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Abstract

Facial aging is the most visible manifestation of aging. People desire to look younger than others of the same chronological age. Hence, perceived age is often used as a visible marker of aging, while biological age, often estimated by methylation markers, is used as an objective measure of age. Multiple epigenetics-based clocks have been developed for accurate estimation of general biological age and the age of specific organs, including the skin. However, it is not clear whether the epigenetic biomarkers (CpGs) used in these clocks are drivers of aging processes or consequences of aging. In this proof-of-concept study, we integrate data from GWAS on perceived facial aging and EWAS on CpGs measured in blood. By running EW Mendelian randomization, we identify hundreds of putative CpGs that are potentially causal to perceived facial aging with similar numbers of damaging markers that causally drive or accelerate facial aging and protective methylation markers that causally slow down or protect from aging. We further demonstrate that while candidate causal CpGs have little overlap with known epigenetics-based clocks, they affect genes or proteins with known functions in skin aging, such as skin pigmentation, elastin, and collagen levels. Overall, our results suggest that blood methylation markers reflect facial aging processes, and thus can be used to quantify skin aging and develop anti-aging solutions that target the root causes of aging.

Introduction

Aging is a multifactorial process. People age at different rates, and chronological age does not always reflect the actual biological age of a person. Therefore, the evaluation of biological age is of high relevance for understanding aging processes and developing interventions to extend healthy aging. Numerous biological clocks based on high-throughput biomarkers, such as methylation markers, gene expression, protein levels, and microbiota, have been proposed to measure a person's biological age.

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The 'first generation' of biological clocks have been trained on chronological age, while the 'second generation' of clocks utilize other age-related measures, including time to all-cause mortality or biomarkers of morbidity. Many biological clocks demonstrate high accuracy in predicting a person's chronological age or age-related measures [1–6]. Novel computational solutions bolster the reliability of biological clocks by extracting the shared aging signal across many CpGs while minimizing noise from individual CpGs [7].

Epigenetic predictors have become the gold standard biomarker for the estimation of human biological age. They are highly reproducible and have been validated in multiple studies. Most existing epigenetics-based biological clocks are developed using penalized regression approaches such as elastic net on a training dataset yielding a subset of CpG (from a few dozen to a few hundred) that are organized into a weighted linear prediction model of biological age. The discrepancy between epigenetic-based biological age and chronological age called

age acceleration is predictive of all-cause mortality and various age-associated diseases.

Skin samples have been utilized to construct various biological clocks. Firstly, the multi-tissue age predictor based on 353 CpGs was developed using 8,000 samples encompassing 51 healthy tissues and cell types including skin [4]. This pan-tissue predictor can be used to estimate the age of most human cell types, tissues, and organs. The skin-blood biological clock utilizes 391 CpGs and it outperforms the pan-tissue clock in all metrics of accuracy in skin samples (including fibroblasts, keratinocytes, and endothelial cells) and blood samples [6]. More recently, a skin-specific biological clock was developed using data from 508 human skin samples, including cultured skin cells and human skin biopsies [8]. This clock was further utilized for identifying a senolytic compound that reduces biological age [9].

Nonetheless, it is not clear whether CpG sites used in these biological clocks are just convenient markers, consequences of aging, or active drivers of aging [10, 11]. Current biological clocks utilize epigenetic biomarkers that are merely correlated with chronological age, and the model that optimally predicts an outcome from a statistical perspective is selected. Generally, no preference is given to the location or possible biological role of the CpG-features used as inputs in the machine learning procedure. While some biological clocks demonstrate high accuracy in predicting a person's chronological age or age-related measures, the molecular mechanisms of how these clocks work are not clear. There is little overlap between CpGs in different biological clocks pointing to the significant redundancy in DNA methylation.

It is therefore critical to identify causal epigenetic biomarkers that are not simply correlated with chronological age or age-related measures but also reflect either damage that accumulates with age or anti-aging protection. This would enable more efficient development of preventative anti-aging measures that slow down, or even reverse, age-related damage while inducing protective anti-aging processes.

In this proof-of-concept study, we explore a hypothesis that CpGs causally associated with perceived facial aging reflect biological processes that underlie skin aging. Perceived facial age is a highly relevant marker of aging. It has been shown to be associated with all-cause mortality, independent of chronological age, and other aging-associated traits [12–15]. Usually, perceived facial age is determined by specialists, such as dermatologists, or by other people. Several studies reported that large-scale data on perceived facial age can provide insights into biological age-related processes. For example, a genome-wide association study of adult participants of the UK Biobank identified 74 independently associated genetic loci which were enriched for cell signaling pathways, including the NEK6 and SMAD2 subnetworks [16]. The NEK6 subnetwork helps govern the initiation of mitosis and progression through the cell cycle and prevents cell senescence. Functional analysis of genes associated with self-perceived facial age revealed a significant over-representation of skin pigmentation, extracellular matrix, and immune-related pathways that are correlated with facial aging [17].

Recalling that "correlation is not causation", we set out to identify CpG sites that exert a causal effect on facial aging. To this end, we integrated genome-wide association (GWAS) data on perceived facial aging of UKBB participants with epigenome-wide methylation quantitative loci (meQTL) from blood, and run Epigenome-Wide Mendelian Randomization (EWMR) (for full details see Methods). We found that candidate methylation markers that causally affect facial aging have little or no overlap with most existing epigenetics-based biological clocks. However, causal CpG sites provide valuable insights into molecular mechanisms of skin aging. Furthermore, our results suggest that blood methylation markers reflect aging processes in the skin, and hence can be utilized to quantify skin aging and to potentially evaluate and develop ingestible anti-aging skin treatments.

Methods

Mendelian randomization

EWMR

As exposures in the Mendelian Randomization (MR), we utilized 11, 165, 559 SNP-CpG associations (methylation quantitative trait loci, meQTL) from whole blood samples identified through genome-wide association analysis from 3, 799 Europeans and 3, 195 South Asians [18]. Summary statistics for the approximately 11.2 million SNP-CpG pairs reaching genome-wide significance are available at [19]. In this study, we used results from the European cohort using genetic instruments at p value threshold $= 10^{-6}$.

Epigenome-wide MR (EWMR) was run on each CpG (exposure) - GWAS (outcome) pair. For the outcomes, facial aging (ukb-b-2148) and telomere length (ieu-b-4879) were obtained from the `MR-Base` GWAS catalog [20]. Additional skin-related outcomes include actinic keratosis (finn-b-L12_ACTINKERA), acute skin changes due to ultraviolet radiation (finn-b-L12_OTHACUSKINSU, finn-b-L12_OTHACUSKINSUNNAS), facial pigmentation (ebi-a-GCST90002283), basal cell carcinoma (ebi-a-GCST90013410). For facial aging and telomere length, the EWMR procedure was run on all available CpGs [18]. For skin-related traits, EWMR analyses were run on CpGs identified as causal ($p < 0.001$) in the EWMR for facial aging. To run EWMR, we used the

`TwoSampleMR` package in `R` statistical language. Pleiotropy was evaluated based on the intercept calculated by MR-Egger regression using `mr_pleiotropy_test` with p value threshold $p = 0.05$. Exposure-outcome relationships that change by at least 5% in the odds ratio (OR $\geq$ 1.05 or OR $\leq$ 0.95) at the significance level $p = 0.001$ are reported [Additional file 1: Table S1A].

