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## DNA methylation-based biomarkers and the epigenetic clock theory of ageing

Steve Horvath<sup>1,2\*</sup> and Kenneth Raj<sup>3</sup>

**Abstract** | Identifying and validating molecular targets of interventions that extend the human health span and lifespan has been difficult, as most clinical biomarkers are not sufficiently representative of the fundamental mechanisms of ageing to serve as their indicators. In a recent breakthrough, biomarkers of ageing based on DNA methylation data have enabled accurate age estimates for any tissue across the entire life course. These ‘epigenetic clocks’ link developmental and maintenance processes to biological ageing, giving rise to a unified theory of life course. Epigenetic biomarkers may help to address long-standing questions in many fields, including the central question: why do we age?

### Chronological age

The calendar time that has passed since birth. Zero is the time at birth. Negative numbers indicate prenatal ages, whereas positive numbers indicate postnatal ages.

### Biological age

Also known as physiological age, organismal age or phenotypic age. This ambiguous concept is held to be dependent on the biological state of the individual.

Since the 1980s, it has been recognized that valid and reliable biomarkers of ageing will be needed to achieve the ancient goal of understanding, slowing, halting or even reversing ageing<sup>1,2</sup>. Instead of using chronological age, which is an imperfect surrogate measure of the ageing process, Baker and Sprott proposed the identification of biomarkers that can accurately and rapidly predict the functional capability of a person or organ and how it changes with age<sup>1,2</sup> — in other words, to identify markers of biological age. In 1988, the US National Institute on Aging initiated a programme with the expressed goal of identifying age-associated biomarkers in model organisms such as mice and rats<sup>3</sup>. The eventual successful development of such biomarkers would require considerable technical as well as cultural breakthroughs. The former included the completion of the Human Genome Project, advances in microarray technology<sup>4</sup> and development of biostatistics (in particular, penalized regression models<sup>4,5</sup>). The latter, which was equally important, was a shift in scientific culture in the form of the open access movement, which led to the availability of large DNA methylation data sets in freely accessible repositories, such as the Gene Expression Omnibus (GEO)<sup>6</sup> and The Cancer Genome Atlas (TCGA)<sup>7</sup>. Indeed, the first accurate multi-tissue biomarker of ageing, which generates an estimated age for multiple tissues or organs of an individual, was developed for the human species through the analyses of publicly available DNA methylation data sets<sup>8</sup>.

Recent evidence from human and mouse studies demonstrates that DNA methylation-based (DNAm) biomarkers satisfy the formerly elusive criteria of a

molecular biomarker of ageing: they apply to all sources of DNA (sorted cells, tissues and organs) and to the entire age spectrum (from prenatal tissue to tissues of centenarians)<sup>8</sup>. Since their discovery, DNAm biomarkers have provided answers to long-standing questions in diverse areas, such as medicine<sup>9,10</sup>, biodemography<sup>11</sup>, endocrinology<sup>12</sup>, dietary studies<sup>13</sup> and cell biology<sup>14</sup>. The field has now moved beyond the question of whether biomarkers of ageing can be developed to why it is possible to do so with DNA methylation data and what these biomarkers teach us about the biology of ageing. For example, investigating how the estimated epigenetic age differs across a group of individuals of the same chronological age could help determine the impact of endogenous or exogenous stress factors on biological ageing. Perhaps the most exciting feature of DNAm biomarkers is that epigenetic changes are reversible, raising the prospect that DNAm age estimates might thus be useful for identifying or validating anti-ageing interventions.

A recent review of six types of potential biological age estimators — epigenetic clocks, telomere length, transcriptomic-based, proteomic-based and metabolomic-based estimators and composite biomarkers — concluded that the epigenetic clock is the most promising molecular estimator of biological age<sup>15</sup>. Similarly, a comparative review of different forensic methods for age estimation concluded that DNA methylation is the most promising age-predictive biomarker<sup>16</sup>. However, the molecular mechanisms underlying DNAm biomarkers need to be better understood before their potential can be fully realized.

We begin by reviewing the development of the most prominently applied types of epigenetic biomarkers of

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## Epigenetic age

The age estimate in years resulting from a mathematical algorithm based on the methylation state of specific CpGs in the genome. Negative numbers indicate prenatal ages.

## CpG dinucleotides

Regions of DNA where a cytosine nucleotide is followed by a guanine nucleotide in the linear sequence of bases along its 5' to 3' direction. Cytosines in CpG dinucleotides can be methylated to form 5-methylcytosine.

## Epigenetic age estimators

Mathematical algorithms that use values assigned to the methylation state of specific CpGs in the genome to estimate the age of a person or biological sample. A multi-tissue age estimator allows one to estimate the age of any nucleated cell, tissue or organ.

ageing. We then discuss how accelerated epigenetic age — that is, when estimated age is higher than expected (on the basis of chronological age) — predicts several age-related phenotypes. Finally, we turn to the question of which mechanisms underlie these epigenetic biomarkers and what they tell us about innate ageing processes in cancer development and across the life course.

## DNA methylation-based age estimators

A rich body of literature, dating back to the 1960s, demonstrates that chronological age has a profound effect on genome-wide DNA methylation levels<sup>17–24</sup>. The methylation states of millions of the 28 million CpG dinucleotides in the human genome were seen to change with age. The advent of DNA methylation array technology enabled the identification of the specific genomic locations of these CpGs<sup>19,22,23,25–32</sup>. Epigenetic ‘age estimators’ are sets of CpGs (also known as ‘clock CpGs’) that are coupled with a mathematical algorithm to estimate the age (in units of years) of a DNA source, such as cells, tissues or organs. This estimated age, also referred to as epigenetic age or more precisely as DNAm age, is not only a reflection of chronological age but also of the biological age of the DNA source, as described below. Owing to their accuracy, DNAm age estimators are often referred to as ‘epigenetic clocks’ (BOX 1).

DNAm age estimators are typically built by regressing a transformed version of chronological age on a set of CpGs using a supervised machine learning method, for example, a penalized regression model, such as lasso (least absolute shrinkage and selection operator) or elastic net. The penalized regression model automatically selects the most informative CpGs for the age prediction or estimation model. Thus, the supervised machine learning analysis yields both a set of

CpGs and a corresponding mathematical algorithm that incorporates the DNA methylation levels into an age estimate. Many of the CpGs that are being used in the algorithm have, on their own, only negligible correlation with chronological age, which illustrates that the whole is greater than the sum of its parts when it comes to composite biomarkers of ageing. Recent years have seen the development of several age estimators that use different sets of CpGs from different tissues and age spectra. Although they exhibit varying levels of accuracy (FIG. 1; Supplementary information), they all embody the same principle described above, which allows them to estimate age and to relate it to age-related conditions.

Why develop a tool to estimate the age of a DNA source on the basis of high-dimensional genomic measurements and aggregate age into a single number? In machine learning, a set of markers can be obtained using a feature selection method (also known as a variable selection method). Although comparisons of multiple individual variables can address detailed questions, for example, about which genomic regions gain methylation as a result of obesity, they cannot address more generalized and overarching questions, such as whether obesity accelerates ageing. Answers to such questions require data reduction. Estimated DNAm age is associated with age-related conditions and predicts lifespan (TABLE 1). Undoubtedly, these biomarkers capture pivotal aspects of biological age.

