale advanced since such laws were made, a distinction between coding and non-coding parts appears outdated, and should therefore not be used any longer when regulating the application of DNA via legislations such as for forensic purposes. Instead the forensic use of DNA shall be regulated on the particular forensic purpose (e.g., individual identification, appearance prediction for investigative purpose etc.) but not the type of markers that likely are changing over time with increasing genetic knowledge.

4.5. Limited scientific knowledge and limited research funding

Besides the current legal burdens in many countries, the main and most significant burden that currently holds-up further FDP progress in the hopes of eventually allowing very detailed overall appearance prediction, is the immense knowledge that is currently missing on the genetic basis of human appearance. For many EVCs that have the potential to be highly useful for FDP purposes due to high heritability, the underlying genes have just not been found yet. Or, the first genes identified only explain very little of the trait variance, while most trait information remains genetically unexplained. In my opinion, the needed fundamental research to identify most, if not all, genetic information that determines human appearance variation in its various forms, can only be achieved by large international collaborations. Large genome-wide studies will be needed to map EVC-underlying genes allowing the generation of candidate markers for EVC prediction studies. Furthermore, at best, an accumulative worldwide database shall be established using standardized EVC phenotypes and genotypes. Such a database will expectedly provide more directly comparable DNA-based prediction accuracy estimates and robust model parameters. This will eventually allow the conclusion of a final set of most informative and robust predictive DNA markers for a given EVC, together with the most suitable prediction approach. From this knowledge, practical FDP tools can effectively be developed at best together with specialized commercial companies.

Besides international research collaborations, what is also strongly needed is the necessary research funding, which is currently not sufficiently available. Here, I am not only calling upon governmental and private research funding agencies around the world to come-up with calls suitable for the respective research projects, but additionally on law enforcement and police agencies as well as forensic institutions to support scientific research in this area. I think it is time to change the typical attitude in forensic science to simply wait until fundamental sciences has discover something with forensic potential, which is then picked-up for further development into practical tools by the forensic community. Many scientific discoveries, including those forensically as important as classical DNA fingerprinting, were/are made by chance reflecting the nature of curiosity-driven fundamental science, and I consider it as ineffective strategy to base efforts for improving the safety of the society on chance events. More effective would it be to invest into specific areas of the fundamental sciences, particularly within human molecular biology and genetics, that bear great potentials to improve forensic analyses to allow dedicated research into forensically-relevant topics from the start in fundamental science all the way to forensic validation of the tool developed from the fundamental knowledge produced. Clearly, one of those areas is the genetics of human appearance for developing FDP tools to solve cases that cannot be solved via other means including conventional DNA profiling, and perhaps eventually – strongly depending on the future progress in the genetic understanding and DNA-prediction of individual-specific facial morphology – replacing comparative forensic DNA profiling including forensic DNA (profile) databases.

Acknowledgements

The author thanks Susan Walsh and Fan Liu and additionally Wojciech Branicki, for useful comments on the manuscript. Susan Walsh and Lakshmi Chaitanya are additionally acknowledged for their help with Fig. 1 and Table 1, respectively, as well as Fan Liu for his help with Box 1. The author is further very grateful to all his departmental, institutional, national, and international colleagues for their collaboration in the field of human appearance genetics and DNA prediction, particularly his colleagues from the International Visible Trait Genetics (VisiGen) Consortium, most notably Tim Spector and Nick Martin. From the current and former members of the Department of Forensic Molecular Biology he specially acknowledges Fan Liu and Susan Walsh as well as Dmitry Zubakov, Mijke Visser, Arwin Ralf, and Lakshmi Chaitanya for their work on appearance genetics and DNA prediction. From Erasmus MC he particularly thanks Andre Uitterlinden, Albert Hofman, Cornelia van Duijn, Ben Oostra, Tamar Nijsten, and Hans Vingerling whose support was essential for the author's achievements in human appearance genetics and DNA prediction. He additionally thanks all other colleagues, whose own work helped improving the knowledge in this field, and which he had the privilege to summarized here, and apologizes to those he could not mention in this review article due to space constrains. The author's original work on the genetics and DNA prediction of human appearance is supported in part by the Erasmus MC University Medical Center Rotterdam (http://www.erasmusmc.nl), by previous funds from the Netherlands Genomics Initiative/Netherlands Organization for Scientific Research (NWO) within the framework of the Forensic Genomics Consortium Netherlands (FGCN) (http://www.forensicgenomics.nl) and the Netherlands Consortium of Healthy Ageing (NCHA), as well as by previous funds from the Netherlands Forensic Institute (NFI).

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