Other MR

EW-GE MR analysis evaluates relationships between CpGs (exposure) and gene expression (outcome). For each CpG and its nearby gene pair, EW-GE MR evaluates whether a CpG causally results in either an increase or decrease of expression of the gene. For this study, EW-GE MR was run for CpGs causal to facial aging as identified in the EWMR step. As above, methylation at a CpG site is proxied by meQTL genetic instruments and gene expression (GE) is proxied by eQTLs from the GTeX data for all tissues as available in the `TwoSampleMR` R package. CpG sites that affect expression of a nearby gene by at least 3% in the odds ratio (OR $\geq$ 1.03 or OR $\leq$ 0.97) at the significance level $p = 0.001$ are reported [see Additional file 5: Table S5A].

To further evaluate whether changes in gene expression (GE) causally affect facial aging (outcome), we ran for genes with available eQTLs for skin tissues (skin not sun-exposed and skin sun-exposed) and whole blood as available in the `TwoSampleMR` R package (GEMR). Genes that affect facial aging in at least one tissue by at least 3% (OR $\geq$ 1.03 or OR $\leq$ 0.97) at the significance level threshold $p = 0.001$ are reported [Additional file 5: Table S5B].

Functional enrichment analysis

Enrichment analysis of causal CpGs was run using the `methylglm` function from the `methylgsa R` package. The `methylglm` function implements logistic regression adjusting for the number of probes in the enrichment analysis. It was run with default parameters using gene ontologies (GO, KEGG, and Reactome). Enrichment analysis on gene and protein levels was run using the FUMA Functional Mapping (https://fuma.ctglab.nl) using the default settings for all coding and noncoding genes. Gene-sets identified at FDR = 0.05 are reported as over-represented.

To assess relationships between CpG and serum protein levels, we used the EWAS catalog (http://www.ewascatalog.org).

Results

CpGs causally affect facial aging

Mendelian randomization (MR) is an established genetic computational approach for causal inference that recapitulates the principle of a randomized clinical trial (RCT). RCTs are very time-consuming and costly as they generally require a direct comparison of the cases and the controls to evaluate the effect of treatment (exposure). The idea behind the MR methodology is that genetic variants (SNPs), randomly assigned at conception, are robustly associated with the exposure and outcome, and not biased by environmental confounders. Thus, the biases can be circumvented by leveraging SNPs as instrumental variables (genetic instruments) to detect causal effects and estimate their magnitude.

The Epigenome-Wide Mendelian Randomization (EWMR) has been used to identify methylation markers that exert causal effects on several complex traits [21, 22] and general aging [11]. The methylation level at each CpG (exposure) is proxied by methylation quantitative trait loci (meQTL) as genetic instruments for exposure. Further integration of eQTL data suggested that genetic variants, responsible for highly significant meQTL associations, also influence gene expression, pointing to a coordinated system of effects that are consistent with causality [21]. Large-scale studies reported that methylation at gene promoters is negatively correlated with gene expression, while methylation within gene bodies can be positively or negatively correlated with gene expression [23]. Furthermore, CpG sites typically regulate multiple transcripts, and while found to predominantly decrease gene expression, this was only the case for 53.4% across $\approx$ 47,000 significant CpG-transcript pairs [22]. The EW-GE MR procedure assesses the causal effect of methylation at a specific CpG site on gene expression proxied by eQTLs across tissues, or in a specific tissue (e.g. skin).

In this proof-of-concept study, we set out to investigate whether methylation markers causally affect facial aging, and to further elucidate their functionalities (Fig. 1). To this end, we ran the EWMR procedure that utilized publicly available summary statistics data on perceived facial aging as an outcome and meQTLs measured in the whole blood as an exposure (Fig. 1 and "Methods"). To understand functions of candidate causal CpGs, functional analyses on the level of CpGs, their nearby genes, and associated proteins were conducted (Fig. 1). Further, to understand whether CpGs causal to facial aging also affect telomere lengths, and other skin-related phenotypes, additional EWMRs were run. To investigate causal effects of causal CpG on the expression of nearby genes, EW-GE MR utilized the GTeX data as outcomes, wherein expression of a gene is proxied by expression quantitative loci (eQTLs) in a specific tissue (e.g. skin and blood). Similarly, causal effect of gene expression (exposure) on facial aging is investigated via the GEMR procedure (see "Methods").

Firstly, the EWMR analysis yielded 1299 candidate CpG sites ($p < 0.001$) that causally affect perceived

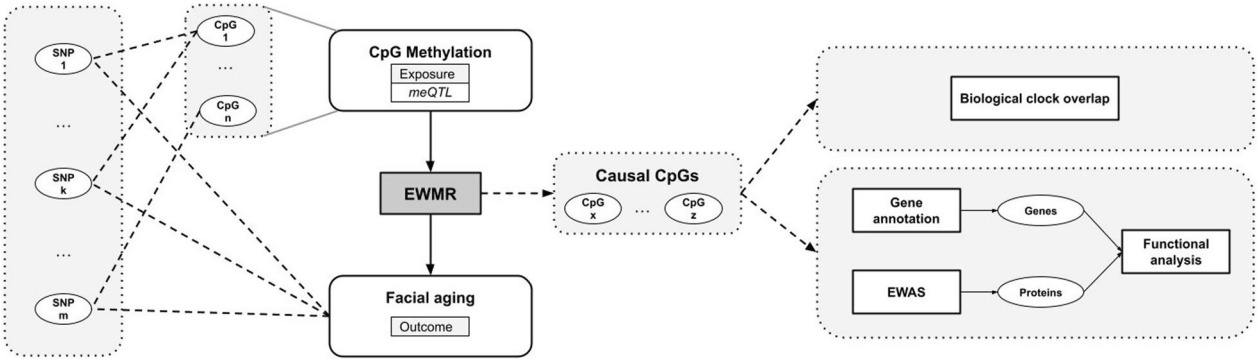

Fig. 1 High-level illustration of the computational framework to identify CpG sites causal to a phenotypic trait, and to elucidate their functionalities. Epigenome-Wide Mendelian Randomization (EWMR) is run to identify CpG sites causal to facial aging utilizing methylation quantitative loci (meQTL) from whole blood as exposure (see "Methods"). Summary statistics for ≈ 11.2 million SNP-CpG pairs reaching genome-wide significance are available [19]. For outcomes, summary statistics from genome-wide association study (GWAS) on perceived facial aging (ukb-b-2148) were obtained from the `MR-Base` GWAS catalog [20]. CpGs causal to facial aging were further interrogated by their overlap with epigenetic biological clocks available in the `methylCIPHER` R library. Functional enrichment analyses on the level of CpG were conducted using the `methylgsa` R library. Functional enrichment analyses on the level of nearby genes and associated proteins, obtained via the EWAS catalog, were conducted using the FUMA Functional Mapping tool.

