INTRODUCTION

Ageing is universally accompanied by a decline in physical and cognitive abilities and an increased disease risk. Advanced age is the strongest risk factor common to cardiovascular, neurodegenerative and malignant diseases (Benayoun et al., 2015). At a cellular level, the hallmarks of ageing include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, de-regulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (reviewed in López-Otín et al., 2013).

Initial evidence that ageing could be decelerated came from genetic studies of short-lived non-vertebrate model organisms (Jia et al., 2004; Kapahi et al., 2004; Kennedy et al., 1995; Klass, 1977; Murphy et al., 2003; Tatar et al., 2001; Tissenbaum & Ruvkun, 1998; Vellai et al., 2003). Nutrient-sensing pathways were identified as regulators of ageing, such as mechanistic target of rapamycin (mTOR) and insulin/insulin-like growth factor (IGF) signalling, which can be manipulated to extend lifespan in mammals such as mice (Blüher et al., 2003; Holzenberger et al., 2003; Johnson et al., 2013; Pearson et al., 2008; Weindruch et al., 1986). Other results based on cellular reprogramming showed epigenetic rejuvenation in mice and humans might be possible (Lu et al., 2020; Manukyan & Singh, 2012, 2014; Ocampo et al., 2016; Olova et al., 2019; Sarkar et al., 2020; Singh & Zacouto, 2010). However, to efficiently quantify the effect of these
interventions, a biomarker that infers biological age is required, that is a biological indicator that predicts the health and lifespan of an individual better than chronological age (chAge) (Baker & Sprott, 1988).

DNA methylation (DNAm), specifically 5-methyl-cytosine (5mC), has emerged as one of the most efficient biomarkers to predict biological age (Benayoun et al., 2015; Jylhävä et al., 2017; Li et al., 2020). In the past decade, a large number of age predictors utilising DNAm have been developed. These DNAm age predictors (more commonly known as epigenetic clocks) are created using CpGs that have tractable changes with age. The majority of these clocks are built using penalised regression models (such as elastic net (Zou & Hastie, 2005) or LASSO (Tibshirani, 1997)), which select a group of CpGs that have a monotonically increasing relationship with age in a given training data (Horvath & Raj, 2018). In other words, key CpGs whose age-related hyper- and hypomethylation correlate with age, are selected and weighted in a linear model. The result is an equation, whereby chronological age can be estimated based on the percentage methylation at these key CpG sites in a given sample.

Epigenetic clocks have become increasingly diverse, with each predictor capturing different aspects of ageing. The expanding repertoire of clocks enable the study of ageing and rejuvenating approaches quantitatively. This review aims to give an overview of the growing toolbox of eAge clocks to inform which approach might best suit to a scientific question.

2 | EPIGENETIC CLOCKS PREDICT BIOLOGICAL AGE

Epigenetic clocks have proven themselves to be accurate at predicting chronological age (chAge), which is commonly referred to as DNAm age or epigenetic age (eAge). When epigenetic clocks first emerged, a fundamental question arose; if eAge deviates significantly from chAge, is this difference due to inaccuracies of the clock itself, or caused by biological factors (e.g. genetics, disease status and environment)? In other words, are the clocks able to predict biological age? This difference between eAge and chAge is referred to as age acceleration and can be calculated as the mean absolute deviation (MAD) or median absolute deviation between eAge and chAge (Horvath, 2013), or as the residual from the linear regression between eAge and chAge (Horvath & Raj, 2018). The reported error of epigenetic clocks can depend on how the test/validation data set was curated. For example, if a pooled data set were split into 90% samples for training and 10% for validation, then the clock would perform better on the validation data set than when applied to a completely independent/external data set. In the various tables summarising epigenetic clocks throughout this review, we will clarify how each clock is validated.

For many of the eAge predictors, age acceleration is associated with a number of age-related diseases and conditions. For example, patients with Down's syndrome (Horvath, Garagnani, et al., 2015), HIV (Horvath & Levine, 2015), obesity (Horvath et al., 2014), Huntington's disease (Horvath, Langfelder, et al., 2016), Werner syndrome (Maierhofer et al., 2017) and Sotos syndrome (Martin-Herranz et al., 2019) tend to exhibit increased age acceleration. eAge acceleration has also been associated with physical and cognitive fitness (Breitling et al., 2016; Marioni, Shah, McRae, Ritchie, et al., 2015; Quach et al., 2017) and neuropathy (Levine et al., 2015; Lu et al., 2017) (for comprehensive lists of age acceleration-associated conditions, please refer to Horvath & Raj, 2018 and Declerck & Berghé, 2018). Variation in epigenetic ageing rates between individuals has been shown to significantly depend on sex and race/ethnicity (Horvath, Gurven, et al., 2016; McCartney et al., 2019). Vitamin D-sufficient individuals have a lower eAge acceleration and longer leukocyte telomere length (LTL) (Chen et al., 2019; Vetter et al., 2020). Smoking has been associated with an increase in eAge in airway cells and lung tissue (4.9 and 4.3 years, respectively) (Wu et al., 2019), and smoking during pregnancy might have an effect on eAge in offspring (Simpkin et al., 2016). The number of studies associating eAge acceleration with diseases, phenotypes and environmental interventions that appear to affect ageing, emphasises eAge as a candidate metric for biological age (Wang et al., 2017). However, some studies have shown no correlation between eAge acceleration and certain diseases or environmental factors, such as type II diabetes (Grant et al., 2017; Horvath, Gurven, et al., 2016), heroin use (Kozlenkov et al., 2017) or depression (Starnawska et al., 2019). Why eAge acceleration tracks with certain age-related disorders and not others are still not well understood. Most epigenetic clocks described in the following section used Illumina DNAm array-based technology and are summarised in Table 1.

3 | DNAM ARRAY-BASED EPIGENETIC CLOCKS

3.1 | Early epigenetic age predictors

The first epigenetic clocks incorporated relatively few CpG sites and samples in their training data sets, in comparison with later clocks. Bocklandt et al., for example created a clock from 68 samples (34 twin pairs) that predicts age in saliva with an average accuracy of 5.2 years (Bocklandt et al., 2011). Koch and Wagner used five CpG sites and predicted age in multiple cell types, but with lower accuracy than the Bocklandt clock (MAD = 11 years) (Koch & Wagner, 2011). The same laboratory produced a six CpG clock that could track passage number in fibroblast cell cultures, regardless of original donor age (Koch et al., 2012). After these initial studies, epigenetic clocks grew in complexity in terms of number of samples, tissues and CpGs implemented.