## Single-tissue DNA methylation-based age estimators.

Bocklandt et al. described the first DNAm age estimator, which was constructed using DNA extracted from saliva<sup>33</sup>. This early incarnation exhibited most of the elements that subsequent and more accurate estimators would go on to possess: it was derived by regressing chronological age (dependent variable) on DNA

### Box 1 | Two interpretations of the term ‘epigenetic clock’

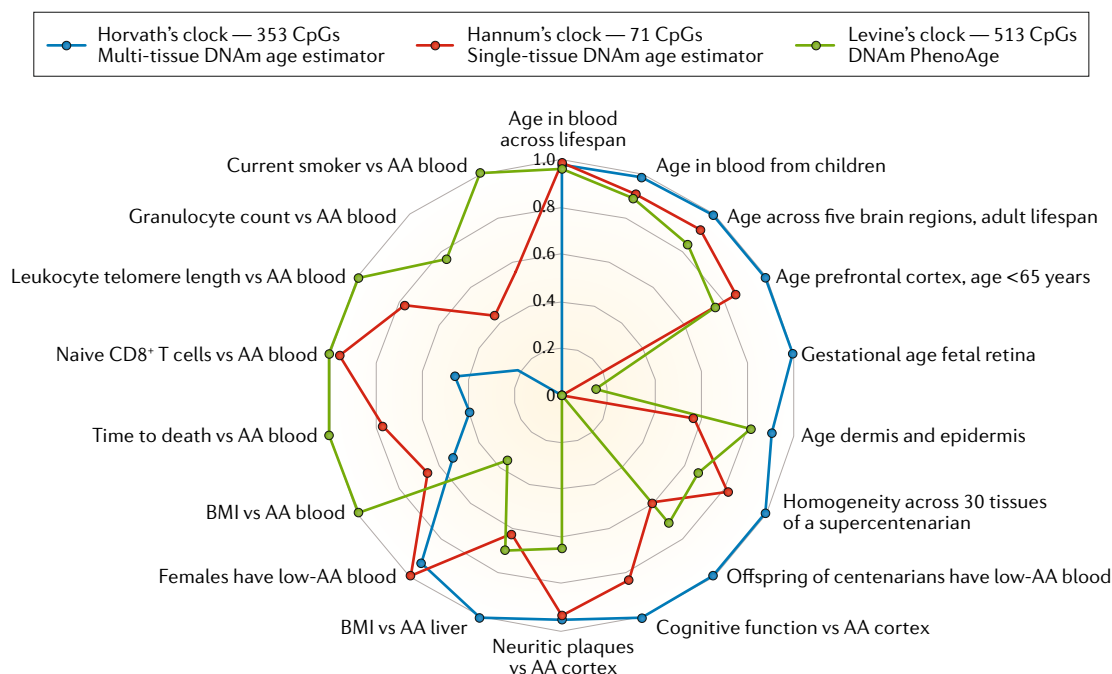
The term ‘epigenetic clock’ is used to denote two distinct but related things. The first is as a synonym of a highly accurate age estimator based on DNA methylation levels, and the second is the concept of an innate process in the body that continues inexorably, resulting in ageing.

#### Age estimators as epigenetic clocks

Distinguishing several measures for assessing the accuracy of an age estimation method is useful because each measure has distinct advantages. The first, referred to as ‘age correlation’, is the Pearson correlation coefficient  $r$ , between DNA methylation-based (DNAm) age (also known as estimated age) and chronological age. The second measure, referred to as (median) ‘error’, is the median absolute difference between the age estimate and chronological age. In our opinion, an age estimator deserves the label ‘ageing clock’ if its age correlation exceeds  $r=0.80$  in a large independent validation data set with a broad range of chronological ages (for example, ranging from age 20 to 100 years). The sample size that is needed for validating an age estimator depends on the accuracy of the age estimator. The age correlation of the multi-tissue age estimator exceeds 0.95 in a data set composed of individuals aged from 0 to 100 years<sup>8</sup>. Sample size calculations show that a test set sample size of  $n=22$  produces a two-sided 95% CI of (0.88, 0.98) if the true age correlation is  $r=0.95$ . Thus,  $n=22$  would result in a low width ( $0.10=0.98-0.88$ ) of the 95% CI if  $r=0.95$ . However, this sample size would be insufficient in the case of lower age correlations. To achieve the same width of the CI, one would need  $n=62$  test samples if  $r=0.90$ ,  $n=205$  if  $r=0.80$ ,  $n=404$  if  $r=0.70$  and  $n=867$  if  $r=0.50$ .

#### Innate biological processes as epigenetic clocks

There is a second interpretation of the term epigenetic clock, namely, a collection of innate biological mechanisms that give rise to age-related DNA methylation changes that underlie highly accurate DNAm age estimators (that apply to the entire lifespan) and play a purposeful role in development and maintenance. The epigenetic clock theory of ageing (described below) views biological ageing as an unintended consequence of both developmental and maintenance programmes for which the molecular footprints give rise to DNAm age estimators.



**Fig. 1 | Comparison of three DNA methylation-based biomarkers of ageing.** The multi-tissue DNA methylation-based (DNAm) age estimator (blue line), also known as Horvath's clock, stands out in terms of its correlation with chronological age across multiple tissue types, its high accuracy in children, its strong correlation with gestational age (differentiation day) in neuronal cell culture models and the homogeneity of its age estimates across tissues, for example, 30 tissue samples collected from a supercentenarian (>110 years)<sup>56</sup>. The phenotypic age estimator (green line), or DNAm PhenoAge stands out in terms of its predictive accuracy for time to death, its association with smoking status and its association with various markers of immunosenescence<sup>59</sup>. In general, DNAm PhenoAge and DNAm age as calculated by the single-tissue age estimator known as Hannum's clock (red line) outperform other blood-based biomarkers in regard to lifespan prediction. Supplementary information contains the data and details on the construction of this radar plot. AA stands for (epigenetic) age acceleration, for example, 'AA blood' denotes age acceleration in blood. BMI, body mass index.

methylation levels (covariates) using a penalized regression model<sup>33</sup>. Similarly, Hannum et al. derived a highly accurate age estimator on the basis of 71 CpGs from DNA of blood, commonly known as Hannum's clock, as well as several distinct age estimators for other tissues<sup>34</sup>. Reflecting the fact that it was trained in whole-blood samples from adults, Hannum's blood-based age estimator is tailor-made for adult blood samples, leading to biased estimates in children<sup>35,36</sup> and in non-blood tissue<sup>34</sup>. Furthermore, it is confounded by age-related changes in blood composition<sup>37</sup>, although this feature is not without merit as it gives rise to more accurate predictions of life expectancy<sup>38</sup>, as described below.

Although it is possible to develop reasonably accurate age estimators with fewer CpGs<sup>33,39–47</sup>, evidence suggests that larger sets of CpGs produce more accurate and robust age estimators<sup>37,48,49</sup>. A striking case in point, Garagnani et al. described an age estimator based on a single CpG in the *ELOVL2* gene<sup>40,50</sup>. However, age estimators with few CpGs are unlikely to be compatible with all tissues and thus would not be accurate multi-tissue age estimators<sup>32</sup>.