facial aging (see "Methods") by at least 5% in the odds ratio (OR >= 1.05 or OR <= 0.95) [see Additional file 1: Table S1A]. There are approximately similar numbers of damaging (659) and protective (640) CpGs (Fig. 2). Damaging, or age-accelerating, CpGs causally increase or accelerate facial aging while protective, or adaptive, CpGs are causally associated with slower facial aging. Protective CpGs may also be referred to as promoting longevity or youthfulness methylation markers. This finding is in line with a recent study on general aging [11] suggesting that biological processes that adapt with age, or protect from early aging, at the epigenetic level are nearly as common as those mechanisms that drive or accelerate aging.

Acknowledging that using unadjusted p values ($p \leq 0.001$) may yield false positive candidate CpGs, we

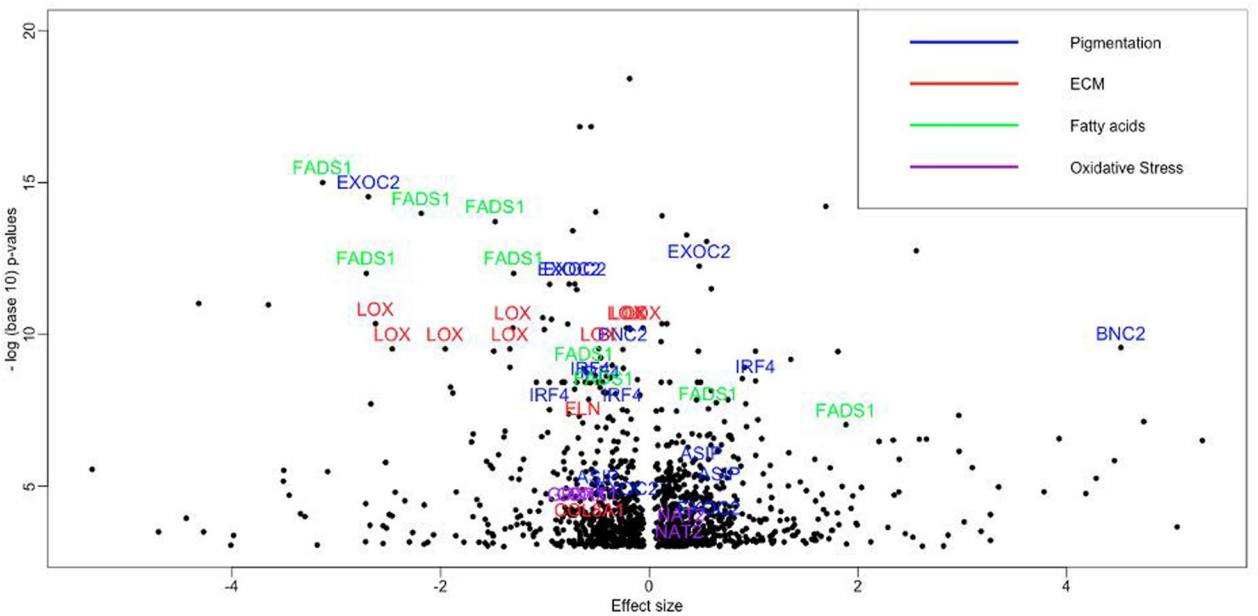

Fig. 2 Volcano plot with effect sizes and p values ($p < 0.001$) from EWMR analyses. Several representative genes from categories related to skin aging are shown. Pigmentation category includes genes IRF4, BNC2, EXOC2, ASIP/RALY. The extracellular matrix category includes genes COL8A1, ELN, LOX; the fatty acids group contains FADS1 and FADS2; the oxidative stress group includes NAT2 and GPX1

further perform various analyses to investigate whether blood-measured CpGs confer information on facial aging mechanisms (Fig. 2). To this end, we first look at overlaps between causal CpGs identified in the EWMR analysis and the published biological clocks. We further perform functional enrichment analyses on the level of causal CpGs and their nearby genes. Using the EWAS catalog, we identify proteins reported to be associated with these CpGs following up with functional analysis.

Little overlap with epigenetic biological clocks

A number of published epigenetics-based clocks are highly predictive of biological age. Interestingly, only a small subset of CpGs are common among biological clocks, while CpG sites that approximate telomere length (DNAmTL) are entirely unique. This may indicate significant redundancy in DNA methylation.

To investigate the overlaps between candidate CpGs causal to facial aging and existing biological clocks, we used the `methylCIPHER` package in `R` [24]. Overall, there is little, or no, overlap between CpGs from epigenetic-based biological clocks and CpGs potentially causal to facial aging. The largest overlap of CpGs causal to facial aging (8 CpGs) is with the updated PhenoAge clock (HRSInChPhenoAge). The effects of these CpGs in the updated PhenoAge clock and their causal effects in our study are anti-correlated ($r = -0.44$). The second largest overlap (7 CpGs) is with the epigenetic-based predictor of chronological age [25] trained on 13, 661 methylation samples from blood and saliva [25].

Only 4 CpGs (cg03473532; rs4731788, cg10586358; rs741702, cg10729426; rs13088318, cg06458239; rs9859077) from the Horvarth skin and blood age predictor [6] are identified as causal to facial aging in the EWMR analysis. Here and below next to the CpG site, we report a leading SNP that genetically controls it as found in our EWMR analysis. Other biological clocks from the `methylCIPHER` package have 2 or 1 CpG in common with the list of causal CpG, while there are no overlaps with the majority of existing biological clocks. This suggests that existing epigenetics-based clocks built using correlated methylation markers are unlikely to reflect early causal age-driving or age-protective events. Similarly, out of the top 1000 CpGs reported to be correlated with facial aging of participants of the Lothian Birth Cohort, 1921 [26], only three CpGs are also identified as causal to facial aging.

The lack of overlap between CpGs causal to aging and epigenetics-based clocks is in line with the recent work on general aging [11]. These authors further suggested that although some epigenetics-based clocks contain CpGs causal to aging, they, by design, favor CpG sites with a higher correlation with age, and thus are not enriched with causal CpGs.