3.2 | Multi-tissue age predictors

The first multi-tissue age predictor (referred to as the Horvath or Pan-Tissue clock) utilised 353 CpGs and has a mean error of 3.6 years, which at that time was unprecedented for any biomarker/age predictor (Horvath, 2013). The training data set used to construct the clock
## TABLE 1 Epigenetic clocks based on Illumina human DNA methylation arrays

| Clock                  | No. CpGs | Error (Years) | Generation of error estimate (type of validation data set used) | No. of samples in training | Method used to find age-associated CpGs | Age range of training | Cell types/Tissue used for training | Additional functional tissues/Cells | Reference                        |
|------------------------|----------|---------------|----------------------------------------------------------------|---------------------------|----------------------------------------|----------------------|------------------------------------|------------------------------------|----------------------------------|
| Bocklandt              | 88       | 5.2           | Leave-one-out                                                   | 68 (34 twin pairs)        | CpGs with q < 0.05 & absolute corr >0.57 with age | 21–55                | Saliva                            | -                                 | Bocklandt et al. (2011)           |
| Koch & Wagner          | 5        | 11            | Independent validation data set                                 | 150                       | Pavlidis Template Matching              | 16–72                | Fibroblasts, keratinocytes, epithelial, peripheral blood | Saliva, breast organoid           | Koch and Wagner (2011)            |
| Passage Number         | 6        | -             | -                                                               | -                         | Pavlidis Template Matching              | -                    | Fibroblasts, mesenchymal stem cells | -                                 | Koch et al. (2012)                |
| Horvath (Pan-Tissue)   | 353      | Median Absolute Deviance 3.6 | Independent validation data set                               | 3931                      | Elastic net regression                  | 0–100                | 51 different tissues/cell types including blood, brain, muscle | -                                 | Horvath (2013)                   |
| Skin & Blood (S&B)     | 391      | No overall MAD for all tissues /cell types                     | Independent validation data set                               | 896                       | Elastic net regression                  | 0–94                 | Fibroblasts, keratinocytes, buccal cells, endothelial cells, lymphoblastoid, skin, blood, saliva | Brain, neurons, glia, liver, bone | Horvath et al. (2018)             |
| Zhang (Elastic Net)    | 514      | RMSE 2.04      | Independent validation data set                               | 13.661                    | Elastic net regression                  | 2–104                | Whole blood, saliva                  | Breast, liver, adipose, muscle, endometrium | Zhang, Vallerga, et al. (2019) |
| Zhang (BLUP)           | 319,607  | RMSE -2.04     | Independent validation data set                               | 13.661                    | Best linear unbiased prediction        | 2–104                | Whole blood, saliva                  | -                                 | Zhang, Vallerga, et al. (2019) |
| Hannum                 | 71       | RMSE 4.9       | Independent validation data set                               | 482                       | FDR to filter significant CpGs then elastic net | 19–101               | Whole blood                          | -                                 | Hannum et al. (2013)             |
| Weidner (102 CpG)      | 102      | 3.3           | Independent validation data set                               | 575                       | CpGs selected by Pearson corr (r > 0.85 or r < -0.85) | 0–78                 | Whole blood                          | -                                 | Weidner et al. (2014)            |
| Weidner (99 CpG)       | 99       | 4.1           | Independent validation data set                               | 656                       | CpGs derived from 102 previous CpGs in Weidner et al. (2014) | 19–101               | Whole blood                          | -                                 | Weidner et al. (2014)            |
| Weidner/Lin (3 CpG)    | 3        | 7.6           | Independent validation data set                               | 656                       | Three CpGs selected from 102 previous CpGs, recursive feature elimination | 19–101               | Whole blood                          | -                                 | Weidner et al. (2014), Lin et al. (2016) |
comprised of 8000 samples from 82 studies, including 51 healthy tissues and cell types. The size of the training data was a step-change in clock design. Hence, the Horvath clock gained popularity in the scientific community since it can predict age in multiple tissues using a relatively small number of CpGs (compared to the rest of the epigenome) and revealed that tissues may age at different rates. For example, brain tissue appears to age slower relative to other tissues in the body, according to the Horvath clock (Horvath, 2013; Horvath, Mah, et al., 2015).

The association between age acceleration and health/disease status was first shown with the Horvath clock in obesity (Horvath et al., 2014) and has since become an established tool to assess biological age (Horvath et al., 2014; Horvath, Garagnani, et al., 2015; Horvath, Langfelder, et al., 2016; Horvath & Levine, 2015; Maierhofer et al., 2017; Martin-Herranz et al., 2019). The Horvath clock has shown some limitations with particular tissues and age-associated disease conditions. One of the most severe premature ageing syndromes, Hutchinson-Gilford Progeria Syndrome (HGPS), did not exhibit age acceleration according to the Horvath clock (Horvath, 2013). Children with multifocal developmental dysfunctions (syndrome X), who appear to age slower, do not decelerate in eAge (Walker et al., 2015). However, being rare genetic disorders, both studies were limited in the number of individuals tested.

The Horvath clock does not work reliably on cultured cells, particularly fibroblasts (Horvath, 2013; Horvath et al., 2018, 2019). Replicative senescence in primary fibroblasts is a widely used model system in cellular ageing (Chandra & Kirschner, 2016; Hayflick, 1965; Hayflick & Moorhead, 1961). More recently, Horvath et al. developed an epigenetic clock that predicts the age of human fibroblasts, keratinocytes, buccal cells, endothelial cells, lymphoblastoid cells, skin, blood and saliva samples, better than the original Horvath clock (Horvath et al., 2018). This clock, known as the skin and blood (S&B) clock, is able to predict both in vivo and in vitro tissues accurately (Horvath et al., 2018, 2019). The S&B clock also detected a modest, but significant age acceleration in HGPS samples (Horvath et al., 2018).

The Zhang clock, while primarily trained to work on blood, is able to predict the ages of breast, liver, adipose and muscle tissue as accurately as the Horvath clock (Zhang, Vallerga, et al., 2019). This clock also outperformed both the Horvath and Hannum clocks in predicting blood age. It is set apart by the size of its training data with over 13,000 samples.

