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HUMAN BIOLOGY TOOLKIT

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"Epigenetic clocks": Theory and applications in human biology

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Abstract

All humans age, but how we age—and how fast—differs considerably from person to person. This deviation between apparent age and chronological age is often referred to as "biological age" (BA) and until recently robust tools for studying BA have been scarce. "Epigenetic clocks" are starting to change this. Epigenetic clocks use predictable changes in the epigenome, usually DNA methylation, to estimate chronological age with unprecedented accuracy. More importantly, deviations between epigenetic age and chronological age predict a broad range of health outcomes and mortality risks better than chronological age alone. Thus, epigenetic clocks appear to capture fundamental molecular processes tied to BA and can serve as powerful tools for studying health, development, and aging across the lifespan. In this article, I review epigenetic clocks, especially as they relate to key theoretical and applied issues in human biology. I first provide an overview of how epigenetic clocks are constructed and what we know about them. I then discuss emerging applications of particular relevance to human biologists—those related to reproduction, life-history, stress, and the environment. I conclude with an overview of the methods necessary for implementing epigenetic clocks, including considerations of study design, sample collection, and technical considerations for processing and interpreting epigenetic clocks. The goal of this review is to highlight some of the ways that epigenetic clocks can inform questions in human biology, and vice versa, and to provide human biologists with the foundational knowledge necessary to successfully incorporate epigenetic clocks into their research.

1 | INTRODUCTION

Aging is a ubiquitous feature of the human experience and a leading predictor of health, disability, morbidity, and mortality (Clark, 2002; Crews, 2003). Nevertheless, individuals of the same chronological age often vary markedly in their age-related physical, physiological, and cognitive decline. Rare genetic disorders such as Werner syndrome provide extreme examples of accelerated aging, but individual differences in cardiovascular, metabolic, musculoskeletal, and neurological decline are present in all populations. The decline in functional capacity that can be decoupled from chronological age is often referred to as "biological age" (BA), and is thought to arise as a function of both genes and the environment (Levine, 2013).

Although the mechanistic underpinnings of BA are still only partly understood, the epigenome is emerging as fundamental component of BA (Benayoun, Pollina, & Brunet, 2015; M. J. Jones, Goodman, & Kobor, 2015). The contribution of epigenetic processes—including DNA methylation (DNAm), chromatin modifications, and

noncoding RNAs—to early development and cellular memory are well-known (Allis, Caparros, Jenuwein, & Reinberg, 2015). More recently, researchers showed that the epigenome not only changes with age, but often does so in a highly predictable manner (Bocklandt et al., 2011). These predictable, age-associated changes in the epigenome have now been used to design a set of tools that are changing how we study aging across the lifespan. These tools are referred to as "epigenetic clocks" (Field et al., 2018).

Epigenetic clocks—typically based on DNA methylation (Box 1)—were initially designed to predict chronological age, and have proven to be the most accurate tool for doing so (Horvath, 2013; Q. Zhang et al., 2019). They accurately estimate age from prior to conception to the most advanced human ages, and even "tick" in isolated cells in vitro (Hoshino, Horvath, Sridhar, Chitsazan, & Reh, 2019). However, they also appear capable of capturing fundamental molecular processes tied to BA that are still not well understood. Epigenetic clocks outperform many other measures, including chronological age, at predicting a long list of age-related health outcomes—from cancer to menopause—as well as all-cause mortality (Horvath & Raj, 2018). Now, newer epigenetic clocks are being explicitly "trained" not only on chronological age, but on a range of other age-related biological and health-related measures. This includes other biomarkers of health and aging such as leukocyte telomere length and leptin levels, in some cases even outperforming the predictive ability of the original biomarker of interest (Lu, Quach, et al., 2019; Lu, Seeboth, et al., 2019).

The predictive capacity of epigenetic clocks allows researchers to study aging and mortality while circumventing many of the logistical or temporal challenges inherent in following a large sample over the long timeframes necessary to obtain clinical or demographic outcomes. Newer epigenetic clocks expand their applications into new territories, solidifying them as a flexible family of tools for tackling long-standing questions in human biology, including plasticity in development, life-history traits, tradeoffs, and the role of the environment in the pace of aging and senescence.