Functional analysis of causal CpGs

Earlier studies reported that the majority of cross-tissue meQTLs showed consistent signs of cis-acting effects across tissues [2, 27] supporting the idea that genetically controlled CpGs are more likely to have common function across tissues compared to those less under direct genetic control. At the same time, a large portion of meQTLs shows tissue-specific characteristics, especially in the brain. To investigate molecular mechanisms underlying facial aging, we performed functional analyses on the level of CpGs inferred from the blood data, as well as on the level of genes and proteins.

Firstly, functional analysis of 1299 candidate CpGs causal to facial aging reveals several highly significantly over-represented GO categories that are known to play critical roles in skin aging. These include semaphorins, DNA repair, elastic fiber, and collagen related gene-sets, mitochondria membrane gene-sets, metabolic processes, and transmembrane transports of vitamins (e.g. thiamine) (Fig. 3). Also see Additional file 2: Table S2A.

Considered separately, damaging CpGs are enriched in collagen binding, multiple mitochondria-related gene-sets, vitamin (thiamine) transmembrane transport, hair cell differentiation, and several others [see Additional file 2: Table S2C]. Protective CpGs are enriched in collagen formation, elastic fiber assembly, DNA biosynthetic process, Platelet-derived growth factor receptor (PDGFR) signaling, bone, muscle, and neuron development processes [see Additional file 2: Table S2B]. Also, response to chemokines is significantly over-represented in protective CpGs. Chemokines are signaling proteins with roles in morphogenesis and wound healing. From an immunological perspective, chemokines and cytokines act in intercellular communicating immunity in skin aging [28].

Annotation of causal CpGs

To get further insight into the mechanistic functions, we first annotated all causal CpGs with nearby genes [see Additional file 1: Table S1A]. This resulted in 412 (unique) genes that correspond to damaging CpGs, and 400 genes that correspond to protective CpGs, with a large overlap (132 genes) between the two lists. The reason for this overlap is that a methylation marker may increase the expression of a nearby gene, decrease it, or have no effect at all, depending on the genomic context [22]. Removal of overlapping genes yields 280 genes that correspond to damaging CpGs, and 268 genes that annotate protective CpGs. We further conducted functional enrichment analyses on the gene level [see "Methods"

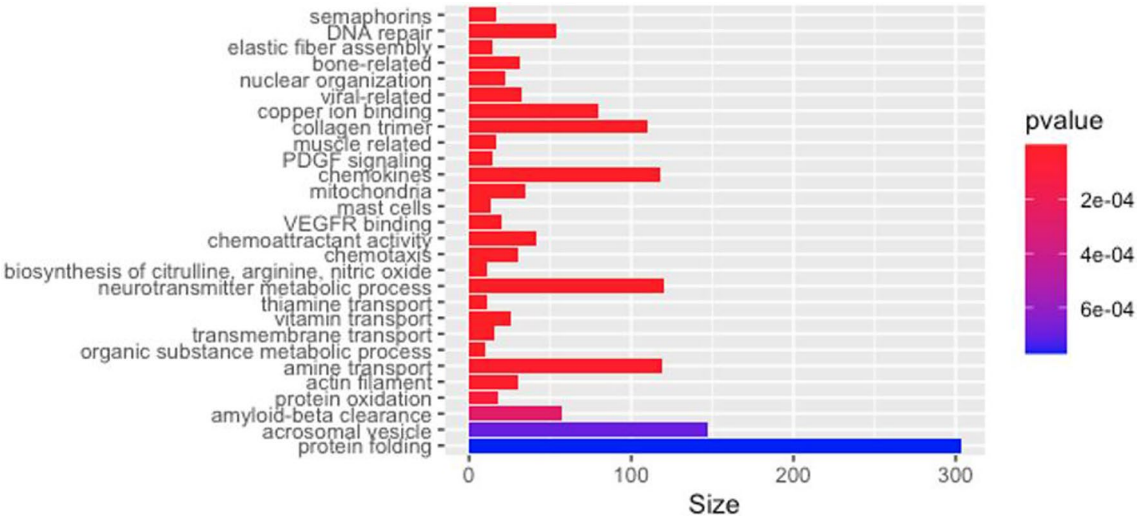

Fig. 3 Bar-plot of adjusted *p* values from the functional analysis of CpGs causal to facial aging. Full data are available [Additional file 2: Table S2A]. Similar categories are lumped together. For example, all significant semaphorin-related gene-sets are shown as one with the most significant adjusted *p* value. The analysis is performed using `methylglm` function from the `methylgsa` R package

and Additional file 3: Table S3]. Results are discussed further in the paper.

Additionally, we utilized the EWAS catalog to connect causal CpGs with proteins whose levels are significantly associated with CpG sites [see Additional file 1: Table S1B]. There is no available data to deduce whether associations between CpGs and protein levels are causal, or whether changes in protein levels drive aging or are the consequences of aging. However, by integrating the results of EWMR analysis and data from the EWAS catalog, we can identify proteins whose levels correlate with age and those that anti-correlate with age.

The group of age-correlated proteins (i.e. protein levels increase with age) includes proteins whose levels anti-correlate with age-protective CpGs, and proteins that correlate with age-damaging CpGs. For example, an age-damaging CpG (cg19588519; rs4131643) located within the CHST15 gene is associated with higher levels of C-reactive protein, an inflammatory biomarker that is an important risk factor associated with age and aging-related diseases. Another example of an age-correlated protein is ECM1 whose levels are negatively related to higher methylation of an age-protective cg15654264 CpG site within the RPRD2 gene. ECM1 contributes to the maintenance of skin integrity and homeostasis. Its expression in human skin depends on age and UV exposure. Intrinsically (UV-protected) aged skin shows a significantly reduced expression of ECM1 in basal and upper epidermal cell layers compared with young skin. However, in photoaged skin, ECM1 expression is significantly increased within the lower and upper epidermis compared with age-matched UV-protected sites [29].

Another group of proteins whose levels become lower with age (age anti-correlated) contains proteins that correlate with age-protective CpGs and those that anti-correlate with age-damaging proteins. This group includes collagens, biotinidase (BTD) involved in biotin metabolism and other proteins enriched in functions related to defense and inflammatory responses, wound healing, immune system categories, and collagen [see Additional file 3: Table S3E]. For example, an age-protective CpG (cg21627412; rs12583161) is associated with higher levels of the collagen protein COL2A1 which has been reported to be markedly reduced in the aged dermis [30]. This age-protective CpG site also protects from melanin hyperpigmentation ($b = -7.4$, $p = 0.016$) and other disorders of pigmentation ($b = -5.84$, $p = 0.007$). Another example is an age-damaging CpG (cg14036479; rs1549082) that anti-correlates with levels of BTD.