#### Multi-tissue DNA methylation-based age estimators.

As its name suggests, a multi-tissue age estimator should apply to all tissues and cell types across the entire duration of the human lifespan. Such an age estimator, which can underpin a 'unified theory of life

course', would be a valuable research tool that links developmental and maintenance processes to innate ageing. The three main conceptual challenges in the development of such a multi-tissue age estimator are the substantial differences in DNA methylation patterns among different cell types or tissues<sup>51–53</sup>, the tissue specificity of age-related DNA methylation changes<sup>23,54</sup> and the fact that changes in DNA methylation early in life might differ from those later in life. As early as 2010, encouraging signs that these hurdles were surmountable began to emerge. In particular, it was recognized that age-related DNA hypermethylation at specific locations — bivalent chromatin domains and targets of Polycomb repressive complex 2 (PRC2) — seemed to be conserved across different tissues and cell types<sup>19,20,31</sup>. Teschendorff et al. described a set of 69 CpGs (mapping to 64 unique PRC2 target genes) that were hypermethylated with age in blood and many other tissues<sup>20</sup>. These 69 CpGs, with positive age correlations across diverse tissues, provided the first indication that a tissue-independent age estimation procedure can indeed be developed. The first multi-tissue age estimator, often referred to as Horvath's clock, was constructed 3 years later<sup>8</sup>. Horvath's clock was trained and validated using 8,000 publicly available microarray samples from over 30 different tissue and cell types collected from children and adults<sup>8</sup>. To better fit the training data, the

Table 1 | Age-related conditions linked to epigenetic age acceleration in specific tissues

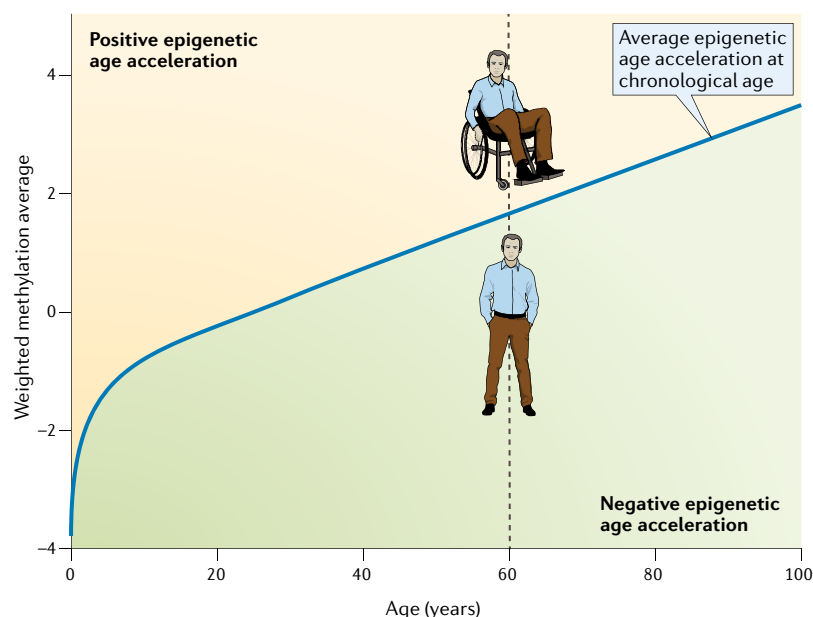
Condition	Source of DNA	Age estimator <sup>a</sup>	Refs
Alzheimer disease	Prefrontal cortex	Horvath's clock	62,87
Amyloid load and neuropathology	Prefrontal cortex	Horvath's clock	62,87
Blood pressure (systolic)	Blood	Hannum's clock	13
Body mass index	Liver	Horvath's clock	85
Cancer	Blood	All clocks	66–68,151
Cardiovascular disease	Blood	DNAm PhenoAge	59
Coronary heart disease	Blood	DNAm PhenoAge	59
Cellular senescence (oncogene-induced)	Various	Horvath's clock	95
Centenarian (offspring status)	Blood	Horvath's clock	65
Cholesterol, HDL (not LDL)	Blood	Hannum's clock and DNAm PhenoAge	13,59
Cognitive performance	Blood and brain	Horvath's clock and DNAm PhenoAge	59,61
C-reactive protein	Blood	All	13,59
Diet (carotenoids)	Blood	Hannum's clock and DNAm PhenoAge	13,59
Dementia	Blood	DNAm PhenoAge	59
Down syndrome	Blood and brain	Horvath's clock	9
Education	Blood	Hannum's clock and DNAm PhenoAge	13,59
Exercise (recreational)	Blood	Hannum's clock and DNAm PhenoAge	13,59
Frailty	Blood	Horvath's clock and DNAm PhenoAge	59,61,64
Gender	Blood and brain	All	11
Gestational week	Blood and brain	Horvath's clock	119,120
Glucose	Blood	All	13,59
Huntington disease	Blood and brain	Horvath's clock	152
Income	Blood	Hannum's clock and DNAm PhenoAge	13,59
Insulin levels	Blood	All	13,59
Menopause	Blood and saliva	Horvath's clock	12
Mortality (all-cause)	Blood	All	3738,73,74
Obesity	Liver and blood	All clocks	13,59
Osteoarthritis	Cartilage	Horvath's clock	153
Parkinson disease	Blood	All	63
Pubertal development	Blood	Horvath's clock	36,121
Sleep	Blood	Hannum's clock	60
Smoking	Blood	DNAm PhenoAge	59
TERT expression	Blood and fibroblasts	Horvath's clock	89
Triglycerides	Blood	All	13,59
Walking speed	Blood	DNAm PhenoAge	59
Werner syndrome	Blood	Hannum's clock and Horvath's clock	10

HDL, high-density lipoprotein; LDL, low-density lipoprotein. <sup>a</sup>Age estimator<sup>a</sup> indicates whether the effect was measured using the multi-tissue 353 CpG methylation-based age estimator, also known as Horvath's clock<sup>8</sup>, the single-tissue (blood) 71 CpG methylation-based age estimator, also known as Hannum's clock<sup>34</sup>, or the DNA methylation-based phenotypic age (PhenoAge) measure based on 513 CpGs<sup>59</sup>.

penalized regression model used a transformed version of chronological age as an outcome measure. This approach revealed an interesting biological phenomenon: the rate of change of DNAm age (the ticking rate of the epigenetic clock) is faster during growth and development<sup>8</sup> (FIG. 2).

The mathematical optimization algorithm automatically selected 353 CpGs by minimizing the error associated with estimating chronological age. The methylation

states of 193 of these CpGs are positively correlated with age, whereas the remaining 160 CpGs have a negative correlation<sup>8</sup>. Of note, although the methylation state of many of these CpGs exhibits only a weak correlation with age individually, their collective effect produces a composite multivariate biomarker that is arguably the most accurate molecular measure of chronological age to date. Horvath's clock is the first multi-tissue age estimator that can accurately measure age using DNA from



**Fig. 2 | Multi-tissue DNA methylation-based age and age acceleration.** The solid blue line shows how an uncalibrated version of the multi-tissue DNA methylation-based (DNAm) age estimate, weighted average of 353 CpGs, changes with age<sup>8</sup>. The rate is very fast in the first year of life, after which it decreases gradually in a nonlinear manner until about 20 years of age. From then on it settles to a slower constant rate. Individuals above the line exhibit epigenetic age acceleration and are older than their peers at the same chronological age. Conversely, individuals below the line exhibit negative epigenetic age acceleration and are younger than their peers at the same chronological age. Positive epigenetic age acceleration is associated with a myriad of pathologies and age-related functional decline (TABLE 1). The multi-tissue DNAm age estimator is a continuous molecular readout of innate processes that accompany development, cell differentiation, tissue homeostasis and, ultimately, ageing.

multiple sources of cells, tissues and organs (with the exception of sperm) across the entire lifespan. The high accuracy of this epigenetic clock has been validated in hundreds of independent data sets and has yet to fail. The degree of its accuracy is such that many laboratories use it as a metric for detecting data entry errors or alignment errors between clinical variables and DNA methylation data.

Many different technological platforms exist to measure DNA methylation, including microarrays, pyrosequencing, quantitative PCR and next-generation sequencing methods<sup>41,55</sup>. The multi-tissue DNAm age estimator is compatible with different technological platforms. Equally important is the robustness of this estimator against missing data points. For instance, although the estimator was trained on data generated by Illumina 27 K and 450 K arrays, it is directly applicable to the latest Illumina EPIC array despite the absence of 17 clock CpGs<sup>8</sup>.