The purpose of this Toolkit is to introduce human biologists and other quantitatively oriented researchers interested in aging—but lacking extensive background in genetics, molecular biology, or bioinformatics—to epigenetic clocks: what they are, what they do, and how to use them. The focus of this article is practical, and the aim is to provide readers with the foundation necessary to evaluate the utility of epigenetic clocks in their own research programs. After a broad introduction to the statistical methods used to "train" epigenetic clocks—which are important for understanding the capabilities and

BOX 1 DNA methylation and epigenetic clocks

DNA methylation (DNAm) is a biological process in which a methyl group (ie, a carbon atom attached to three hydrogen atoms, CH_3) is covalently attached to DNA (Allis et al., 2015). In mammals, the most well-characterized form of DNAm is when the methyl group is attached to the cytosine (C) base directly adjacent to a guanine (G) base, separated by the phosphate (p) backbone, shortened to "CpG" site. In general, the methyl group projects into the "groove" of the double helix, impeding binding of transcription factors and decreasing gene expression, but DNAm can have other effects (eg, altering RNA splicing, increasing gene expression) depending on the genomic context (eg, region of the gene, CpG content of the genomic region) (P. A. Jones, 2012). There are roughly 28.3 million CpG sites in the human genome, which often occur in clusters of hundreds to thousands known as CpG "islands" found in gene promoter regions. Even the newest array-based methods used in the design of many epigenetic clocks cover only a small proportion of these sites. As their names suggest, Illumina's Infinium HumanMethylation 27 Beadchip quantifies DNAm at 25578 CpG sites, while the HumanMethylation 450 (450 k array) covers 482 421 CpG sites (Bibikova et al., 2011). Most of the original epigenetic clocks were designed using these discontinued legacy arrays, but newer clocks use data from the most recent EPIC beadchip array, which covers 863 904 CpG sites. Despite the fact that the EPIC array still only covers 3% of CpG sites in the genome, this includes at least one probe in >99% of all annotation RefSeq genes (Pidsley et al., 2016). Nevertheless, methods such as whole-genome bisulfite sequencing that cover all CpG sites require high-depth next-generation sequencing and alignment, making array-based methods less expensive and technically demanding. Because cytosine for a given locus is either methylated (1) or not (0), values from these methods represent the proportion of methylated CpG sites across all cells from which DNA has been extracted. These proportions of methylated/unmethylated sites are the "raw data" used in epigenetic clocks.

limitations of these tools—I provide an overview of both established and emerging applications of epigenetic clocks for research in human biology and aligned fields. These include studying the impact of nutrition, exercise, and lifestyle; reproductive investment and tradeoffs; stress and resilience; and the environment and ecology on aging and age-related decline. I then discuss aspects of study design, sample collection, technical processing of DNAm, and interpretation of results for studies using epigenetic clocks. I include key references and resources that will facilitate the incorporation of epigenetic clocks into human biology research.

1.1 | What are "epigenetic clocks"?

Epigenetic clocks can refer to two distinct but related phenomena. The first refers to epigenetic changes—typically DNA methylation (DNAm)—as they unfold predictably over time (M. J. Jones et al., 2015). Specifically, this definition describes an innate biological process as it relates to an age-related outcome of interest (eg, chronological age, mortality risk, etc.). The pace or "ticking rate" for some clocks may be influenced by genetic oscillators and circadian genes, but "epigenetic clock" is not synonymous with other circadian "clocks." The second definition of "epigenetic clock" describes a statistical model that uses predictable epigenetic variation—often DNAm—to estimate an age-related outcome of interest (Horvath & Raj, 2018). The predictable age-related changes in DNAm that are used for epigenetic clocks can be determined using two methods: supervised machine learning or epigenome-wide association.

Most well-known epigenetic clocks are constructed using supervised machine learning methods (Horvath & Raj, 2018). This kind of clock construction entails a "training" stage and a "testing" stage. The clock is "trained" by compiling a large dataset comprised of DNAm values for many samples and the outcome of interest (eg, the chronological age of the individual the sample comes from). Sample variety and size is important for training a model that can later be generalized to other contexts (James, Witten, Hastie, & Tibshirani, 2013). Training starts with an oversaturated model in which the number of predictors (ie, scaled DNAm for all available sites; Box 1) greatly exceeds the number of observations (ie, individual samples). Penalized regression methods (eg, ridge, lasso, or elastic net) then either shrink (ie, regularize) coefficients or "soft-threshold" coefficients below an absolute value to zero. Regularizing coefficients in this way reduces the bias of the final model, making it more robust when applied in other contexts. Soft-thresholding to zero is also a form of feature selection, such that only the most informative predictors (ie, CpG sites) are retained in the final model. The shrinkage penalty, which determines the strength of regularization and thresholding, can be "tuned" by fitting models across a grid of penalty values and calculating the error using resampling methods such as bootstrapping or k-fold cross-validation (James et al., 2013). The result is an equation—both a subset of CpGs and their associated coefficients—that can be then used to predict the age-related outcome of interest (eg, chronological age) in a new sample of individuals or tissues.