Connecting CpGs with proteins results in a sparse CpG-protein-facial aging network that contains 90 damaging proteins and 61 protective proteins. Overlapping proteins, removed from further analysis, are likely due to false positives in the results of EWMR, or the EWAS studies. Age-correlated proteins are also enriched in defense and immune responses (innate in this case), and proteins with the roles in matrisome [see Additional file 3: Table S3D]. In the next sections, we will zoom into several skin aging-related categories.

Deciphering causal molecular chains

To search for molecular chains spanning from SNPs to CpGs to gene expression to facial aging, we conducted additional MR studies (see "Methods"). The EW-GE MR

study utilized genetically proxied CpGs as exposures and genetically proxied expressions of nearby genes as outcomes [see "Methods" and Additional file 5: Table S5A]. Further, the GEMR study used genetically proxied gene expressions in two skin tissues and blood (considered separately) as exposures and facial aging data as outcome [see "Methods" and Additional file 5: Table S5B].

By conducting an EW-GE MR study, we identified 143 CpG-Gene pairs, where a CpG may causally affect expression of a nearby gene [Additional file 5: Table S5A]. GEMR reported several genes whose change in expression in at least one tissue may causally affect facial aging [Additional file 5: Table S5B].

To establish causal molecular chains, we examined the shared genes obtained from both EW-GE MR and GEMR. Only six genes overlap, including IRF4, CRELD2, PSMA4, SMG1, MLH3, RERE. Two genes IRF4 and CRELD2 have been implicated in skin aging measurement. The SMG1 gene, expressed in skin, has been reported to be associated with general mortality and aging, immune system and hematopoietic system phenotypes, while the RERE gene is mentioned as related to general mortality and aging (according to GeneCards).

Within the context of gene expression, only the changes in expression of the IRF4 gene exert a significant impact on facial aging. This gene is further discussed in the next section "Causal CpGs and skin pigmentation". The effects of other gene expressions on facial aging are marginal, measuring less than 5% [see "Methods" and Additional file 5: Table S5], and hence not considered further. In general, more high-throughput studies and additional analyses are needed to infer directions and sizes of the effect of methylation at CpG sites on gene expression.

Causal CpGs and skin pigmentation

Several top CpGs causal to facial aging are located within genes that have roles in skin pigmentation. Specifically, there are 6 protective CpGs in the IRF4 gene, controlled by different SNPs [Additional file 1: Table S1A] whose (hyper)methylation is highly significantly associated with lower facial age. The interferon regulatory factor, IRF4, is expressed in melanocytes in the skin. It is strongly associated with human pigmentation, sensitivity of skin to sun exposure, freckles, blue eyes, and hair color. The EW-GE MR analysis identifies an age-protective CpG (cg26422851; rs9378808) in the IRF4 gene that may causally lower expression of this gene ($b = -9.4$, $p = 4 \times 10^{-5}$ that in turn, as found by the GEMR analysis, may have a protective effect on facial aging ($b = 0.2$, $p < 10^{-8}$). Hence, a plausible molecular chain spans from a SNP (rs9378808) in the IRF4 gene that controls an age-protective CpG cg26422851 that slows down facial aging via lowering expression of the IRF4 gene.

Another highly damaging CpG (cg14044465; rs71513271) is found in the BNC2 gene, while another CpG (cg03291755; rs7861573) in this gene is an anti-aging candidate marker that also causally lowers its expression ($b = -11.8$, $p = 4.8 \times 10^{-8}$). The BNC2 gene has been reported to be associated with skin color variation in Europeans [31] and East Asians [32]. Interestingly, an age-damaging CpG in the BNC2 gene (cg14044465) causally increases the odds of actinic keratosis ($b = 0.36$, $p = 0.0003$), while an age-protective CpG (cg03291755) has a protective effect against actinic keratosis ($b = -3.64$, $p = 4.5 \times 10^{-6}$), and basal cell carcinoma ($b = -2.12$, $p = 3.72 \times 10^{-7}$).

Furthermore, both IRF4 and BNC2, as well as RALY/ASIP are implicated in facial pigmented spots during aging through pathways independent of basal melanin production [33]. The ASIP gene harbors two causally damaging CpGs (cg16440058; rs6059723 and cg16655240; rs4012155) and an age-protective causal CpG (cg05267394; rs819178). This protective CpG may also causally protect from facial pigmentation ($b = -19.65$, $p = 0.048$), basal cell carcinoma ($b = -3.49$, $p = 0.003$), and actinic keratosis ($b = -7.67$, $p = 0.0002$).

Another pigmentation-related gene worth mentioning is the EXOC2 gene which is associated with hair color, skin pigmentation, and other pigmentation phenotypes [34]. Our EWMR analysis finds multiple protective and damaging CpGs in the EXOC2 gene. Specifically, two protective CpGs (cg20223322 and cg26187313) both controlled by one SNP (rs4959926) are also identified to causally lower the odds of basal carcinoma ($b = -3.58$, $p = 0.0008$), while a damaging methylation marker (cg17645074; rs6597037) in the EXOC2 gene increases the odds of this skin cancer ($b = 2.26$, $p = 0.0013$).

The MC1R gene coding for melanocortin 1 receptor, dubbed a 'youthfulness' gene [35], has been found to contribute to facial pigmented spots [33] and skin cancers, including melanoma. Indeed, SNP (rs72813445) in the MC1R gene genetically controls an age-protective CpG cg01161216 in the TCF25 gene ($b = -1.54$, $p = 0.0009$).

Overall, the EWMR analysis of multiple skin aging-related phenotypes confirms that age-protective CpGs in skin pigmentation genes generally protect against age-related skin conditions and skin cancer, while age-damaging CpGs contribute to the risks. Functional analysis, using the FUMA tool, of all annotated genes associated with causal CpGs revealed chr16q24 as highly significantly ($p = 3.4 \times 10^{-9}$) over-represented positional set that contains 17 genes [see Additional file 3: Table S3A]. Remarkably, the region chr16q24.3 was identified to be associated with skin pigmentation and skin aging in a recent comprehensive meta-analysis of 44 GWAS and

gene expression studies [36]. This region is also over-represented in genes associated with protective genes ($p = 0.0002$) [see Additional file 3: Table S3C].

Further, the facial pigmentation GWAS trait is also over-represented ($p = 0.004$) in all genes with causal CpGs. Other pigmentation-related over-represented traits include tanning ($p = 0.012$ based on three genes ASIP, IRF4, EXOC2) and low tan response ($p = 10^{-5}$ based on ASIP, BNC2, IRF4, SPIRE2, AFG3L1P, DBNDD1, TRPS1). Further, causal genes are enriched in skin cancer-related traits, such as basal cell carcinoma ($p = 3.4 \times 10^{-5}$), cutaneous squamous cell carcinoma ($p = 7.6 \times 10^{-5}$), non-melanoma skin cancer ($p = 0.01$), and melanoma ($p = 0.016$).