Since its inception, the multi-tissue age estimator has been used in a wide range of studies, including the determination of ageing rates of different parts of the body<sup>8</sup>, which revealed that most tissues and organs from the same body exhibit broadly similar ages. This synchronicity of DNAm age across all tissues is perhaps the most intriguing feature of the epigenetic clock, as best highlighted by brain and blood cells. These cell types represent opposite spectral ends of cellular proliferation

frequency and regeneration and yet yield similar epigenetic age estimates. Nonetheless, some very interesting unexpected exceptions exist. For example, the cerebellum is epigenetically younger than other parts of the brain<sup>56</sup>, and female breast tissue is epigenetically older than other parts of the body<sup>8,57</sup>. Although the multi-tissue DNAm age estimator does not apply to sperm cells<sup>8</sup>, evidence suggests that a specific epigenetic age estimator for sperm can be constructed<sup>58</sup>.

**Phenotypic age estimator.** The first generation of DNAm age estimators exhibited only weak associations with clinical measures of physiological dysregulation, such as blood pressure or glucose levels<sup>13</sup>. Physiological dysregulation, which is more closely related to clinical biomarkers of ageing than to chronological age, is the result of not only age-related molecular alterations but also endogenous or exogenous stress factors, for example, obesity. To develop even more powerful DNAm-based estimators of biological age that incorporate these features, several strategies can be employed (BOX 2). One of these strategies involves the replacement of chronological age with a surrogate measure of biological age ('phenotypic age') that differentiates morbidity and mortality risk among individuals of the same age, as exemplified by the phenotypic age estimator built by Levine et al.<sup>59</sup>. This phenotypic age estimator, referred to as DNAm PhenoAge, was constructed by first generating a weighted average of 10 clinical characteristics: chronological age, albumin, creatinine, glucose and C-reactive protein levels, lymphocyte percentage, mean cell volume, red blood cell distribution width, alkaline phosphatase and white blood cell count<sup>59</sup>. These values were then regressed on DNA methylation levels in blood using a penalized regression model. This approach resulted in the automatic selection of 513 CpGs<sup>59</sup>, the weighted average of which is effectively an estimate of phenotypic age.

DNAm PhenoAge greatly outperforms the first generation of DNAm age estimators for predicting mortality, health span or cardiovascular disease, as well as in terms of its strong relationship with various measures of multi-morbidity<sup>59</sup>. Overall, DNAm PhenoAge is an attractive composite biomarker based on DNAm levels in blood that captures organismal age and the functional state of many organ systems and tissues, above and beyond what is explained by chronological time. However, similar to Hannum's clock, DNAm PhenoAge can lead to biased age estimates in children and in non-blood tissues.

### Epigenetic age acceleration

As would be expected, the application of DNAm age estimators to the general population invariably reveals outliers, that is, individuals whose chronological and epigenetic ages are divergent. In simple operational terms, those with an epigenetic age that is older than expected (on the basis of their chronological age) are described as exhibiting positive epigenetic age acceleration, whereas the reverse situation would be described as negative age acceleration (FIG. 2). Positive epigenetic age acceleration suggests that the underlying tissue ages faster than expected on the basis of chronological age,



**Box 2 | Statistical strategies for building DNA methylation-based estimators of biological age**

The development of a DNA methylation-based (DNAm) age estimator requires three major decisions: the statistical prediction method (for example, penalized regression), the outcome measure (that is, a surrogate marker of biological age) and the covariates (a subset of CpGs). Alternative statistical methods are not likely to lead to substantial improvements because both theoretical and empirical studies show that elastic net regression works extremely well when the number of predictors ( $p$ ) is much larger than the number of observations ( $n$ )<sup>5</sup>.

Larger sets of CpGs should in theory result in more accurate biomarkers. However, our empirical studies indicate that relatively little is gained by looking at ever larger sets of CpGs. The reason for this phenomenon of diminishing returns is probably because DNAm biomarkers of ageing measure global properties of the methylome that can be characterized by moderate numbers of CpGs.

The multi-tissue DNAm age estimator uses chronological age as a surrogate for biological age because chronological age is highly correlated with biological age and is arguably a near-optimal surrogate of biological age during development<sup>8</sup>. However, other outcome measures can lead to substantial improvements in regard to mortality and morbidity prediction in adults, as can be seen from the success of the DNAm phenotypic age (PhenoAge) estimator<sup>59</sup>. Defining biologically meaningful surrogate measures of biological age, beyond chronological age, is conceptually challenging because of the dangers of confounding<sup>142</sup>.

Another strategy for developing more powerful DNAm estimators of organismal age consists of aggregating the DNAm age estimates of multiple organs. Future research should explore how to define powerful composite biomarkers of ageing on the basis of DNAm age estimates of different accessible tissues such as skin, buccal epithelium, and adipose tissue or fluids such as blood or urine.

whereas a negative value suggests that the tissue ages slower than would be expected.

**Intrinsic versus extrinsic measures of epigenetic age acceleration.** An important feature of blood that accompanies ageing is the change in cell-type composition. The proportions of naive or senescent cytotoxic T cells change with age. Although the age imputed by Horvath's clock is largely unperturbed by and independent of these changes, the ages measured by the Hannum estimator and DNAm PhenoAge estimator are more reflective of changes in cell-type composition. As such, the multi-tissue DNAm age estimator is said to measure (cell-)intrinsic ageing, whereas the Hannum and DNAm PhenoAge estimators also measure extrinsic ageing<sup>38,59</sup>. These operational definitions are naturally extended to describe age accelerations as intrinsic age acceleration and extrinsic age acceleration on the basis of the estimators used. For some disorders, such as immunosenescence or HIV infection, differences in blood cell subpopulations are the hallmark of the disorder itself and correcting for this would remove the true biological signal. For example, sleep disturbances have been associated with a decrease in naive CD8-positive T cells and an increase in extrinsic epigenetic age acceleration<sup>60</sup>. Although intrinsic measures seem to exhibit greater consistency across cell types and organs, extrinsic measures seem to be better suited for assessing age-related decline of tissue performance<sup>38</sup> as they exhibit stronger predictive associations with time to death than intrinsic measures of age acceleration (based on multi-tissue DNAm age)<sup>38,59</sup>. Regardless of the type, age acceleration is of particular interest and importance because it pertains to outliers or deviations from the norm, which is more interesting and revealing than the average.

**Relevance for age-related conditions.** The relevance of measures of epigenetic age acceleration can be appreciated by the fact that they are associated with a great number of age-related conditions and diseases (TABLE 1). Both DNAm age and DNAm PhenoAge acceleration

are linked to neuropathology in elderly individuals<sup>61,62</sup>, Down syndrome<sup>9</sup>, Parkinson disease<sup>63</sup>, Werner syndrome<sup>10</sup>, physical and cognitive fitness<sup>61,64</sup> and centenarian status<sup>65</sup>. Certain conditions, notably Down syndrome, are associated with strong epigenetic age acceleration in both blood and brain tissue<sup>9</sup>. In addition to being predictive of all-cause mortality, DNAm age and DNA PhenoAge acceleration in blood are associated with the risk of developing certain types of cancer<sup>59,66–69</sup>.

Given the existence of the blood–brain barrier, it is striking that associations can be observed between epigenetic age acceleration of blood (based on multi-tissue DNAm age) and white matter integrity in the brain<sup>70,71</sup>, as well as with various measures of cognitive or memory functioning in older individuals<sup>61,72</sup>. Similarly, DNAm PhenoAge acceleration is associated with dementia status and various measures of cognitive functioning<sup>59</sup>.