### 3.3 Tissue-specific age predictors

A number of CpG clocks have been developed for single tissues, aiming at an increased accuracy for a given cell type or specialised applications. Multiple clocks have been developed for blood, the first of which was the Hannum clock (Hannum et al., 2013; Horvath & Raj, 2018; Weidner et al., 2014; Zhang et al., 2017; Zhang, Kamath, et al., 2019; Zhang, Vallerga, et al., 2019). A later study found 102
| Clock                   | Sequencing          | No. CpGs | Error (Years) | Generation of error estimate (type of validation data set) | No. of samples in training | Method used to find age-associated CpGs                                                                 | Age range of training | Cell types/Tissue used for training | Reference                  |
|------------------------|---------------------|----------|---------------|------------------------------------------------------------|--------------------------|--------------------------------------------------------------------------------------------------------------------------------|----------------------|-----------------------------------|------------------------------|
| Weidner 3 CpG          | Bisulphite pyrosequencing | 3        | 4.5           | Independent validation data set                             | 82                       | 3 CpGs selected from 102 previous CpGs by recursive feature elimination                                               | 0–78                | Whole blood                       | Weidner et al. (2014) |
| Eipel Buccal           | Bisulphite pyrosequencing | 5        | 5.1           | Independent validation data set                             | 55                       | 3 CpGs from Weidner et al. (2014), plus two additional buccal-specific CpGs                                        | 1–85                | Saliva                            | Eipel et al. (2016)   |
| Zbieć-Piekarska (ZP) Clock | Bisulphite pyrosequencing | 5        | 3.9           | Independent validation data set                             | 420                      | 8 CpGs from Hannum et al. (2013) then multivariate linear regression                                              | 2–75                | Peripheral blood                  | Zbieć-Piekarska et al. (2015) |
| Cho Model 2            | Bisulphite pyrosequencing | 5        | 4.2           | Independent validation data set                             | 100                      | Similar CpGs to ZP clock (same associated genes but different CpGs), trained in multivariate regression model          | 20–74               | Whole blood                       | Cho et al. (2017)      |
| Jung-Blood             | SNaPShot             | 5        | 3.5           | Independent validation data set                             | 100                      | CpGs used by Cho Model 2 (with different Clorf132 CpG) retrained in multivariate linear model                      | ~19–70              | Whole blood                       | Jung et al. (2019)    |
| Jung-Saliva            | SNaPShot             | 5        | 3.6           | Independent validation data set                             | 100                      | **                                                                                                              | ~19–70              | Saliva                            | Jung et al. (2019)    |
| Jung-Buccal Swab       | SNaPShot             | 5        | 4.3           | Independent validation data set                             | 100                      | **                                                                                                              | ~19–70              | Buccal epithelial cells           | Jung et al. (2019)    |
| Jung-Mixed Tissue      | SNaPShot             | 5        | 3.8           | Independent validation data set                             | 300                      | **                                                                                                              | ~19–70              | Whole blood, saliva, buccal epithelial cells | Jung et al. (2019)  |
| Dias-Deceased Clock    | Bisulphite PCR       | 5        | 8.8           | Independent validation data set                             | 51 (Deceased)            | PCR of CpGs from previous studies, trained in multivariate linear model                                             | 24–86               | Blood                             | Dias, Cordeiro, Corte Real et al. (2020a) |
| Dias-Multi-Locus Model | Bisulphite PCR       | 4        | 5.4           | Random segregation of validation data set from training      | 53                       | Using CpGs/regions previously used in Dias, Cordeiro, Corte Real et al. (2020a), trained in multivariate linear model | 1–95                | Peripheral blood                  | Dias, Cunha, et al. (2020) |
| Dias-Blood (5 CpG)     | SNaPShot             | 5        | 4.3           | Random segregation of validation data set from training      | 59                       | Same CpGs used by Jung et al. (2019), retrained in multivariate linear model                                     | 1–94                | Peripheral blood                  | Dias, Cordeiro, Pereira, et al. (2020) |
| Dias-Blood (3 CpG)     | SNaPShot             | 3        | 4.8           | Random segregation of validation data set from training      | 59                       | 3 of 5 CpGs used by Jung et al. (2019) were retrained in multivariate linear model                                 | 1–94                | Peripheral blood                  | Dias, Cordeiro, Pereira, et al. (2020) |

Note: Epigenetic clocks created using a low number of CpGs (typically under 10), usually from preselected CpGs/regions known to have high age correlation. Error is based on mean absolute deviation (MAD).
CpG sites that can predict age in blood, 99 of which were adapted for a separate clock that works on the Illumina 450 K array (Weidner et al., 2014). It was demonstrated that three of the 102 CpGs alone (selected by recursive feature elimination) can predict age in arrays and pyrosequenced samples (Lin et al., 2016; Weidner et al., 2014). A minimal approach, such as this that uses as few CpGs as possible, is a sought after technique for affordable use in clinical and forensic fields (see "Minimised CpG Clocks").

A study by Boroni et al. has produced an accurate skin age predictor, based on 2266 CpGs (one of the largest number of CpGs used to create an eAge clock) selected by elastic net regression (Boroni et al., 2020). It was trained on dermis, epidermis and whole skin biopsies (40, 99 and 110 samples, respectively) and had a root mean squared error (RMSE) of 4.98 when tested on an external validation data set of whole skin biopsies (by comparison, the Horvath and S&B clocks had RMSEs of 15.74 and 7.64, respectively) (Boroni et al., 2020). Inaccuracies in epigenetic clocks are apparent when predicting the age of younger individuals (under 20 years old) (Simpkin et al., 2020). This might be due to insufficient numbers of young individuals in training data sets, or due to the linear models used to construct the epigenetic clocks (see "Inaccuracies and Tick-Rate of Epigenetic Clocks"). The Pediatric-Buccal-Epigenetic (PedBE) clock was developed for use in 0- to 20-year-olds and trained on a large number of buccal swab samples (1,032, aged 0–19.5 years old) (McEwen et al., 2016). It was demonstrated that three of the 102 CpG sites alone (near ELOVL2 and FHL2 (Garagnani et al., 2012; Bacalini et al., 2017)) and is designed for common tissues found at crime scenes, such as blood, saliva, buccal swabs and semen (Table 2).