Validating the model in new contexts with known outcomes is referred to as "testing" (James et al., 2013). Testing epigenetic clocks allows the researcher to determine the accuracy of the clock for predicting the age-related outcome of interest. For example, one of the most well-studied clocks was designed by Steve Horvath (Horvath, 2013), who trained his clock using 21 369 probes from 3921 samples derived from 39 datasets comprised of 27 different tissues. His model converged on a clock that uses variation in DNAm from 353 CpG loci (Box 1). This clock was then "tested" in 3211 new samples derived from 42 different studies and from 22 tissues (Horvath, 2013). Because it was trained using data from many different tissues, Horvath's clock shows remarkable accuracy across a range of contexts and tissue types. Pearson's correlation coefficients between predicted age and chronological age are high ($r = .96$, $P < 1.0e{-}200$), while median error between predicted age and chronological age are low (3.6 years across range of 0-100 years) (Horvath, 2013). Once trained and tested, the clock can be used to predict the age-related outcome of interest in other new study contexts.

While regularized regression and the methods described above have generated the most well-known epigenetic clocks, other approaches of selecting age-associated CpG loci have also been successfully employed. Several clocks have been derived by using the strongest correlations between DNAm and chronological age from epigenome-wide association studies (EWAS) (Lin et al., 2016; Vidal-Bralo, Lopez-Golan, & Gonzalez, 2016; Weidner et al., 2014; Y. Zhang et al., 2017). Because they may rely on relatively few CpG loci in their age calculations, such clocks can be more economical and do not require technical bioinformatic skills to use. However, it is not known whether clocks built in this way exhibit the same accuracy and robustness across contexts (eg, across different genetic backgrounds, tissue types, age ranges) as clocks using a larger number of CpG sites. Regardless of the methods used, researchers interested in using epigenetic clocks to predict chronological age or other age-related phenomenon should understand the context in which the clock was trained and how it

performs under a range of contexts relevant to the hypothesis or application. In this respect, human biologists also have the opportunity to play an important role in assessing performance of various clocks in different ecological and social settings as the field develops (eg, Horvath et al., 2016).

2 | APPLICATIONS AND UTILITY OF EPIGENETIC CLOCKS

2.1 | Established applications of epigenetic clocks

2.1.1 | Predicting chronological age

Epigenetic clocks trained on chronological age have proven to be the most accurate markers of chronological age devised (Horvath, 2013; Q. Zhang et al., 2019). This accuracy is highly desirable for forensics or for estimating chronological age in isolated populations that may not have detailed or accurate birth records. However, a "perfect" chronological epigenetic clock is by design uninformative with respect to variation in health, development, and BA (Q. Zhang et al., 2019). Quite early on it became obvious that one of the most interesting features of certain epigenetic clocks was not simply that they are accurate—but what these clocks can tell us when they are wrong! It turns out that the discrepancy between an individual's actual and predicted chronological age—the "error" in the clock—captures a range of normal and pathological variation in biological aging and development (Chen et al., 2016; Marioni, Shah, McRae, Chen, et al., 2015). This discrepancy, often referred to as "epigenetic age acceleration" (AgeAccel) expands the applicability of chronological epigenetic clocks beyond simple "time-keepers" and into groundbreaking tools for studying BA.

2.1.2 | Predicting morbidity and mortality

Human biologists and researchers in allied fields are often concerned with the long-term health of their study population, and researchers may wish to understand how healthspan and lifespan are affected by tradeoffs, constraints, lifestyle, behavior, or psychosocial stress. However, following subjects over the long periods of time necessary to record clinical and mortality outcomes can be costly to initiate and maintain (Ng et al., 2012). The ability of epigenetic clocks to predict morbidity and mortality far in advance of their clinical endpoints makes them powerful tools for studying health and BA even without long-term follow-up studies.

Horvath's clock—especially accounting for chronological age and cell-type variation in the blood sample (referred to as "intrinsic epigenetic age acceleration" or Horvath-IEAA; Table 1), predicts all-cause mortality. The predictive accuracy remains even after correcting for a range of mortality-associated risk factors (ie, chronological age, social class, educational level, body mass index, alcohol intake, smoking pack-years, self-reported recreational physical activity, hypertension, history of diabetes, history of cancer, hypertension status, cardiovascular disease, and APOE e4 status) (Chen et al., 2016; Marioni, Shah, McRae, Chen, et al., 2015). Similarly, Hannum's clock—including correcting for chronological age and weighted to incorporate additional information about age-related blood immune cell composition ("extrinsic epigenetic age acceleration" or Hannum-EEAA) has yielded similar results (Chen et al., 2016). The relationships between epigenetic age and mortality risk reported in these studies is not trivial; individuals in the top 5% of epigenetic age were at an almost 50% higher risk of mortality during follow-up than an individual with the average epigenetic age (Chen et al., 2016).