Both intrinsic and extrinsic measures of epigenetic age acceleration in blood are associated with an increased risk of death from all-natural causes even after accounting for known risk factors<sup>37,38,73–75</sup>. A meta-analysis of blood DNA methylation data from more than 13,000 individuals belonging to three racial groups (non-Hispanic white, Hispanic and African-American individuals) found that all measures of age acceleration considered were able to predict life expectancy<sup>38</sup>. DNAm PhenoAge acceleration stands out in terms of its strong predictive accuracy for time to death (due to all-cause mortality), cause-specific death, coronary heart disease and disease-free status. DNAm PhenoAge is correlated with the number of comorbidities, various measures of physical functioning or frailty, age-related dementia and cognitive impairment, and it relates to a host of blood-based biomarkers, including C-reactive protein, insulin, fasting glucose, triglyceride and high-density lipoprotein (HDL) cholesterol levels. DNAm PhenoAge is also associated with lifestyle and demographic variables, including educational level, physical exercise, income, systolic blood pressure, body mass index, markers of fruit and vegetable intake (for

example, carotenoid levels) and smoking status. By contrast, DNAm age acceleration as determined by the multi-tissue DNAm age estimator exhibits much weaker associations with lifestyle factors and markers of inflammation, which probably reflects the fact that it relates to an innate ageing process that is under genetic control<sup>13</sup>, as discussed below.

### Linking DNAm age to biological function

The use of advanced machine learning methods to analyse large sets of DNA methylation data has generated DNAm age and DNAm PhenoAge estimators that outperform existing molecular biomarkers of ageing in terms of their strong relationship with chronological age<sup>15</sup> as well as with a large number of age-related conditions. However, this unbiased data-driven approach presents challenges for understanding what exactly is being measured by these estimators. What do the clock CpGs represent? Are methylation changes of these CpGs driving ageing or are they consequences of ageing? Several approaches described below can be used to address this challenge, including determining the cellular function of the clock CpGs, determining correlative changes in gene expression or carrying out genome-wide association studies (GWAS) to identify genetic factors associated with epigenetic age acceleration. Other approaches not discussed in detail include *in vitro* studies, longitudinal mediation analyses of human cohorts and studies in model organisms.

**Genetic oscillators and circadian rhythm.** Most clocks are based on an oscillator. It is therefore plausible that DNAm age relates to a genetic oscillator, for example, a cell cycle oscillator, a (developmental) segmentation clock or the circadian clock<sup>76–78</sup>. Indeed, emerging evidence suggests that DNAm age estimators relate to circadian rhythm; for example, 1 of the 353 CpGs of the multi-tissue DNAm age estimator is located in the 5' untranslated region of the *CLOCK* gene, and cytosines with circadian epigenetic oscillations considerably overlap with cytosines exhibiting age-related changes<sup>79</sup>.

**Function of clock CpGs.** The identification of clock CpGs naturally leads to the assumption that these CpGs are the drivers of epigenetic ageing. However, clock CpGs used in age predictors are those CpGs that collectively produce the most accurate readout of age. They are neither exclusive nor comprehensive of all age-related CpGs. Overall, it is best to interpret epigenetic age estimates as a higher-order property of a large number of CpGs much in the same way that the temperature of a gas is a higher-order property that reflects the average kinetic energy of the underlying molecules. This interpretation does not imply that DNAm age simply measures entropy across the entire genome. Instead, analyses of the clock CpG sites underlying Horvath's clock revealed that a statistically significant number of the 193 CpGs that correlate positively with age are located in poised promoters, whereas the 160 negatively correlated CpGs are located in strong enhancers<sup>8</sup>. Of note, histone 3 lysine 4 trimethylation (H3K4me3) and H3K27me3, which are characteristic

of poised promoters, also characterize many promoters in embryonic stem cells that control developmental genes. Although they are transcriptionally repressed in this bivalent state, they can be activated upon appropriate stimulation. During cellular differentiation, some of these promoters become methylated, leading to the speculation that their plasticity is replaced by stable repression as part of the process of lineage determination<sup>25</sup>. These features suggest that the multi-tissue DNAm age estimator, and thus its underlying clock CpGs, has at least an indirect relationship with biological processes underlying development, cell differentiation and the maintenance of cellular identity. It has been proposed that age-related methylation changes of clock CpGs reflect the workings of an epigenetic maintenance system in the service of these biological processes<sup>8</sup>, as discussed below.

**Gene expression and age-related changes in DNA methylation.** Providing direct evidence linking age-related methylation changes to transcriptional changes has been difficult for several reasons<sup>25,32,80</sup>. First, it is important to appreciate that epigenetic ageing is not a concerted change of all the cells in a tissue at the same time from one epigenetic state to another. Instead, epigenetic ageing comprises DNA methylation changes that occur in a small number of cells at any one time (BOX 3). To be precise, the average difference of methylation between DNA from people aged <35 years and those >55 years old is 0.032 (3.2%). Unsurprisingly, such minute changes are not expected to be reflected in RNA or protein levels in bulk cell population analyses. With the advent of single-cell technologies, which enable measurements of RNA and DNA methylation in individual cells, the relationship between DNA methylation levels and transcriptional changes might be revealed<sup>81,82</sup>. Second, the low correlation between DNA methylation and gene expression levels may reflect fairly high fluctuation of RNA levels, which can change from one hour to the next. By contrast, age-related DNA methylation states seem to be far more stable<sup>32</sup>. Third, age-related changes in methylation of CpGs might not act on proximal genes<sup>83</sup> but distal ones, increasing the complexity of detecting correlative changes. Fourth, it is possible that age-related methylation changes have only limited effects on gene expression<sup>84</sup>.

Unlike DNAm age estimators, DNA PhenoAge exhibits strong correlations with gene expression levels in sorted blood cells. DNA PhenoAge acceleration was found to be associated with increased activation of pro-inflammatory and interferon pathways and decreased activation of the transcriptional and translational machineries, the DNA damage response and nuclear mitochondrial signatures<sup>59</sup>. Nuclear mitochondrial genes were also found to be inactivated in liver tissues with positive epigenetic age acceleration<sup>85</sup>.

**Genetic studies of epigenetic age acceleration.** The notion that epigenetic ageing is at least affected, if not regulated, by genetics is intuitive and supported by twin studies and single-nucleotide polymorphism (SNP)-based estimates of the heritability of epigenetic age



### Box 3 | DNAm age as a measure of clock cells

To understand the nature of DNA methylation-based (DNAm) age estimators, it is necessary to appreciate what is being measured. Each clock CpG takes a value between 0 and 1, whereby 1 indicates methylated and 0 indicates unmethylated. Importantly, the values of the clock CpGs (and the vast majority of all CpGs) are almost never 0 or 1 but a value in between. For example, a value of 0.66 indicates that 66% of the thousands of copies of that particular CpG, which were derived from thousands of cells, are methylated. This non-binary value occurs because DNA methylation measured by the Illumina Infinium platform is not from a single cell but a population of cells (in the thousands). Hence, the non-binary methylation values reveal that cells from a particular population or tissue are epigenetically heterogeneous, even if they seem morphologically homogeneous. This intercellular heterogeneity gives rise to the methylation values used by age estimators to measure epigenetic age. Of note, this change in heterogeneity is very small: the average change of a clock CpG between the ages <35 years and >55 years is only 3.2%. These age-related changes probably reflect both intracellular changes and changes in cell composition. The changes in cell composition could reflect systematic changes in a subset of cells, which we term 'clock cells'. DNAm age might track the loss of somatic stem cells in some tissues. However, this interpretation of DNAm age is not directly applicable to sorted neurons, which lend themselves to very precise DNAm age estimates<sup>8</sup>. The DNAm age of neurons (and other cells) probably reflects intracellular DNAm changes that result from entropy, the failure of DNAm maintenance and/or the actions of an epigenomic maintenance system.

acceleration. These analyses produced fairly high heritability estimates ( $h^2$ ) of approximately 40%<sup>8,34,37,49,86,87</sup>. This finding is consistent with the fact that the rate of epigenetic ageing is highly stable across the lifespan<sup>88</sup>. Hence, identifying the genes that affect the pace of epigenetic ageing represents a new frontier in GWAS that may help to characterize the underlying biological process of ageing.