Minimised clocks use a variety of technologies such as the Qiagen platform for pyrosequencing (referred to as pyrosequencing from here), which is more cost-effective for profiling the methylation of select CpGs. The Weidner 3 CpG clock (see "Tissue-Specific Age Predictors") for example, can predict age in blood samples using pyrosequencing (Weidner et al., 2014), but over-predicts age in saliva (a common source of DNA at crime scenes) by 14.6 years on average (Eipel et al., 2016). When adapted for saliva by adding two additional buccal-specific age-associated CpGs, eAge prediction was improved (Eipel et al., 2016).

Pyrosequencing had its own limitations (e.g. multiplexing; allowing a large number of samples and CpGs to be pooled and sequenced in a single run); however, new approaches increasing multiplex capabilities in pyrosequencing are emerging (Fleckhaus & Schneider, 2020). Another assay, termed SNaPshot, can multiplex 10 CpG sites (Thermo Fisher, 2020) and is used for many minimised epigenetic clocks.

The use of minimised clocks in forensics is just developing and for most clocks, cross-validation is missing (Cho et al., 2017). However, the clock by Zbiec´-Piekarska et al. has been validated and adapted in other studies. It is based on 5 out of 8 CpGs previously identified by Hannum et al. as showing the strongest age association (Hannum et al., 2013; Zbiec´-Piekarska et al., 2015) and has a standard error of 4.5 years and an MAD of 3.9 years. The genes associated with these CpGs are ELOVL2, C1orf132, TRIM59, KLF14 and FHL2. A clock based on five CpGs (located near the same previous genes) was created by Cho et al. and has been validated in multiple tissues (Cho et al., 2017; Dias, Cordeiro, Pereira, et al., 2020; Jung et al., 2019). These CpGs not only operate adequately with SNaP-shot assays as tissue-specific age predictors, but also as a multi-tissue age predictor for common forensic tissues (blood, saliva and buccal swab) (Jung et al., 2019). Three of the CpG sites (near ELOVL2, FHL2 and C1orf132) have also proven sufficient to predict age efficiently (Dias, Cordeiro, Pereira, et al., 2020). These studies have demonstrated the versatility and accuracy predictors based on a few select CpGs can have and might be good candidates to increase the scale of eAge prediction.

### Table 3 Composite and mortality epigenetic clocks

| Clock                  | No. CpGs | Method used to obtain CpGs | No. of samples in training | Reference                      |
|------------------------|----------|-----------------------------|-----------------------------|--------------------------------|
| PhenAge                | 513      | Elastic net                 | 9,926                       | Levine et al. (2018)           |
| GrimeAge               | 1,113    | Elastic net                 | 1,731                       | Lu, Quach, et al. (2019)       |
| Zhang Mortality Clock  | 10       | LASSO Cox regression        | 548                         | Zhang, Wilson, et al. (2017)   |
| DunedinPoAm            | 46       | Elastic net                 | 810                         | Belsky et al. (2020)           |
| Telomere Clock         | 140      | Elastic net                 | 2,256                       | Lu, Seebold, et al. (2019)     |

Note: All clocks in this table are composite clocks, i.e. CpGs that correlate with physiological or cellular ageing are used to create a biological age predictor (except the Zhang Mortality Clock, where mortality data were directly regressed on DNAm).
Epigenetic clocks have proven capable of estimating not only chAge, but also time-to-death. Marioni et al. first showed that the higher difference between eAge and chAge, the greater the risk of all-cause mortality (mortality independent of health status, known genetic factors, and lifestyle factors) (Marioni, Shah, McRae, Chen et al., 2015). This finding was further validated in other studies (Chen et al., 2016; Christiansen et al., 2016). Positive age acceleration was also shown to predict cause-specific mortality in cancer and cardiovascular disease (Perna et al., 2016). These mortality associations were found using clocks that were not designed to directly predict mortality. Various composite approaches have been developed, whereby CpGs that correlate with metrics of physiological or cellular ageing (e.g. cholesterol or protein abundance) are used to construct a clock to predict age (Table 3). These clocks were built with the potential of capturing more of age-relevant biology than clocks trained on chAge alone.

The first composite biomarker age predictor (created independent of DNAm data) was based on 23 years of mortality data (Levine, 2013). This predictor incorporated ten biomarkers (e.g. C-reactive protein, glycated haemoglobin, systolic blood pressure, total cholesterol) that significantly correlated with age (Levine, 2013). Using a similar process, Levine et al. combined chAge plus nine other biomarkers. The resulting phenotypic clock was regressed on DNA methylation data using elastic net regression, resulting in 513 CpGs forming the DNAm PhenoAge clock (Levine et al., 2018). This clock predicts all-cause mortality, cancer, healthspan, physical functioning and Alzheimer’s disease more accurately than previous age predictors (Levine et al., 2018).

The GrimAge clock developed by Lu et al. uses the methylation of CpGs associated with smoking (pack-years) and levels of 7 plasma proteins previously associated with mortality (Ignjatovic et al., 2011; Ridker et al., 2003), as surrogates for physiological risk factors (Lu, Quach, et al., 2019). The age acceleration of GrimAge was not only found to be associated with age-related conditions and lifestyle factors, but outperformed previous attempts at predicting time-to-death, time-to-coronary heart disease and time-to-cancer (Lu, Quach, et al., 2019). A significant association has been shown between GrimAge acceleration and lifelong trauma, but not childhood trauma (Katrinli et al., 2020), which is consistent with other studies showing Hannum (Wolf et al., 2016) and Horvath (Yang et al., 2012) clock age accelerations in post-traumatic stress disorder. GrimAge acceleration is also significantly associated with cortical atrophy (Katrinli et al., 2020), shorter pregnancy periods and lower birthweight (Ross et al., 2020).

Zhang et al. created a mortality-specific predictor, where they performed an epigenome-wide association study (EWAS) on a cohort with up to 14 years follow-up data. 58 CpGs were found that correlate with all-cause mortality, from which a predictor was constructed using only ten of the CpGs (Zhang, Wilson, et al., 2017).
multiple tissues using a relatively small number of CpGs (compared to the rest of the epigenome; Horvath, 2013). The fact that such a clock can be constructed provokes the question, is there a functional significance that correlates these CpGs with age in multiple tissues? If ageing is a phenomena that we are “programmed” to undergo, then are these CpGs an integral part of that machinery? To understand the nature of eAge/epigenetic clocks, we must understand the aspects of physiological ageing they capture, the CpGs that constitute these clocks, and any causative relationships with ageing.