In addition to all-cause mortality, acceleration of Horvath and Hannum clocks (and their IEAA and EEAA extensions) accurately predict leading causes of morbidity and mortality including lung function (Marioni, Shah, McRae, Ritchie, et al., 2015), frailty and cognitive decline (Breitling et al., 2016; Marioni, Shah, McRae, Ritchie, et al., 2015), and cancer (Levine, Hosgood, et al., 2015; Zheng et al., 2016). As discussed in more detail below, both clocks are also associated with a range of environmental exposures and developmental outcomes. However, neither the Horvath nor Hannum clocks were originally designed to predict these outcomes; that is, both were "trained" on chronological age alone. Indeed, a recently devised epigenetic clock of chronological age using larger sample sizes is so accurate that it no longer reflects BA, and the error in this clock does not show a discernable relationship with mortality (Q. Zhang et al., 2019). Accordingly, other epigenetic clocks have been developed, trained on outcomes ranging from gestational age and leukocyte telomere length to markers of inflammation and all-cause mortality itself (Table 1). These new clocks are filling in the gaps left by the Horvath and Hannum clocks and are expanding the utility of epigenetic clocks into a family of molecular tools for studying aging and development across the lifespan.

While a comprehensive review of all epigenetic clocks is beyond the scope of this article, several clocks have been devised specifically to predict BA and mortality risk, and have demonstrated exceptional capacity to do so.

TABLE 1 List of major epigenetic clocks, how they were derived, what they were trained on, the number of CpG sites used for estimation, and relevant references

Major clocks	Derived from	Trained on	#CpG	References
Horvath AgeAccel	21 369 CpGs	Chronological age	352	Horvath, 2013
Hannum AgeAccel	EWAS on 473 034 CpGs, feature selection on 70 387 age-associated CpG sites	Chronological age	71	Hannum et al., 2013
Horvath Intrinsic Epigenetic AgeAccel (IEAA)	Same as Horvath AgeAccel plus additional sites used for white blood cell estimation	Chronological age (estimated white blood cell proportion down-weighted)	~30 k	Chen et al., 2016
Hannum Extrinsic Epigenetic AgeAccel (EEAA)	Same as Hannum AgeAccel plus additional sites used for white blood cell estimation	Chronological age (estimated white blood cell proportion up-weighted)	~30 k	Chen et al., 2016
Levine-DNAmPhenoAge (AgeAccelPheno)	20 169 CpGs	Composite of clinical measures of health and lifespan	513	Levine et al., 2018
Lu-DNAmGrimAge (AgeAccelGrim)	DNAm clocks for seven candidate biomarkers and DNAm smoking pack years, age, and sex	Time to death	1030	Lu et al., 2019
Lu-DNAmTL	~450 k CpGs found on both Illumina 450 k and EPIC arrays	Telomere length	140	Lu et al., 2019
Weidner's 3-CpG age clock	EWAS on ~27 k CpGs, feature selection on 102 CpGs	Chronological age	3	Weidner et al., 2014
Zhang's 10-CpG mortality clock	EWAS on 430 363 CpGs, feature selection on 58 replicated age-associated CpGs	All-cause mortality	10	Zhang et al., 2017

Levine et al. (2018) trained their "PhenoAge" (Table 1) clock by selecting a subset of clinical markers predictive of age-related mortality. A mortality score (in years) was then calculated, which was used as the outcome of interest for selecting CpGs in a supervised machine learning approach similar to that described for Horvath's clock (James et al., 2013). Thus, although it includes chronological age as a covariate, the resulting DNAm clock is based on a clinically based mortality score, rather than chronological age itself (Levine et al., 2018). Using similar approaches, the GrimAge clock created by Lu et al. (Table 1) is based on chronological age, sex, and DNAm clocks for smoking pack years and seven other clinical markers of mortality (Lu, Quach, et al., 2019). This means that GrimAge is an epigenetic clock built on sex, age, and eight other epigenetic clocks!

Although the derivation of these newer clocks may strike some readers as unintuitive, their predictive ability and accuracy across contexts is remarkable. Accelerated PhenoAge is highly predictive of cardiovascular disease risk, number of coexisting morbidities, poorer likelihood of being disease free, poorer physical and cognitive function, and likelihood of dying of lung cancer among both smokers and nonsmokers (Levine et al., 2018). In one study, the mortality hazard of individuals in the top 5% of AgeAccelPheno was found to be >2.5 times greater than those in the bottom 5% (Levine et al., 2018). Lu's GrimAge has produced even more striking results. AgeAccelGrim predicts hypertension, type II diabetes, poorer physical functioning, time-to-coronary heart disease, time-to-cancer, chronic obstructive pulmonary disease, and computed tomography for visceral adiposity and fatty liver (Hillary et al., 2020; Lu, Quach, et al., 2019). A large meta-analysis using GrimAge found that individuals in the top 5% of AgeAccelGrim had a mortality risk >4.5 times greater than those in the bottom 5% (Lu, Quach, et al., 2019). More recently, a clock was developed using *change* in 18 age-associated biomarkers rather than cross-sectional measurements (Belsky et al., 2020). The authors refer to this tool as a "speedometer" of aging rather than a "clock," stemming from the longitudinal nature of the training data and what appears to be a capacity to measure the pace—not static state—of BA. This clock or others using longitudinal data may be more useful for studying changes in environments or health interventions, such as caloric restriction (Belsky et al., 2020).