Causal CpGs and extracellular matrix

The most visible hallmark of aging skin is the formation of wrinkles due to the loss of skin elasticity. Our unbiased EWMR analysis identified several causal CpG sites in genes with functions in elastic fiber formation, collagen biosynthesis and modifying enzymes, ECM glycoproteins, and semaphorin interactions.

Specifically, a highly protective CpG (cg05010648; rs2301994) in the ELN gene coding for the elastin protein has a large protective effect on facial aging (OR = 0.59). While currently there is no information on the downstream effect of this CpG on the level of elastin, it is an excellent candidate for an age-protective methylation marker.

A number of causal CpGs in two enzymes, LOX and LOXL, responsible for elastin cross-linking are also identified. While the elastin mRNA level generally remains quite stable at all ages, the levels of lysyl oxidase LOX and lysyl oxidase-like LOXL are affected by age and therefore have been suggested as targets to induce elastogenesis in adult skin [37]. There are 9 age-protective CpGs within the LOX gene: 4 CpGs (cg08623535, cg23913350, cg01824804, cg07233159) are genetically controlled by one SNP (rs840462) in the LOX gene, and 5 CpGs (cg20703137, cg12580445, cg21215899, cg21482489, cg01191064) are affected by two SNPs (rs2914598 and rs2972344) in the SRFBP1 gene. Further GEMR analysis identified that higher expression of the LOX gene in both exposed and not-exposed skin may causally lower facial age. However, the EW-GE MR analysis did not identify significant causal relationship between any of these CpGs and the expression of the LOX gene. Another related result is that a damaging CpG (cg02812767; rs16958477) causally increases expression of the LOXL1 gene($b = 6.47$, $p = 0.0009$).

The EWMR analysis also identifies an age-protective methylation marker (cg08464402; rs13317800) in the collagen gene COL8A1. Extending annotations to the protein space, via the EWAS catalog, points to an additional age-protective CpG (cg21627412; rs12583161) that increases levels of the collagen protein (COL2A1), while an age-damaging CpG (cg13280184; rs7725097) correlates with levels of the MMP3 enzyme which is known to get upregulated with age and sun exposure. The MMP3 enzyme degrades ECM proteins, such as type IV, V, IX, and X collagens, gelatin, fibrillin-1, fibronectin, laminin, and proteoglycans, contributing to photoaging, wrinkle formation, and skin laxity.

These are potentially important findings on causal methylation markers that may slow down skin aging via higher levels of collagen proteins (e.g. COL8A1) or accelerate aging via reducing levels of collagen proteins (e.g. COL2A1) or increasing rate of collagen degradation (e.g. MMP3).

Functional analysis revealed significantly over-represented ECM-related gene-sets in the list of genes with causal CpGs [see Additional file 3: Table S3A]. These gene-sets include ECM organization ($p = 0.0006$ based on 15 genes, including COL8A1, DAG1, DDR1, EFEMP1, ELN, FN1, GDF5, LAMB2, LOX, LOXL1, LTBP2, MMP2, P4HA2, SDC1, TNXB); elastic fiber formation ($p = 0.0036$); ECM glycoproteins ($p = 0.0044$; BGLAP, CRELD2, EFEMP1, ELN, FN1, LAMB2, LTBP2, POMZP3, PXDNL, RSPO2, SLIT1, THBS2, TNXB, VWA7). Further, causal genes are significantly enriched in semaphorin receptor binding ($p = 0.04$; SEMA6C, SEMA4B, SEMA4C, SEMA4D) and response to wounding ($p = 0.0004$; based on 31 genes) [see Additional file 3: Table S3A-C].

Similar results are obtained on the protein level [see Additional file 3: Table S3D-E]. For example, age-correlated proteins contain 16 proteins with general matrisome functions (NABA_MATRISOME, $p = 0.01$; ECM1, F13B, LEFTY2, HPX, MMP3, IGF1, SERPINA12, SERPINF2, CCL11, REG3G, CST8, CXCL11, ESM1, SFTA2, PLXNA4, SVEP1), while anti-correlated proteins are enriched in collagen containing ECM ($p = 0.009$; PRG2, COL2A1, POSTN, SERPINA1, GH1, SERPINE2, PTN, L1CAM) as well as wound healing ($p = 0.011$; MPL, CD44, POSTN, SERPINA1, NOG, SERPINE2, LARGE, HMOX1, KLKB1) [see Additional file 3: Table S3D-E].

Causal CpGs and Other Skin Aging Processes

There are multiple causal CpGs in other genes or proteins with known roles in skin health and skin aging. Below, we consider two skin-related processes, fatty acid metabolism, and oxidative stress.

Firstly, there are multiple causal CpGs in the FADS1 and FADS2 genes that code for fatty acid desaturases (FADS) with critical roles in fatty acid metabolism. Fatty acids impact a wide range of biological activities,

including immune signaling and inflammation. They contribute 15% of the total lipid content within the skin surface lipids that regulate skin barrier permeability and modulate the risk of infection by interacting with skin microbiota. FADS have been reported to impair skin barrier function contributing to skin pathology and reducing total lipid content in the skin of older adults. This is believed to contribute to dryness, increased susceptibility to infection, and impaired wound healing in response to injury. A recent study demonstrated that the phenotypic associations of rs174537 are likely due to methylation differences that may directly impact FADS activity [38]. Specifically, rs174537 is anti-correlated with cg27386326 (rs174604) which is identified as an age-protective causal CpG in our EWMR analysis.

Another study [39] examined the relationship between keratinocyte fatty acid composition, lipid biosynthetic gene expression, gene promoter methylation, and age. They found that expressions of FADS genes and elongases (ELOVL6 and ELOVL7) were lower in adult versus neonatal keratinocytes and were associated with lower concentrations of n-7, n-9, and n-10 polyunsaturated fatty acids in adult cells. Hence, causal CpGs in the FADS cluster represent potentially actionable methylation markers for treating early signs of skin aging. A recent MR analysis reported causal associations between genetically proxied unsaturation degree of fatty acids and the pace of facial skin aging [40].

Several GWAS traits related to fatty acids are significantly over-represented in genes with CpGs causal to facial aging, including plasma omega-3 polyunsaturated fatty-acid levels (docosapentaenoic acid, eicosapentaenoic acid, alpha-linolenic acid); plasma omega-6 polyunsaturated fatty-acid levels (adrenic acid, arachidonic acid) and very long-chain saturated fatty-acid levels (fatty acid 22:0) [see Additional file 3: Table S3A].

Another skin aging-related category is increased oxidative stress. The oxidative stress theory of aging states that age-associated functional losses are due to the accumulation of Reactive Oxygen Species (ROS). Overall, the endogenous antioxidant capacity (enzymatic and non-enzymatic) of the skin is lowered with age, and the aged skin is more vulnerable to external factors, especially UV radiation, pollution, and microorganisms. Since the epidermis is more exposed to external stimuli than the dermis, the ROS load is higher in the epidermis compared to the dermis [41].