5.1 | Inaccuracies and tick-rate of epigenetic clocks

As with the Horvath clock, most clocks that followed after were also built on penalised linear regression models. However, are there intrinsic inaccuracies in the Horvath clock, and the approach used to construct epigenetic clocks? El Khoury et al. analysed previously published DNAm data sets and found that both the Horvath and Hannum clocks systematically underestimate the age of older individuals (El Khoury et al., 2019). If age acceleration is dependent on chAge itself, biological interpretation of age acceleration at very old age becomes difficult. Centenarian peripheral blood mononuclear cells are predicted 8.6 years younger than their chAge, according to the Horvath clock (Horvath, Pirazzini, et al., 2015). Similar findings were also found in analysis of cerebellum tissue from supercentenarians (Horvath, Mah, et al., 2015). The interpretation has been that the younger age predicted for centenarians reflects survival bias, where the lower biological age enabled the centenarians to live long. However, with the clock possibly underpredicting older age systematically, this assumption might need to be reexamined. Alternatively, this discrepancy might be the result of a regression to the mean effect, where very high values (eAges) are underestimated by regression models.

While negative age acceleration (eAge predicted lower than chAge) was highest in the cerebellum, this underestimation was also observed in other tissues (including blood) from multiple data sets (El Khoury et al., 2019; Marioni et al., 2019; Martin-Herranz et al., 2019). It was also found that when accounting for age as a cofactor, the correlation between age acceleration and amyloid plaque load in brain tissue is attenuated (El Khoury et al., 2019), which is inconsistent with previous findings (Levine et al., 2015). It is possible that 5-hydroxymethyl cytosine (5hmC, an epigenetic modification more prevalent in brain tissue and indistinguishable from 5mC after bisulphite conversion) could cause age prediction offset in brain tissue (El Khoury et al., 2019; Lunnon et al., 2016). However, 5hmC is not prevalent in blood and therefore does not explain the negative age acceleration in blood detected by Marioni et al. (2019), El Khoury et al. (2019). These alterations in predictive accuracy of the clock in older individuals could be due to intrinsic changes in the rate of biological ageing during certain time points. The rate of change, or “tick” rate, was explored earlier in the Horvath clock study (Horvath, 2013). By looking at the weighted averages of the 353 CpGs compared with chAge, the tick rate was exponential between 0 and 20 years old, after which it continued linearly. As such, the Horvath clock applies a logarithmic transformation to ages <20 years, while the linear model is unaltered for ages >20 years (Horvath, 2013; Snir et al., 2019). The study suggested that a higher organismal growth and cell division rate at early age might explain the initial acceleration in ageing (Horvath, 2013). A later study found a faster eAge tick rate during puberty in girls (Binder et al., 2018). However, no decrease in the tick rate of older subjects was observed (Horvath, 2013), which could be due to a lack of older individuals in the training data set used to construct the Horvath clock (El Khoury et al., 2019). Differences in tick rate could also be sex-specific. The Horvath, Hannum, and Zbiec-Piekarska clocks show slightly faster ageing in men than women (Bergsma & Rogaeva, 2020).

A recent study found that simple multiple linear regression outperforms more involved machine learning techniques (Lau & Fung, 2020). However, if there is indeed a non-linear progression of age acceleration, then other models might be worth exploring to predict eAge. Deep learning and support vector regression are other alternatives to penalised linear regression that have been used (Aliferi et al., 2018; Galkin et al., 2020, 2021; Levy et al., 2020; Xu et al., 2015). The epigenetic pacemaker (EPM) is another algorithm where predicted age follows a logarithmic trend (Snir et al., 2016, 2019). Whether EPM or other non-linear models predict eAge in centenarians more accurately has not been determined.

5.2 | What aspects of physiological ageing does eAge capture?

eAge acceleration (eAge higher than chAge) or deceleration (eAge lower than chAge) is reflected in many diseases (e.g. Down syndrome) and environmental factors (e.g. smoking) that appear to increase or decrease ageing at a physiological level (Chen et al., 2016; Higgins-Chen et al., 2020; Horvath et al., 2014; Horvath, Langfelder, et al., 2016; Horvath et al., 2018; Horvath & Levine, 2015; Horvath, 2015; Maierhofer et al., 2019; Marioni, Shah, McRae, Chen, et al., 2015; Martin-Herranz et al., 2019; Simpkin et al., 2016; Wu et al., 2019). What remains unclear is whether eAge reflects or measures known physiological/cellular ageing phenomena (e.g. telomere length, senescence).

Consistent eAge prediction between tissues of an individual suggests that eAge is not a measure of cellular proliferation, since different tissues have variable proliferation rates (Horvath, 2013; Horvath et al., 2019; Horvath, Mah, et al., 2015). Indeed, multiple studies have shown that while eAge changes with cell passage number, the Horvath age predictor does not rely on cell division since it can track eAge in non-proliferative tissues (e.g. neuronal cells; Horvath, 2013; Yang et al., 2016; Horvath et al., 2019). A mitotic clock (EpiTOC) has been developed specifically to track cell divisions, and acceleration of this clock correlates with cancer status (Yang et al., 2016). It would be intuitive to assume that eAge reflects other known aspects of ageing such as senescence, since an increase in senescence cells is considered a hallmark of ageing...
(Horvath et al., 2019; López-Otin et al., 2013). However, this has not been shown; instead, both replicative and damage-induced senescence do not correlate with increased eAge in vitro (Horvath et al., 2019; Lowe et al., 2016). It is possible that the accumulation of senescent cells in tissues with age remains proportionally low and that it is the effect on surrounding cells that is registered in eAge. Human telomerase reverse transcriptase (hTERT) expressing cells continue to epigenetically age despite never being able to enter replicative senescence (Kabacik et al., 2018). Leukocyte telomere length (LTL) erosion is one of the first biological phenomena that showed potential as biomarkers of ageing (Frenck et al., 1998; Harley et al., 1990; Hastie et al., 1990; Lindsey et al., 1991) and could be a physiological sign of ageing that correlates with eAge. However, like cellular proliferation and senescence, multiple studies have shown that eAge has no association with telomere length (Cypris et al., 2020; Horvath et al., 2019; Kabacik et al., 2018; Lowe et al., 2016; Marioni et al., 2016).