The relationships between many of the aforementioned clocks and morbidity and mortality have been replicated using large (n = 7300-9500) meta-analyses across

multiple populations, even controlling for sample heterogeneity and a range of additional biological, social and environmental risk factors (but see Kresovich, Xu, et al., 2019). Furthermore, the number of sites used (71-1030 CpGs) means that these clocks appear to be relatively robust to some technical variation and/or some missing data. However, the Horvath, Hannum, Levine and Lu clocks were trained and tested on DNAm derived from the Illumina Infinium microarray platform, which may still be cost-prohibitive for some projects (see "Choosing a clock and measuring DNAm," below). A more affordable approach includes a clock by Zhang et al. that uses a score based on DNAm at only 10 CpG sites which can be measured cheaply and easily using methods such as bisulfite pyrosequencing. Zhang's 10 CpG mortality clock (Table 1) is able to predict frailty, cardiovascular disease mortality, cancer mortality, and all-cause mortality with astonishing accuracy: individuals with aberrant DNAm levels for ≥5 of 10 sites have mortality hazard ratios 7 times greater than that of individuals with no evidence of aberrant DNAm for any of the 10 sites (Y. Zhang, Saum, Schöttker, Holleczek, & Brenner, 2018; Y. Zhang et al., 2017). The reliance of this clock on relatively few CpG sites for estimating morbidity and mortality risk could make this clock more sensitive to the influence of genetic or technical variation, although so far there is no evidence for the former (Y. Zhang et al., 2017). Furthermore, while Zhang's clock has not been tested in as many populations as the microarray-based clocks, these findings have been replicated in at least one other population, lending support to Zhang's 10 CpG mortality clock for questions pertaining to mortality and morbidity in human populations (Gao et al., 2019).

2.1.3 | Predicting growth, development, and maturation

Senescence, morbidity, and mortality represent one end of the continuum of BA. But epigenetic clocks also appear to be able to track early growth, development and maturity, making epigenetic clocks useful to researchers interested life history tradeoffs that involve growth and development (eg, between growth and immunity [Urlacher et al., 2018]). Horvath's clock begins "ticking" just weeks after conception, with the onset of cellular differentiation (Hoshino et al., 2019). Epigenetic aging also progresses normally in embryonic tissues grown in vitro and in pluripotent stem-cell derived organoids (Hoshino et al., 2019), consistent with the postulation that Horvath's clock captures a fundamental property of development and BA. Given that development involves the interplay of both genes and the environment, it is not surprising that the pace of epigenetic clocks appears to be determined by both genetic and early life maternal and environmental effects.

Indeed, Horvath's epigenetic clock "ticks" most rapidly during infancy, childhood, and adolescence (Horvath, 2013). In contrast to the other clocks discussed so far, Horvath's training set (range 0-100 years) included infants and children, making it more applicable to younger individuals, and most early studies of epigenetic age conducted in infants and children used this clock. Using Horvath's clock, Simpkin et al. found that infants born by cesarean section or to mothers who smoke have accelerated epigenetic age at birth (Simpkin et al., 2016). However, some maternal effects may not manifest until childhood and adolescence. Birthweight does not predict Horvath-AgeAccel at birth, but it positively predicts Horvath-AgeAccel during childhood (∼7-9 years of age) and *negatively* predicts Horvath-AgeAccel during adolescence (∼14.5-19 years of age) (Simpkin et al., 2016). This "reversal" of epigenetic age trends between childhood and adolescence is an important component of epigenetic aging: children with *faster* Horvath-AgeAccel may exhibit *slower* Horvath-AgeAccel after adolescence. It is also during childhood and adolescence when the difference in Horvath-AgeAccel between males and females emerges (Simpkin et al., 2016), with males showing markedly more advanced epigenetic ages than females by early adulthood, consistent with the higher mortality rates and shorter average life expectancy of men (Crimmins, Shim, Zhang, & Kim, 2019).

One possible explanation for the apparent reversal of Horvath-AgeAccel at puberty traces to the fact that prior to adulthood, children with favorable developmental conditions exhibit accelerated Horvath epigenetic age (Simpkin et al., 2016). Indeed, accelerated epigenetic age during childhood has been found to predict height (Gettler et al., 2020), menarcheal age and maturational tempo (time between thelarche and menarche) (Binder et al., 2018; but see Simpkin et al., 2017). However, accelerations of Horvath's epigenetic age during childhood has also been associated with stress (Davis et al., 2017; but see Marini et al., 2020), exposure to violence (Jovanovic et al., 2017), and allergies (Peng et al., 2019), suggesting that Horvath-AgeAccel is more than simply a reflection of healthy development. Life history theory could provide a useful framework for clarifying the divergent patterns in epigenetic aging in growing children. Children exposed to high levels of stress, violence, poor nutrition, infectious disease or extrinsic mortality may facultatively adjust maturational tempo to match presumed future prospects of survival and reproduction (Ellis & Del Giudice, 2019; Kuzawa & Bragg, 2012). Such adjustments could result in tradeoffs that will affect health and

lifespan in adulthood, consistent with broader expectations of work describing the developmental origins of health and disease (DOHaD). This framework might explain the apparently paradoxical finding that children in both favorable and unfavorable environments may exhibit epigenetic age acceleration.