One of the supporters of the antioxidant capabilities of the cell is glutathione. Age-related decline in the activity of the antioxidant enzyme glutathione peroxidase (GPX1) may contribute to increased free radicals and GPX1 was proposed as a potential therapeutic target for the enhancement of the regenerative capacity in old subjects [42]. The EWMR analysis identifies three causally age-protective CpGs (cg24011261, cg05551922, cg05055782) proxied by one SNP (rs62259939) in the GPX1 gene. These CpGs are also found to be candidates for causal positive effects on the telomere length [see Additional file 4: Table S4A] while at the same time increasing the odds of basal cell carcinoma (e.g. $b = 4$, $p = 0.01$ for cg24011261).

Another xenobiotic enzyme that may lose its antioxidant capacity with age is NAT2. It is involved in the metabolism of drugs, environmental toxins, and carcinogens, and also affects levels of cholesterol and triglycerides. According to the EWMR analysis, NAT2 harbors two age-damaging CpGs (cg14494313; rs1041983 and cg24555670; rs4646267) that may be related to the decreased antioxidant capacity of aging skin.

Furthermore, genes with age-protective CpGs are significantly enriched in the cellular response to oxidative stress ($p = 0.04$; based on genes GPX1, ERO1L, MGMT, TPM1, PDE8A, MMP2, CBX8, NR4A2, PLA2R1, PARK2). Additionally, all causal genes are significantly enriched in the oxidation-reduction process ($p = 0.0036$; based on 36 genes) and the response to oxidative stress gene-set ($p = 0.0096$; based on 20 genes) [see Additional file 3: Table S3C].

Among serum proteins correlated with lower perceived facial age, at least three proteins (AKR1C3, ASL, HMOX1) have functions in xenobiotic metabolism. Overall, proteins correlated with more youthfulness are enriched in adhesion, response to bacteria, viruses, nutrients, growth factors, and acute inflammatory and immune response, while proteins correlated with older age are enriched in adaptive immune response, adaptive response to stress, as well as several metabolic pathways, including vitamin, phosphorus, carbohydrate, and small molecule metabolic processes [see Additional file 3: Table S3D, Table S3E].

Causal CpGs and telomeres

Cellular senescence and telomere shortening are molecular hallmarks for aging in general and play important roles in skin aging. As senescent cells accumulate with age in various tissues, including skin, they secrete factors called the senescent-associated secretory phenotype (SASP) that trigger inflammation and remodel the extracellular matrix (ECM) contributing to premature aging phenotypes. Cell senescence is often approximated by a reverse of telomere length (TL) as shorter telomere length (more senescence) has been reportedly associated with age-related diseases and also with mortality.

However, the relationship between skin aging and TL is more complex. Specifically, longer telomeres have been reported to be associated with an increased risk of

melanoma [43]. This link was first discovered because of a strong association between longer telomeres and a high number of naevi. A recent MR study found evidence that genetically proxied TL shortening may reduce the risk of cutaneous melanoma and non-melanoma skin cancers while slightly speeding up the pace of skin aging [44]. In contrast, for cutaneous squamous cell carcinomas and basal cell carcinomas, the relationship is reversed with a higher risk in individuals with shorter telomeres [45, 46].

We set out to explore the relationship between methylation markers causal to facial aging and TL. To this end, we ran the EWMR analysis on meQTLs from blood as exposure and TL as the outcome (see "Methods"). Firstly, there is a large overlap between CpGs causal to facial aging and CpGs causal to telomere length TL (235 CpGs, [see Additional file 4: Table S4]). In contrast, the overlap between CpGs causal to facial aging and CpGs from the epigenetic estimator of telomere length (DNAmTL from the `methylCIPHER R` package) is very small (only 4 CpGs). It is worth noting that while the DNAmTL estimator is highly correlated with cellular replication rate, it contains only one CpG (cg00029246; rs79880068) in the DGKI gene that causally affects TL. This again points out that CpG sites in the DNAmTL estimator correlate with the length of telomeres and may be the consequences of telomere shortening rather than drivers of it.

Among common CpGs that are causal to both facial aging and TL, there are 19 age-damaging and TL-shortening CpGs located in the TNXB gene genetically controlled by several SNPs [see Additional file 4: Table S4] that encodes a member of the tenascin family of extracellular matrix glycoproteins with anti-adhesive effects. The TNXB gene plays a role in ECM organization and collagen fibrin formation. It also functions in matrix maturation during wound healing. Deficiency of TNXB has been associated with the connective tissue disorder Ehlers-Danlos syndrome. Six age-damaging CpGs in the TNXB gene are also associated with lower levels of the CRISP2 protein which is involved in cell-cell adhesion, regulation of ion channels' activity, and is implicated in male fertility. Other common genes are GPSM3 and GPX1 with roles in regulating inflammatory response and protecting cells against oxidative damage. Specifically, the GPX1 gene that harbors three age-protective CpGs has a function in glutathione conjugation and fatty acid metabolism. Another gene in common is PSORS1C1 known to confer susceptibility to psoriasis and systemic sclerosis. It harbors an age-damaging and TL-lengthening CpG (cg01016122; rs3095300).

To further investigate complex relationships between epigenetic markers causal to facial aging and TL, we relaxed the p value cut-off in the EWMR procedure ($p = 0.01$) and obtained 486 CpGs causal to both traits [see Additional file 4: Table S4A]. Interestingly, two groups of causal CpGs emerge in this dataset. The first group contains CpGs that exhibit behavior consistent with the prevailing views that short telomeres correlate with aging. This "consistent" group contains CpGs that may causally shorten TL and accelerate facial aging, or causally lengthen TL and slow down facial aging. The "reverse" group contains CpGs that causally affect TL and aging in opposite directions, e.g. longer TL and accelerated facial aging. Interestingly, correlations of the effect sizes from the EWMR analyses for facial aging and TL for the consistent and reverse groups taken separately are very strong (Pearson correlation coefficient in the consistent group $= -0.89$, and in the reverse group $= 0.87$).

CpGs in the consistent group are significantly enriched in several processes related to skin development and maintenance including keratinization, keratinocyte differentiation, regulation of keratinocyte proliferation, epithelial cell differentiation, epidermal cell differentiation, cornified envelope, desmosome; vitamin D signaling pathway; responses to extracellular stimulus and nutrients, vitamins, interferon-gamma, and EGF [see Additional file 4: Table S4C]. CpGs in the reverse group are significantly enriched in the regulation of signaling receptor activity, protein localization to cell surface, several metabolic processes (citrulline, arginine, nitric oxide, amino acids, glutamine), and pathways related to synaptic plasticity and transmission [see Additional file 4: Table S4D].