A plausible alternative is that eAge is governed by cellular differentiation. As stem cells divide during development, they differentiate into different cell types as the embryo matures, which could be reflected by changes in eAge. One study tested the influence of tissue identity on eAge by growing keratinocytes in a media that encourages differentiation. No increase of eAge was observed in the differentiating keratinocytes compared to the non-differentiating, proliferating keratinocytes (Horvath et al., 2019). A separate study transdifferentiated fibroblasts to neurons using miRNAs. The reprogrammed neurons not only had a similar eAge as the donor fibroblasts but also similar telomere length, oxidative stress and DNA damage (Huh et al., 2016), suggesting that direct reprogramming had no effect on eAge.

It has been hypothesised that eAge-related changes are reflected in intracellular alterations and changes in cell composition in a subset of cells termed “clock cells” (Horvath & Raj, 2018). eAge might therefore capture the loss of somatic cells in some tissues (Horvath & Raj, 2018) or the loss of stem cells, which do decline during ageing (Hernando-Herraez et al., 2019). A caveat is that eAge can be captured in neuronal cells, which are terminally differentiated cells and lack a stem cell pool (Horvath, 2013; Horvath, Mah, et al., 2015; Horvath & Raj, 2018).

It also possible that eAge measures aspects of age-related epigenetic drift or deregulation (Yu et al., 2020). Demethylation can occur in either a passive manner (e.g. via inhibition of DNMT1 during cell replication; Wolffe et al., 1999; Mayer et al., 2000), or actively via methyl-CpG binding domain protein 4 (MBD4; Hendrich et al., 1999) or TET enzymes (Ichiyama et al., 2015; Jin et al., 2014). However, there is little evidence to suggest that active processes, such as TET, directly demethylate with age and affect eAge prediction (Wallace, 2014; Yu et al., 2020; Zhang et al., 2016). In addition, eAge can be measured in nonproliferating tissues (Horvath, 2013; Horvath et al., 2019; Yang et al., 2016) meaning passive demethylation is an unlikely mechanism. It is possible that actively dividing tissues accumulate somatic mutations in DNA methylation machinery during ageing, resulting in epigenetic drift observed as aberrant eAge prediction (Robertson et al., 2019).

The precise aspects of physiological ageing that eAge captures remain to be discovered, but further investigations into genes associated with eAge/clock CpGs and associations with other ageing biomarkers may disclose clues to the true nature of eAge.

5.3 Causality of clock CpGs in ageing

DNA methylation became apparent as a potential biomarker of ageing with the discovery of strong age-associated CpGs, such as those in the CpG islands of ELOVL2, FHL2 and PENK1 (Bacalini et al., 2017; Garagnani et al., 2012). ELOVL2 is a strong biomarker for ageing in multiple tissues in both human and mouse (Bacalini et al., 2017; Chen et al., 2020; Garagnani et al., 2012; Hannum et al., 2013; Slierker et al., 2018). The CpGs neighbouring ELOVL2 strongly hypermethylate with age (Garagnani et al., 2012) and have been used in multiple forensic clocks (see “Minimised CpG Clocks”). ELOVL2 is an enzyme involved in elongation of long-chain polyunsaturated fatty acids, and also in the production of docosahexaenoic acid (DHA). DHA is the main polyunsaturated fatty acid in the retina and brain, and is necessary for healthy retinal function. Chen et al. showed that the Elovl2 promoter is more highly methylated in the retina of aged mice and that demethylation of this site recovers age-related decline in visual function via increased expression of Elovl2 (Chen et al., 2020). This is one of few studies to test a causal link of age-associated CpGs with phenotypic ageing.

5.4 Transcriptional associations with eAge

One approach to functionally annotating CpGs is to analyse gene expression changes that correlate with the methylation of age-associated CpGs. In the Horvath clock, the 193 CpGs (out of 353) that hypermethylate with age are more likely to be located in poised (bivalent) promoters. The 160 (out of 353) CpGs that hypomethylate with age are more likely to be in either weak promoters or strong enhancer regions (Horvath, 2013). However, linking the activity of age-related CpGs with specific gene expression has proven difficult (Horvath & Raj, 2018; Jung & Pfeifer, 2015; Yin et al., 2017; Zheng et al., 2016). The most likely reason is that many age-associated CpGs might not be related to gene expression. Another reason might be that the epigenetic state of cells in any given tissue is heterogeneous, making associations between methylation and gene expression difficult to find. Dual transcriptomic and epigenetic sequencing at a single cell level could help to establish a functional link between the two (Horvath & Raj, 2018) (Angermueller et al., 2016). A recent study by Hernando-Herraez et al. used scMT-seq to assess ageing in mouse muscle stem cells (MuSCs). They isolated young and old quiescent MuSCs and determined that epigenetic drift (specifically,
stochastic methylation heterogeneity at promoters is associated with age-associated transcriptional heterogeneity (Hernando-Herraez et al., 2019). They also predicted eAge by aggregating single cells by individual (two young and two old mice, with 35 cells per individual). Their age predictor performed accurately on the young MuSCs; however, their old MuSCs had a similar eAge to the young samples (~10 weeks, while the chAge of the old MuSCs were ~100 weeks). To compensate for this error, they estimated eAge using different combinations of cells and permutations, by removing 5% of cells and calculating eAge of the subsequent sample. The old MuSCs were still ~90 weeks lower than the chronological age (Hernando-Herraez et al., 2019).  