To more precisely model the epigenetic changes that occur during childhood, several clocks that capture gestational (Knight et al., 2016) and pediatric (McEwen et al., 2019) epigenetic age have been developed. These clocks are providing noninvasive biomarkers for understanding prenatal developmental trajectories and will be increasingly useful for researchers working with infants and children as more data on these clocks accumulates. An important next step in this area is to link early life exposures that affect epigenetic clocks during childhood with health and functional decline during adulthood.

2.1.4 | Epigenetic clocks as surrogates for other biomarkers

An important characteristic of epigenetic "clocks" is that they can be trained on any feature that is accompanied by predictable changes in DNAm. As already described for Levine's PhenoAge and Lu's GrimAge clocks, this includes using DNAm as surrogate predictors for age-related changes in other clinical or biomarkers. In addition to the GrimAge clock, Lu and colleagues generated an epigenetic clock for leucocyte telomere length, a measure genome stability and cellular senescence and biomarker for a range of health and age-related risk factors (Bakaysa et al., 2007; Blackburn & Gall, 1978; Rej et al., 2020). While the correlation between telomere length and the clock for leukocyte telomere length (DNAmTL) in validation populations was modest ($r = .44$), Lu et al. found that DNAmTL outperformed telomere length alone in its associations with age, sex, and self-reported racial identity (Lu, Seeboth, et al., 2019). DNAmTL also outperformed telomere length alone in predicting time-to-death, time-to-coronary heart disease, time-to-congestive heart failure, and history of smoking, even after adjusting for a range of classical risk factors (body mass index, educational history, alcohol intake, history of diabetes, history of cancer, and hypertension status) (Lu, Seeboth, et al., 2019). While DNAmTL does not appear to be capturing telomere length itself, it is thought to measure biological outcomes that are themselves associated with shortening telomere length in adults (Lu, Seeboth, et al., 2019).

The fact that the DNAmTL based surrogate clock can outperform the measure it was trained on is an unusual characteristic that has been observed for other surrogate clocks. For example, Lu's clock for smoking pack years (DNAm-PACKYRS, used for calculating GrimAge) predicts lifespan better than the self-reported smoking pack years (Lu, Quach, et al., 2019). Surprisingly, the DNAm-PACKYRS clock predicts lifespan even among non-smokers! The reasons behind this finding are unclear but have been validated in multiple independent datasets comprised of thousands of individuals (Lu, Quach, et al., 2019). One possible explanation is that DNAm-PACKYRS is capturing some of the intrinsic biological impacts of smoking. This would make it more accurate than self-reported pack years, or better able to detect individual differences in the susceptibility to the effects of smoking itself. In most cases, the causal relationships between clinical biomarkers and their DNAm surrogate clocks remain to be elucidated. In the meantime, DNAm-based surrogate clocks for clinical biomarkers may lead to opportunities for human biologists. Situated at the interface of genes and the environment, DNAm surrogates may provide measures of health, development, and BA that are more proximal to the aging process itself. Furthermore, unlike some of the clinical measures they provide proxies for, DNAm can be measured in banked and/or dried blood spot samples, as will be discussed in more detail below.

2.2 | Emerging applications for epigenetic clocks

2.2.1 | Studying the impact of nutrition and lifestyle

Individual trajectories in epigenetic aging appear to take shape quite early in the lifecourse. Some of this individual variation in the "ticking" of epigenetic clocks is likely set prenatally by genetic variation, which has been studied through both heritability and genome-wide association studies (Gibson et al., 2019; Lu et al., 2018; Marioni, Shah, McRae, Chen, et al., 2015). Not surprisingly therefore, longitudinal trajectories for some epigenetic clocks (ie, GrimAge Li et al., 2020) are relatively stable. However, a robust measure of BA should be sensitive to many of the nutritional, behavioral, ecological, and social factors that affect the aging process (Crews, 2003). Several epigenetic clocks appear to demonstrate such flexibility, which makes them excellent tools for studying the factors that influence BA without the need for long-term health and mortality-related outcomes.