Discussion

The gigantic anti-aging cosmetic industry has largely been focused on figuring out the most effective products that conceal, or temporarily mitigate the visible downstream effects of aging, rather than proactively address the upstream root causes of aging [47]. It has already been proposed that the prerequisite for an anti-aging rejuvenation treatment is a robust reduction in biological age, which can be assessed by biomarkers of aging, such as epigenetic clocks [48]. Skin-specific biological clock based on methylation markers [8] has already been used to identify a novel senotherapeutic peptide that improves skin health, reduces skin biological age, and decreases the expression of senescence markers [9].

However, the current generation of biological clocks is based on methylation sites that are correlated with chronological age, and hence their causal effect on skin aging is difficult to infer. To understand the causes of skin aging, and develop efficient senotherapeutic treatments, it is important to distinguish epigenetic drivers of aging from the consequences of aging. Furthermore, it is crucial to identify age-damaging methylation sites from age-protective ones.

In this study, we systematically evaluated the relationship between DNA methylation and facial aging. We utilized the EWMR procedure and identified hundreds of potentially causal methylation markers that affect facial aging. Subsequent functional analysis revealed several interesting insights. Firstly, it appears that age-protective, or adaptive, methylation changes are nearly as common as those methylation changes that drive, or accelerate, aging. This is consistent with a recent report on general aging [11]. Hence, identifying CpGs that causally impact youthfulness is as important as finding age-damaging causal CpGs. The development of rejuvenating skin treatments should target both groups of methylation markers to reverse damaging methylation sites on one hand while inducing, or maintaining, the protective methylation sites.

Secondly, our study reports little overlap between existing epigenetics-based biological clocks and CpGs causal to facial skin aging. One explanation is that causal damaging or adaptive CpGs may not change as much with age, and thus have not been included as input features in machine learning models for biological clocks. Indeed, consistent with an earlier study on general aging [11], we found no correlation between the size of the causal effect on facial aging and the magnitude of age-related changes.

Thirdly, our study demonstrates that methylation data from blood can be utilized to uncover damaging and protective CpGs causal to facial aging. This finding provides further scientific support for the burgeoning 'beauty from within' industry that develops ingestible anti-aging skin supplements. Furthermore, identified causal CpGs shed light on biological processes that underlie facial skin aging and other dermatological traits. For example, age-protective CpGs in skin pigmentation genes generally protect against age-related skin conditions and skin cancer, while age-damaging CpGs contribute to the risks. Significant overlap in causal CpGs that contribute to facial aging and senescence further confirms that CpGs causal to aging underlie biological mechanisms that causally affect the pace of aging rather than those that are mere consequences of aging. At the same time, our study points out that the relationship between telomere length and facial aging is not linear, as some CpGs that contribute to longer telomeres may exert damaging effects on the pace of facial aging.

Our study has several limitations. Firstly, the usage of the SNP-CpG dataset measured in whole blood raises questions about tissue-specificity of causal CpGs. Indeed, the list of causal CpGs identified in this study is likely missing causal skin-specific CpGs. However, it is worth noting that the EWMR approach focuses on genetically controlled methylation sites that were reported to have consistent effects across different tissues [2, 27] providing further evidence that genetically controlled CpGs are more likely to have common functions across tissues compared to those with less direct genetic control. Moreover, blood is one of the top tissues that shares a significant number of CpGs and meQTLs with other tissues, including skin.

Some skin-specific CpGs may not be genetically controlled but rather influenced by environmental factors, for example pollution levels or UV radiation. In these cases, the EWMR procedure would not capture the effect of such CpGs, even if the meQTLs data is collected from skin samples. Thus, a meQTL dataset from blood is good proxy for studying facial aging,

Another limitation of this study is that we used a higher p value threshold in the EWMR procedure that may have resulted in a higher proportion of false positives. The reason for this is to showcase the integration of blood methylation data with the summary statistics from GWAS on perceived facial age that is based on replies to the question of whether a person thinks others perceive her/him as looking younger or older than their real chronological age. It is worth noting that less than 5% of the UKBB participants reported "looking older than actual age".

Finally, another limitation is that the perceived facial data is self-reported. Future studies should be based on more precise estimates of perceived facial age estimated by specialists such as dermatologists or aestheticians. Finally, this study utilizes the lumped data from females and males. Furthermore, it largely contains data from white British participants. To understand the epigenetics of facial aging, future studies should be based on separate analyses of female and male datasets as well as consideration of other ethnicities.

Overall, this is the first study that uncovers epigenetics markers causal to facial aging. It provides proof-of-concept for utilizing blood-measured CpGs in elucidating biological processes that underlie facial aging. Novel causal CpGs can be utilized as input features for building the biological clock of facial aging, predicting personalized face aging trajectories (ageotypes), and developing novel rejuvenating topical, and 'beauty from within' senotherapeutic solutions that target root causes and early signs of aging.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-023-01590-x.

Additional file 1. Table S1A: Methylation markers causal to facial aging annotated with nearby genes, **Table S1B:** CpG sites with protein annotations.

Additional file 2. Table S2A: Enrichment analysis of CpGs causal to facial aging for all CpGs, **Table S2B:** Enrichment Analysis of protective CpGs, **Table S2C:** Enrichment Analysis of damaging CpGs.

Additional file 3. Table S3A: Functional analysis for all genes, **Table S3B:** Functional analysis for damaging genes, **Table S3C:** Functional analysis for protective genes, **Table S3D:** Functional analysis at the protein level (age-correlated).

Additional file 4. Table S4A: Common CpGs causal to facial aging FA and telomere length TL, **Table S4B:** Enrichment Analysis of common CpGs causal to FA and TL, **Table S4C:** GO enrichment analysis of causal CpGs from the 'consistent' group: e.g. shorter telomeres accelerated aging, **Table S4D:** GO enrichment analysis of causal CpGs from the 'reverse' group: e.g. longer telomeres accelerated aging.

Additional file 5. Table S5A: EW-GE MR. Methylation markers affecting expression of a nearby gene, **Table S5B:** GE MR. Gene expression causally affecting facial aging in three tissues: whole blood, skin exposed to sun and skin not exposed to sun.

Author contributions

RK contributed to conceptualization; KV, MMP and RK contributed to methodology; MMP, KV and RK contributed to software, resources and data curation; MMP, KV, MŠ, AM and RK involved in validation; KV, MMP, and RK contributed to writing—original draft preparation; KV, MMP, MŠ, AM and RK contributed to writing—review and editing; KV and RK involved in visualization; RK involved in supervision; MŠ and RK involved in project administration; all authors reviewed the manuscript.

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Availability of data and materials

All used datasets and tools are publicly available (more information in "Methods" section).

Declarations

Ethical approval and consent to participate

Not applicable.

Competing interests

Authors have no competing interests, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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