### 5.5 Genetic variants associated with eAge

A genome-wide association study (GWAS) is a method that could reveal genes that regulate eAge by finding genetic polymorphisms that correlate with eAge. A GWAS of cerebellum tissue found variants near an mTOR complex 2 gene (MLST8) and in an RNA-helicase gene (DHX57) that are associated with age acceleration. Many genes associated with cerebellar age acceleration also had overlap with neurodegenerative conditions such as Alzheimer’s disease (Lu et al., 2016). Another GWAS revealed that one of the loci associated with intrinsic eAge acceleration (IEAA), which adjusts for both chAge and blood cell counts; Horvath, Gurven, et al., 2016; Quach et al., 2017) co-locates with hTERT (Lu et al., 2018). Variants of hTERT were found that associated with both IEAA and longer telomeres. Moreover, it was shown in vitro that higher hTERT expression (which is normally associated with cellular longevity) appears to cause a linear increases of eAge. By comparison, control cells pashed with no hTERT experienced an initial increase in eAge after 33 days in culture that eventually plateaued. These findings further enforce that eAge is not governed by cell division, replicative senescence or telomere length per se (Cypris et al., 2020; Horvath et al., 2019; Kabacik et al., 2018; Lowe et al., 2016; Marioni et al., 2016), since short telomeres are indicative of high proliferation and triggers replicative senescence. This paradoxical result could explain previous observations where during embryonic development and early postnatal life, the rate of epigenetic ageing is more rapid (Hiyama & Hiyama, 2007; Lu et al., 2018; Simpkin et al., 2016, 2017). These are periods of fast organismal growth coupled with high hTERT expression and cell division, which in turn would result in a higher eAge prediction.

Another approach to identify eAge-associated genetic variants involves screening for developmental disorders that cause an acceleration or deceleration of eAge. This was conducted by Martin-Herranz et al., who screened 367 genetic disorders, and found that Sotos syndrome significantly accelerated eAge (Martin-Herranz et al., 2019). Sotos syndrome is caused by a loss-of-function mutation in NSD1, which encodes a histone H3 lysine 36 (H3K36) methyltransferase (Chouflani et al., 2015; Kurotaki et al., 2002). Methylated H3K36 recruits DNMT3A/B and promotes methylation of surrounding regions. The authors hypothesised that H3K36 methylation machinery might break down with age, leading to an altered epigenome and increased eAge. The NSD1 mutation Martin-Herranz et al. observed might simulate an ageing effect that occurs naturally. An updated study with more samples (particularly of Sotos syndrome) is required to corroborate their findings (Martin-Herranz et al., 2019).

### 6 NON-HUMAN EPIGENETIC AGE PREDICTORS

Since the advent of DNA methylation age prediction for humans, age predictors have been created for other species; mice (Table 4), rats (Horvath, Singh, et al., 2020; Levine et al., 2020), dogs (Thompson et al., 2017; Wang et al., 2020), wolves (Thompson et al., 2017), humpback whales (Polanowski et al., 2014), chimpanzees (Guevara et al., 2020; Ito et al., 2018), marmosets (Horvath, Zoller, Haghani, Lu, et al., 2020), naked mole rats (Lowe et al., 2020), sea bass (Anastasiadi & Pifer, 2020) and zebrafish (Mayne et al., 2020) (see Table 5 for a list of non-human/mouse epigenetic clocks). In 2017, three mouse epigenetic clocks were developed primarily using reduced representation bisulphite sequencing (RRBS) data. Wang et al. 2017 used 148 CpGs from liver tissue (using both RRBS and whole genome bisulphite data, WGBS) and found a moderate conservation of age-related CpGs between human and mouse. Their clock also showed an age reduction for calorie restriction, rapamycin and Prop1<sup>delta</sup> dwarfism (which results in lifespan extension up to 1.5 fold) (Brown-Borg et al., 1996; Cole et al., 2017; Wang et al., 2017). Petkovich et al. built a mouse epigenetic clock using 90 CpGs from blood and detected that calorie restriction reduces epigenetic age according to their clock (Petkovich et al., 2017). The first mouse multi-tissue age predictor was constructed based on 329 unique CpGs with a median absolute error of 3.33 weeks, mainly trained on young- and middle-aged mice (0.2–9.5 months) (Stubbs et al., 2017). A recent multi-tissue age predictor in mouse has been developed by Meer et al. that uses 435 CpGs, and predicts age across a wide age range (1–35 months) (Meer et al., 2018). It operates on multiple tissues including blood, liver, brain and heart (Meer et al., 2018). Thompson et al. created four mouse RRBS clocks to compare statistical methods and found the most accurate clock resulting from elastic net regression (Thompson et al., 2018).

The Wang, Stubbs and Petkovitch mouse clocks mentioned here show little overlap in CpGs used (Field et al., 2018). This is probably due to the variability of RRBS data, where the regional genome coverage differs between protocols and enzymes used, rather than different statistical methods applied (Field et al., 2018; Thompson et al., 2018). Transferability of these clocks to data sets outside of the original studies has therefore been difficult. WGBS at a high enough coverage for eAge prediction is expensive, and most mouse clocks are trained on RRBS. Another alternative has been developed by FOXO BioScience, who have collaborated with Van Andel Institute and Illumina to create a cost-effective Infinium Mouse Methylation Array (FOXO BioScience, 2020).
### Table 4: Mouse epigenetic clocks

| Clock          | Number of CpGs | Correlation ($R^2$) | Generation of error estimate (Type of validation data set) | Number of samples in training data | Method used to find age-associated CpGs | Age range of training samples (Months) | Cell types/Tissue used for training | Reference                  |
|----------------|----------------|---------------------|-----------------------------------------------------------|-----------------------------------|------------------------------------------|----------------------------------------|-------------------------------------|-----------------------------|
| Wang           | 107            | 0.91                | Independent validation data set                           | 148                               | Elastic net                             | 0.2–26                                 | Liver                               | Wang et al. (2017)            |
| Petkovich      | 90             | >0.90               | Independent validation data set                           | 141                               | Elastic net                             | 3–35                                   | Partial blood                      | Petkovich et al. (2017)       |
| Stubbs         | 329            | 0.7                 | Training data sets partitioned and mixed with two external data sets to make up validation data set | 129                               | Elastic net                             | 0.2–9.5                                | Liver, lung, heart, muscle, spleen, cerebellum, cortex | Stubbs et al. (2017)          |
| Meer           | 435            | 0.89                | Random segregation of validation data set from training    | ~333                              | Elastic net                             | 0.2–35                                 | Blood, heart, cortex, liver, lung, muscle, spleen, cerebellum, pro B cells, follicular B cells | Meer et al. (2018)            |
| Thompson All CpGs (Ridge) | 582            | 0.79                | Leave-one-batch-out                                       | 893                               | Ridge Regression                        | 0–30                                   | Various tissues including adipose, blood, kidney, liver, lung, muscle, spleen | Thompson et al. (2018)        |
| Thompson All CpGs (Elastic Net) | 582            | 0.82                | Leave-one-batch-out                                       | 893                               | Elastic net                             | 0–30                                   | ""                                 | Thompson et al. (2018)        |
| Thompson Conserved CpGs (Ridge) | 273            | 0.64                | Leave-one-batch-out                                       | 893                               | Ridge Regression                        | 0–30                                   | ""                                 | Thompson et al. (2018)        |
| Thompson Conserved CpGs (Elastic Net) | 273            | 0.68                | Leave-one-batch-out                                       | 893                               | Elastic net                             | 0–30                                   | ""                                 | Thompson et al. (2018)        |
| Wood Mouse Clock | 9              | 0.88                | Same data set used for training                            | 48                                | LASSO                                    | 3–16                                   | Ear punch samples                  | Little et al. (2020)          |