GWAS of epigenetic age acceleration have identified several loci within the genome that typically exhibit tissue-specific associations, including the *MLST8* gene in the cerebellum, the *EFCAB5* gene in multiple brain regions and the *TERT* gene in blood<sup>86,87,89</sup>. Of note, *MLST8* encodes a subunit of the mammalian target of rapamycin (mTOR), which regulates cell growth and survival in response to nutrient and hormonal signals. How different *MLST8* alleles affect epigenetic ageing in the cerebellum remains to be determined. Although the paucity of information prevents speculation on how the *EFCAB5* protein product might influence epigenetic ageing, the same cannot be said of telomerase reverse transcriptase (TERT), the catalytic subunit of telomerase, which has been implicated in ageing and cancer.

**Telomerase reverse transcriptase and telomere length.** Numerous large-scale epidemiological studies have shown that telomere length has only a weak negative association with markers of biological age and life expectancy<sup>64,90–92</sup>. This finding could be because telomere attrition does not have marked effects on cell physiology until a critical telomere length is reached<sup>93</sup>, at which point the cell becomes senescent<sup>91</sup>. Leukocyte telomere length exhibits weak negative correlations with extrinsic epigenetic age acceleration (based on Hannum's clock) and DNAm PhenoAge acceleration, which probably reflects age-related changes in blood cell composition such as the decline of naive T cells<sup>59,94</sup>. However, telomere length has at best a negligible correlation with intrinsic epigenetic age acceleration (that is, multi-tissue DNAm

age adjusted for blood cell counts)<sup>64,92,94</sup>. Genetic studies have discovered an unexpected relationship between telomere maintenance genes, notably *TERT*, and intrinsic epigenetic age acceleration. *TERT* alleles that are associated with longer telomeres are also associated with increased intrinsic epigenetic age acceleration in leukocytes<sup>89</sup>. This surprising association could be confirmed using ex vivo experiments in which TERT-expressing cells continued to grow and age epigenetically long after the nontransduced control cells had senesced<sup>89,95</sup>. These studies demonstrate that biological ageing cannot be arrested by *TERT* expression alone.

Although the epigenetic clock does not relate to telomere length in differentiated cells, several lines of evidence suggest that telomere biology relates to epigenetic ageing in stem cells (for example, the above mentioned GWAS studies in blood). Embryonic stem cells and induced pluripotent stem (iPS) cells with short telomeres exhibit reduced expression of DNA (cytosine-5)-methyltransferase 3A (DNMT3A), lower levels of DNA methylation and unstable differentiation<sup>96,97</sup>. Overall, DNA methylation has profound effects on the ability of stem cells to differentiate and sustain their committed state owing to an inefficiency in methylating and repressing the promoters of pluripotent factors such as NANOG (reviewed in REF<sup>97</sup>). Increasing evidence points to stem cell differentiation as one of the molecular mechanisms underlying the epigenetic clock (BOX 3), which also has implications for cancer development.

**Mitotic age.** In stark contrast to telomere length<sup>95</sup>, the DNAm age of differentiated cells does not reflect their proliferative history, as evidenced by one of the most notable features of the multi-tissue DNAm age estimator, which produces similar age estimates within the same individual for highly proliferative tissues, for example, blood and colon, and less-proliferative ones, such as neurons<sup>8</sup>. Thus, DNAm age is not the same as mitotic age, which enumerates the number of times a cell has divided. Supporting the notion that DNAm age does not measure mitotic age<sup>98</sup>, some cancer types, such as luminal breast cancer, are associated with positive age acceleration, as determined by the multi-tissue DNAm age estimator, whereas others, such as basal breast cancer, are associated with negative age acceleration<sup>8,99</sup>. That is, malignant tissue can be younger than expected. Despite the strength of in vivo evidence described above, a correlation between DNAm age and cellular proliferation has nevertheless been observed in certain ex vivo cultures of mesenchymal stem cells<sup>8</sup> and primary fibroblasts<sup>89</sup>. Whether this observation is specific only to ex vivo culture conditions or whether it hints at the possibility that DNAm age is associated with the proliferation of specific cells, such as stem cells or progenitor cells, in vivo remains to be determined.

Although the multi-tissue DNAm age estimator does not measure cellular proliferation, this does not imply that there are no DNA methylation changes that occur in function of cellular division. Indeed, several proposals have been put forward exploring the feasibility of using the DNA methylation changes to develop a mitotic clock,

for example, for measuring the number of cell divisions incurred by long-lived adult stem cells<sup>32,100–102</sup>. The first such mitotic-like clock recorded increased proliferation universally in cancerous and precancerous lesions<sup>98</sup>, as would be expected. The different sets of CpGs that underpin the mitotic clock and the epigenetic clock make it clear that these two clocks are distinct and that they measure different cellular properties.

**Cellular senescence.** Although it is clear that epigenetic age is not a measure of cellular proliferation in differentiated cells, it is affected by certain triggers of senescence. Cellular senescence can be triggered by multiple factors, including, but not limited to, critically short telomeres, epigenetic derepression of the cyclin-dependent kinase inhibitor 2A (CDKN2A; also known as p16<sup>Ink4A</sup> or ARF) locus, overexpression of oncogenes and DNA damage<sup>103</sup>. Senescent cells lack replicative capacity and, therefore, cannot contribute to tissue homeostasis<sup>104–108</sup>, but they remain metabolically active and secrete a myriad of pro-inflammatory cytokines<sup>109</sup>. The important role of cellular senescence in ageing is elegantly demonstrated in experiments that recorded rejuvenation or delayed ageing in mice that were engineered to eliminate senescent cells<sup>110–112</sup>. Although senescent cells exhibit methylation changes of some specific CpGs, these are in general different from age-related CpGs<sup>113</sup>.

In general, cellular senescence and epigenetic ageing are distinct phenomena<sup>95</sup>. For example, cells induced to senesce by irradiation do not exhibit epigenetic ageing<sup>8,95</sup>. Conversely, TERT-immortalized cells, which do not undergo replicative senescence, continue to exhibit epigenetic ageing<sup>89,95</sup>. Although the distinctiveness and uncoupling of epigenetic ageing and senescence are clear, they can nevertheless converge under some circumstances. For example, it was observed that oncogene-induced cellular senescence was accompanied by epigenetic ageing<sup>95</sup>. Why, when and how these two different features of ageing can become entwined remain to be elucidated. It is possible that cellular senescence and epigenetic ageing might serve different but complementary roles when it comes to suppressing potential cancer development (for example, overexpression of oncogenes). Whereas cellular senescence aims to inhibit cellular proliferation, epigenetic ageing might protect against dedifferentiation signals and epigenomic and/or genomic instability.

**The epigenomic maintenance system and cancer.** When the multi-tissue DNAm age estimator was first reported, it was hypothesized that the methylation changes represent actions carried out by cells to maintain epigenetic stability. In other words, epigenetic age could potentially be the historical record of the activity of an epigenomic maintenance system. Upon sensing epigenomic and/or genomic instability, the epigenomic maintenance system is hyperactivated to limit somatic mutations and to reinforce stable cell differentiation. The more rigorous the activation of the epigenomic maintenance system, the more likely that further instability and mutation acquisition is mitigated. As a

consequence, this would generate a greater degree of epigenetic age acceleration. Interestingly, this projection is consistent with the observation that in some cancers, tumours with fewer somatic mutations, indicative of more stable genomes, have greater epigenetic age acceleration and vice versa<sup>8,99</sup>. This hypothesis is also consistent with the observation that expression of several oncogenes is associated with increased DNAm age, as is oncogene-induced cellular senescence in ex vivo studies<sup>95</sup>. Hence, the inverse relationship between the number of mutations and epigenetic age acceleration of some tumours may be a tell-tale sign of the intensity of the epigenomic maintenance system to mitigate epigenomic instability. From this hypothetical perspective, the molecular processes underlying the increase in DNAm age in adults are probably not accidental but beneficial: they accompany processes that suppress epigenomic instability and concomitant erroneous transcriptional changes that compromise cellular identity and tissue homeostasis. The components and mechanisms of the epigenomic maintenance system remain to be characterized but may involve DNMTs.