As examples, greater self-reported consumption of fish, fruits, and vegetables are associated with lower Hannum-EEAA and AgeAccelGrim, while greater reported consumption of red meat is associated with

higher AgeAccelPheno and AgeAccelGrim (Levine et al., 2018; Lu, Quach, et al., 2019). These measures of self-reported dietary intake are supported by blood measures of dietary intake; carotenoids such as lutein, lycopene, zeazanthin, α-tocopherol, and α- and β-carotenes all show negative relationships with age-corrected IEAA, EEAA, AgeAccelPheno and AgeAccelGrim (Levine et al., 2018; Lu, Quach, et al., 2019; Quach et al., 2017). Similarly, blood measures of inflammation (C-reactive protein), glucose metabolism (Insulin, glucose), systolic blood pressure, and obesity (waist-to-hip ratio, BMI) show the expected positive relationships to epigenetic aging measures, while HDL cholesterol shows similarly predicted negative relationships (Levine et al., 2018; Lu, Quach, et al., 2019; Quach et al., 2017).

Other behavioral and lifestyle characteristics that affect life expectancy are linked to epigenetic clocks. Self-reported smoking status predicts more rapid AgeAccel for mortality-trained clocks (PhenoAge and GrimAge), whereas alcohol consumption and exercise predict slower AgeAccel for these measures (Levine et al., 2018; Lu, Quach, et al., 2019; Zhao et al., 2019). There is also evidence for a beneficial effect of exercise on most of the epigenetic clocks described thus far (Levine et al., 2018; Lu, Quach, et al., 2019; Quach et al., 2017), but the impact of physical activity may be less relevant among older individuals, perhaps because aging trajectory at that point is relatively "set" (Sillanpää et al., 2019). Furthermore, the relationship between exercise and epigenetic age may be more complex even among young individuals. Compared to controls, young elite athletes—especially power athletes—exhibited acceleration in a less commonly used 5-CpG epigenetic clock (Spólnicka et al., 2018). Whether differences in epigenetic AgeAccel between physical training regimes (power vs endurance) exist for more well-established epigenetic clocks (IEAA, EEAA, AgeAccelPheno, or AgeAccelGrim)—and if so, whether they are tied to the distinct metabolic or endocrinological profiles that accompany training—is an open question.

The observation that behavior and lifestyle affect the ticking of epigenetic clocks, which are themselves indicative of morbidity and mortality risk, means that epigenetic clocks may provide new tools for studying the immediate impacts of tradeoffs or health interventions. Although research in this area is still in its early phases, there is some evidence that the ticking of epigenetic clocks can be "slowed" through changes in behavior or pharmacological means. Using a protocol designed to rejuvenate the thymus—which shrinks with age and is essential for proper immune function and cancer defense (Bilder, 2016)—Fahy et al. were able to decelerate epigenetic age acceleration for four clocks (Horvath-IEAA, Hannum-EEAA, AgeAccelPheno, AgeAccelGrim) by an average of 2.5 years (Fahy et al., 2019). These changes were also linked to positive indices of immune function and inflammation (eg, C-reactive protein) and persisted at least 6 months after discontinuation of the protocol (Fahy et al., 2019).

A protocol involving the prolonged administration of metformin, dehydroepiandrosterone (DHEA), and recombinant human growth hormone (rhGH) like that used by Fahy et al. is unlikely to fit into research programs carried out by most human biologists. However, the treatments themselves may provide insights into broader theories about the role of hormones in mediating tradeoffs in immunity and somatic maintenance (ie, the immunosomatic metabolic diversion hypothesis; Micheal P. Muehlenbein, 2004).The experimental use of GH or DHEA also raises questions about the extent to which natural variation in commonly studied hormones (ie, testosterone, estrogen, cortisol, prolactin) might also affect BA as measured through epigenetic clocks (more on estrogen below). Furthermore, Fahy et al.'s intervention study raises the possibility that natural variation in social, environmental, or behavioral exposures might have similar impacts. Human biologists with expertise in biosocial and behavioral aspects of human health—from meditation and religious practices to seasonal changes in diet or exposure to sunlight—could find innovative ways to incorporate epigenetic clocks into their research.

2.2.2 | Studying reproductive investment and trade-offs

Few physiological transitions are as extreme as breastfeeding and pregnancy in women, which entail massive alterations in metabolism, immune function, and hormone levels (eg, estradiol, progesterone, and human chorionic gonadotropin) (Albrecht & Pepe, 2015; Anderson, MacLean, McManaman, & Neville, 2015; Robertson, Petroff, & Hunt, 2015). Investment into reproduction—particularly in conditions of resource scarcity—are expected to lead to tradeoffs with somatic maintenance that will manifest as accelerated BA. Evidence for a relationship between these "costs of reproduction" and epigenetic clocks is accumulating. While pregnant, women appear "younger" than expected using Horvath's clock, and "older" than expected for both PhenoAge and GrimAge clocks (Ryan et al., 2018). Consistent with theorized costs of reproduction that are expected to draw resources away from somatic maintenance and accelerate BA, both Horvath and GrimAge clocks also increase with parity or gravidity (Kresovich, Harmon, et al., 2019; Ryan et al., 2018).