Note: All clocks were trained on mouse RRBS data (with the exception of Wang et al., which used both RRBS and WGBS, and the Wood Mouse Clock, which used a targeted PCR approach combined with Oxford Nanopore).
Indeed, other studies have created a similar custom array to accurately predict age in model organisms. Currently available as a preprint, the Horvath laboratory has published an epigenetic clock that works on both rats and humans (Horvath, Singh, et al., 2020). This was created using a custom Illumina methylation array called the HorvathMammalMethylChip40, made up of 36,000 CpGs conserved among 50 mammalian species (Arneson et al., 2021; Horvath, Singh, et al., 2020). The MAE for human and rat data was 0.03, and a correlation of 0.95. Three single tissue clocks were also created for rat liver, brain and blood, as well as a multi-tissue clock combining all three tissues (Horvath, Singh, et al., 2020). Another preprint has been released of a sheep epigenetic clock, using the same array, with a median error of 5.1 months (~3.5–4.2% of expected sheep lifespan). The study reported that castrated sheep had a higher age acceleration than age-matched controls, and a dual human and sheep clock was constructed with an additional 1,848 human samples (Sugrue et al., 2020). A rat clock has also been developed using 134 RRBS whole blood samples (Levine et al., 2020). Elastic net selected 68 CpGs, and had a correlation of $r = 0.9$ in their test data set. It appears to work in mice, where it predicted reduced age acceleration after calorie restriction.

Many age-associated CpGs are conserved between different species (Horvath, 2013; Horvath, Singh, et al., 2020; Wang et al., 2017, 2020), meaning the development of pan-species clocks is plausible. For example, an epigenetic clock has been created using 394 CpGs from modules of developmental genes with conserved, age-related methylation changes, between mouse, human and dogs (Wang et al., 2020). Recent preprints have shown various universal pan-tissue epigenetic clocks that predict age across 9 tissue types from 128 different mammalian species (Lu et al., 2021), and models that predict maximum lifespan, gestation time and sexual maturity (Li et al., 2021). The CpGs used to construct the clocks were also associated with genes that are enriched during mammalian development (Lu et al., 2021). These clocks further enforce the idea that ageing is a conserved evolutionary process intertwined with mammalian development.

### 7 | CONCLUSION

eAge prediction is a powerful approach that has revolutionised experimental gerontology. As the number and diversity of epigenetic clocks increases, so too does our understanding of biological age.
Depending on how these clocks are constructed, they appear to capture different aspects of ageing. These differences depend on the tissues, number of samples, age range and algorithms used in their construction.

Whether the change of methylation is causal to ageing remains to be shown and herein lies a caveat studying diseases or interventions that directly affect DNAm. Studying a process that interacts with DNAm might alter age prediction, without changing the actual ageing trajectory. For example, it is possible that a global increase or decrease in methylation caused either by technical errors (Olova et al., 2018) or mutations in oncogenes (such as DNMT3A or TET2; Robertson et al., 2019), could result in false-positive shifts of eAge. It remains to be tested how stable epigenetic clocks are against global sweeps of DNAm.

Linear models have proven effective predicting eAge of individuals between the ages of 20 and ~70, but drop in accuracy outside of these ages. Clocks trained on specific age groups, such as PedBE, are valid approaches to this issue (McEwen et al., 2020). Alternative non-linear models may be better aligned with the actual trajectories of methylation changes with age. However, it is also possible that training eAge on chAge alone is not enough to explain biological age, as demonstrated by composite approaches such as PhenoAge and GrimAge clocks.

Multiple studies have shown that it is possible to build accurate minimised clocks using only a few highly age-associated CpGs (Cho et al., 2017; Daunay et al., 2019; Dias, Cordeiro, Pereira, et al., 2020; Jung et al., 2019; Weidner et al., 2014; Zbiec’s-Piekarska et al., 2015). Many of these clocks use CpGs nearby ELOVL2 and FHLL2, and work in saliva and blood (Cho et al., 2017; Dias, Cordeiro, Pereira, et al., 2020; Jung et al., 2019; Zbiec’s-Piekarska et al., 2015). They have yet to be tested in other scenarios, such as clinical applications. On the other hand, clocks might be more robust when utilising a large number of CpGs (Boron et al., 2020; Zhang, Kamath, et al., 2019).

The approach used by Horvath to develop epigenetic clocks has spawned not only an abundance of similar DNAm age predictors, but also other novel approaches, such as transcriptional (Bryois et al., 2017; Peters et al., 2015), proteomic (Lehalier et al., 2019; Tanaka et al., 2018) and cellular biophysical/biomolecular (Phillip et al., 2017) clocks. Indeed, DNAm can be regressed with health co-factors such as smoking and alcohol consumption to produce predictors of complex traits and mortality (McCarty et al., 2018). While DNAm is one of the most accurate and versatile biomarkers for ageing and disease, our understanding of it is still developing. Perhaps looking at DNAm in combination with other non-DNAm based biomarkers will broaden our understanding and predictive power of biological ageing and mortality. Composite clocks such as PhenoAge and GrimAge are first steps in that direction. Transcription clocks may reveal regulators of biological ageing, for example, if key ageing genes are found to be linked with eAge either by correlating with age acceleration or directly with methylation changes of key clock CpGs.

To us, the key areas to emerge will be in a) understanding the different aspects of ageing captured by distinct clocks and b) testing causality of DNAm in age acceleration through interventional epigenetics and other approaches. As epigenetic clocks become more sophisticated and commonplace, caution must be considered when inferring the biological significance of age acceleration. Research must continue regarding the nature of eAge and the aspects of ageing captured.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

D.J.S. and T.C. both wrote and conceived the manuscript and are both corresponding authors.

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