### Epigenetic clock in development and ageing

The role of DNA methylation in the processes of development (embryogenesis and cell differentiation) is well described<sup>114</sup>. The following lines of evidence link the multi-tissue DNAm age estimator to molecular processes that have a role in cellular differentiation. First, a considerable proportion of the 353 clock CpGs of Horvath's clock are located near genomic sites that are known to control the expression of genes in development and differentiation (enhancer regions and targets of PRC2)<sup>8</sup>. Second, DNAm age correlates strongly with differentiation status in neuronal cell cultures and distinguishes neural precursors from more mature neurons<sup>8</sup>. Third, the DNAm age of adult somatic cells can be reset to that of stem cells — resulting in negative DNAm age — by expressing Yamanaka factors<sup>8</sup>. At the other end of the age spectrum, DNAm changes might be related to the decline of stem cell function<sup>19,20,25,115</sup>, myeloid skewing of an ageing haematopoietic system<sup>24,100,101</sup>, immunosenescence<sup>116</sup>, cryptic transcription<sup>117</sup> or the expression of transposable elements<sup>118</sup>. From the outset, developmental epigenetic processes seem to have little in common with these age-related processes later in life. However, the continuous molecular readout provided by the multi-tissue DNAm age shows that purposeful molecular processes during development and cell differentiation are at least indirectly linked to detrimental processes later in life (FIG. 2).

Although positive epigenetic age acceleration has been linked to a myriad of age-related conditions later in life (TABLE 1), it might be beneficial early in life, as indicated by the following results: gestational week correlates with DNAm age<sup>19,120</sup>; children who are epigenetically older at birth are taller and have a higher fat mass throughout childhood and adolescence even after adjusting for sex<sup>35</sup>; and epigenetic age acceleration in children correlates with certain measures of pubertal timing, including female breast density and age at menarche<sup>36,121</sup>. The antagonistic pleiotropic effect

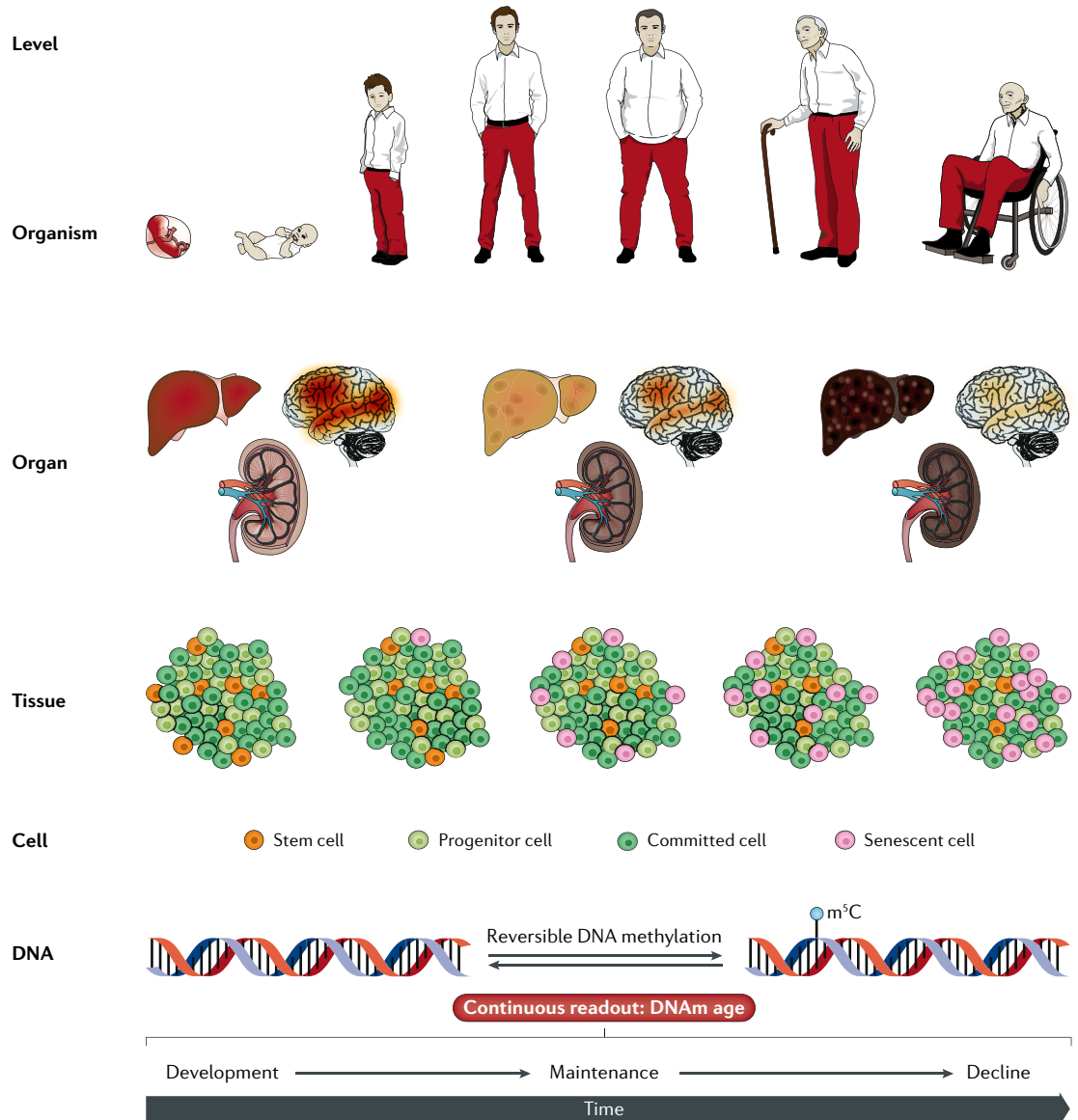
## Quasi-programme theories of ageing

Several variations of a theory that posits that ageing is not the intended outcome of biological processes but that some programmed processes nevertheless result in ageing. Therefore, the process of ageing can be viewed as quasi-programmed.

of DNAm age is illustrated by its relation to gender: boys are epigenetically older than girls at a young age, possibly reflecting larger birthweight, and this sex difference persists to old age where it accompanies higher mortality risk<sup>11,35</sup>.

Several quasi-programme theories of ageing have explored the potential link between development and ageing<sup>122–132</sup>. The proposed epigenetic clock theory of ageing views biological ageing as an unintended consequence of both developmental programmes and maintenance programmes, the molecular footprints of

which give rise to DNAm age estimators. The precise mechanisms linking the innate molecular processes (underlying DNAm age) to the decline in tissue function probably relate to both intracellular changes (leading to a loss of cellular identity) and subtle changes in cell composition, for example, fully functioning somatic stem cells (BOX 3). At the molecular level, DNAm age is a proximal readout of a collection of innate ageing processes that conspire with other, independent root causes of ageing to the detriment of tissue function (FIG. 3). The genetic studies have revealed that the innate ageing



**Fig. 3 | Tissue function versus DNA methylation-based age.** DNA methylation-based (DNAm) age is a continuous readout of molecular processes that play a role in development, tissue maintenance and, ultimately, decline. DNAm age increases as stem and progenitor cells undergo differentiation to produce more committed cells for growth during the early developmental years and for replenishment of committed cells during the maintenance years (after 20 years). The precise mechanisms linking the innate molecular processes to the decline in tissue function probably relate to subtle changes in cell composition, for example, a decline in somatic stem cells, and/or the loss of cellular identity. Independently, senescent cells, which are not measured by the multi-tissue DNAm age estimator, begin to accumulate in later years owing to numerous factors unrelated to epigenetic ageing. In time, these collective changes at the cellular level compromise tissue fitness, leading to the decline of organ functions and the manifestation of physical ageing. m<sup>5</sup>C, 5-methylcytosine.

## Box 4 | Effect of stress on age acceleration

During the inexorable passage of time throughout life, the body is exposed to multiple stress factors that sometimes affect DNA methylation levels and DNA methylation-based age estimates. Many stress factors seem to have a tissue-specific effect (for example, metabolic stress resulting from obesity greatly accelerates the epigenetic age of the liver but much less so of blood)<sup>13,85</sup>. Long-term exposure to air pollution has shown weak associations with increased epigenetic age acceleration in blood<sup>143,144</sup>. HIV infection is associated with increased epigenetic age in blood and brain tissue<sup>145,146</sup>, whereas cytomegalovirus infection and *Helicobacter pylori* infection increase the epigenetic age of blood<sup>147,148</sup>.

Cumulative lifetime stress (but not acute psychological stress) has been linked to epigenetic ageing<sup>149,150</sup>. Higher income and educational levels are associated with lower extrinsic age acceleration<sup>13</sup>. Of note, not every stress factor that affects mortality and morbidity is associated with biological ageing. Many toxins kill within short time periods without affecting innate ageing processes. The fact that external stress factors have only a weak effect on intrinsic age acceleration highlights that the latter is under strong genetic control<sup>13,89</sup>.

processes underlying DNAm age are to a considerable extent under genetic control. As such, it is not surprising that apart from some notable exceptions, extrinsic stress factors have only a weak impact on intrinsic epigenetic age acceleration (BOX 4). Wear-and-tear theories of ageing seem to be inconsistent with epigenetic ageing effects early in life but are consistent with epigenetic ageing effects later in life.

### Anti-ageing interventions

The great promise of DNAm age biomarkers of ageing is the identification and/or validation of effective anti-ageing interventions in humans. The view that epigenetic surrogate biomarkers of mortality or morbidity will address this challenge is supported by findings that a healthy diet (high vegetable and fish intake), avoidance of obesity and physical exercise are associated with slower extrinsic epigenetic age acceleration in blood<sup>13,59</sup>. These unsurprising results are remarkable in that they were obtained using cross-sectional studies. However, DNAm age and DNAm PhenoAge are not particularly dynamic markers of lifestyle changes; that is, short-term lifestyle interventions seem to have only a small effect on epigenetic ageing rates<sup>13,85</sup>. For example, within a 9-month follow-up period, the substantial weight loss resulting from bariatric surgery was not associated with a reduction in epigenetic age of human liver tissue samples<sup>85</sup>. Moreover, it is likely that anti-ageing interventions will have tissue-specific effects. For example, postmenopausal hormone replacement therapy is associated with epigenetic age acceleration in buccal epithelial cells but not in blood cells<sup>12</sup>.

To date, the most effective in vitro intervention against epigenetic ageing is achieved through expression of Yamanaka factors, which convert somatic cells into pluripotent stem cells, thereby completely resetting the epigenetic clock<sup>8</sup>. In vivo, haematopoietic stem cell therapy resets the epigenetic age of blood of the recipient to that of the donor<sup>133,134</sup>. The dynamics of this process reveal a striking and short-term added rejuvenation effect<sup>134</sup>. For example, 6 months after a 50-year-old individual receives blood from a 20-year-old individual, the blood of the recipient becomes approximately 14 years old. However, this additional rejuvenation effect

of 6 years disappears within the next 6 months, at which point the age of blood in the recipient becomes about 21 years old. Five years after transplantation, the blood of the recipient will have a DNAm age of 25 years unless graft versus host disease develops, which accelerates epigenetic ageing<sup>134</sup>.

DNAm-based biomarkers will probably not replace existing clinical biomarkers that inform patient care. For example, it is hard to imagine that an epigenetic age measurement would replace a blood pressure measurement for the evaluation of anti-hypertensive therapeutics. Nonetheless, DNAm age or DNAm PhenoAge estimators may be useful for identifying drugs that block early ageing processes that give rise to the multitude of ageing comorbidities. It remains unknown whether it is possible to slow biological ageing by directly targeting age-related DNA methylation levels.

### Conclusions and perspectives

Perhaps the most profound revelation of the multi-tissue DNAm age estimator is the existence of innate epigenetic clock processes that have a role in development, cell differentiation, tissue homeostasis and, ultimately, ageing. In the language of Greek mythology, DNAm age is Clotho's 'thread of life,' connecting development from conception to post-maturity maintenance and ultimately death. As such, the epigenetic clock reveals that biological ageing is intricately woven into the very biological processes that initiate, develop and maintain life.

The existing DNAm age and DNAm PhenoAge estimators have demonstrated that, collectively, these epigenetic biomarkers of ageing satisfy the formerly elusive properties of molecular biomarkers of ageing. These human DNAm biomarkers have ushered in a new era of molecular ageing studies that leverage large-scale epidemiological studies surrounding the organism that we most care about. These studies have established that DNAm age or DNAm PhenoAge age acceleration is associated with congenital conditions, age-related conditions, lifestyle, environment, longevity and mortality. Further epidemiological studies will increase the number of associations to include many more health conditions and pathologies. In particular, longitudinal human cohort studies will enable the rigorous evaluation of the extent to which epigenetic clock processes mediate the relationship between chronological age and phenotypic manifestations of ageing. Future epidemiological studies should consider other sources of DNA (for example, buccal cells), because more powerful estimates of organismal age can be obtained by evaluating multiple tissues (BOX 2).

To understand how epigenetic clock processes connect with the diverse hallmarks of ageing, experimental studies in cells and animal models will be pivotal and, satisfyingly, many are already in progress<sup>95,135–138</sup>. The most rigorous studies on cause-and-effect relationships will probably leverage epigenetic clocks in vertebrate animal models. To build multi-tissue DNAm age estimators for species that exhibit cytosine methylation patterns (notably, vertebrates) should be straightforward, as evidenced by existing DNAm age estimators for whales<sup>139</sup>, dogs<sup>140</sup> and mice<sup>135–138</sup>. Of note, owing



to high sequence conservation, the human multi-tissue DNAm age estimator also applies to chimpanzees, albeit not to other primates<sup>8</sup>. Phylogenetic and other comparative biological studies of epigenetic ageing rates across species promise to advance our understanding of the epigenetic determinants of maximum or median lifespan. Moreover, DNAm age estimates for model organisms might become useful biomarkers for preclinical studies of anti-ageing interventions. This view is supported by the finding that lifespan-extending interventions in mice, such as early caloric restriction or full-body knockout of the growth hormone receptor gene *Ghr*, slow epigenetic ageing<sup>135–138</sup>. The worm *Caenorhabditis elegans* lacks 5-methylcytosine<sup>141</sup>, but

other types of epigenetic modifications such as adenine methylation or histone modifications may lend themselves for developing epigenetic age estimators in this widely used model organism.

We anticipate many developments in the coming years. Soon there will be much material for evolutionary biologists to contemplate why and how epigenetic ageing has come to be, for biologists to understand every single cog, gear and spring that constitute the epigenetic clock and for clinicians to investigate how to incorporate epigenetic age in their decisions for anti-ageing treatments.

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## Author contributions

The authors contributed equally to all aspects of this article.

## Competing interests

The Regents of the University of California is the sole owner of several patent applications directed at the invention of measures of epigenetic age estimation for which S.H. is a named inventor. K.R. declares no competing interests.

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## Supplementary information

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