The effect of reproduction on epigenetic clocks may be partly tied to changes in immune cell composition of blood, but hormone levels also appear to be an important contributor to epigenetic clock ticking rate. Accelerated epigenetic age using Horvath's clock is linked to earlier age at menopause and time since menopause in women who have already experienced this transition (regardless of the age of menopause) (Levine et al., 2016; but see Gibson et al., 2019). Furthermore, accelerated epigenetic age is observed among women who have undergone surgical menopause (bilateral oophorectomy), and appears to be decelerated with menopausal hormone therapies (Levine et al., 2016). Similarly, the severity of vasomotor symptoms ("hot flashes") among older women was associated with AgeAccelPheno, and women with accelerated PhenoAge and GrimAge were more likely to exhibit hot flashes (Thurston et al., 2020). These findings point to reproduction and hormones as important components in women's epigenetic aging, and to the potential utility of epigenetic clocks for studying other key life history traits and transitions.

Less is known about epigenetic clocks and men's reproductive health and investment, although an accelerated average epigenetic age relative to women emerges shortly after puberty for several clocks (Simpkin et al., 2017), consistent with higher mortality rates among men compared to women more generally (Horvath, 2013). Given the importance of testosterone in men's life history and health (Michael P. Muehlenbein & Bribiescas, 2005), future work examining the relationship between testosterone and epigenetic clocks is warranted.

2.2.3 | Studying psychosocial stress and resilience

Chronic stress is thought to contribute to "wear and tear" on the body, accelerating BA (Seeman, McEwen, Rowe, & Singer, 2001). Accordingly, Hannum, PhenoAge, and GrimAge clocks are accelerated among individuals with lower household income and education levels (Levine et al., 2018; Lu, Quach, et al., 2019; Quach et al., 2017; Zhao et al., 2019). These effects may be partly tied to the effects of diet, exercise, and lifestyle described above. However, trauma and stress may also mediate the effect of these environmental factors on epigenetic age (Liu et al., 2019). A significant proportion of Horvath's clock CpGs are in glucocorticoid response elements (Zannas et al., 2015). Furthermore, the administration of a synthetic glucocorticoid (dexamethosome) in living humans leads to changes in DNAm and RNA levels for genes near these CpGs (Zannas et al., 2015). Consistent with these findings, a study in adolescent girls found that greater diurnal cortisol production was linked to Horvath-AgeAccel (Davis et al., 2017). In a separate study, the number of lifetime stressors was associated with acceleration of Horvath-AgeAccel in a cohort of African American women (Zannas et al., 2015). However, these effects were blunted among women with severe experiences of child abuse (Zannas et al., 2015), possibly mirroring blunted glucocorticoid responses often associated with such traumatic experiences (Adam, Klimes-Dougan, & Gunnar, 2007).

In fact, several other studies have found that "resiliency" to stress might come at the cost of accelerated epigenetic age. Higher measures of self-control predict slower Horvath- and Hannum-AgeAccel, but only among individuals who were raised in the context of high socioeconomic status (SES). Individuals with higher measures of self-control raised in low SES contexts showed the opposite effect, leading the authors to conclude that resilience may only be "skin-deep" (Miller, Yu, Chen, & Brody, 2015). Several studies among veterans also support harmful effects of resiliency on epigenetic age. Veterans exposed to traumatic events who did not show any symptoms of post-traumatic stress disorder (PTSD) were more—not less—likely to have increased Horvath-AgeAccel (Boks et al., 2015). In another study, veterans with PTSD exhibited accelerated epigenetic age compared to those without PTSD only if they scored high on feelings of self-efficacy and resilience (Mehta et al., 2018). Collectively these studies suggest that stress and trauma contribute to accelerated epigenetic age, but also that those who appear to be managing it the best from the outside may experience the negative impacts on health and BA. More research is needed in this area, however, and antidepressant medications—common among individuals who score high on PTSD and major depressive disorder scales—may also accelerate Horvath's clock (Verhoeven et al., 2018).

2.2.4 | Studying environmental and ecological variation

Given the connection between epigenetic clocks and nutrition, physical activity, reproduction, and stress, it is not surprising that epigenetic aging rate for several clocks varies across socioecological contexts (Fagny et al., 2015; McEwen et al., 2017). In some cases, clocks display divergent age-related trends depending on the context, which may be informative about how environmental and ecological variation affects the aging process. Tsimane forager-horticulturalists of Bolivia show slower Horvath-IEAA and faster Hannum-EEAA than Caucasian or Hispanic counter-parts (Horvath et al., 2016). Slower

Horvath-IEAA and faster Hannum-EEAA have also been reported for African forest-dwelling (Baka and Batwa) hunter-gatherers and forest-dwelling Agrarian Bantus, but not urban-dwelling Bantus (Gopalan et al., 2017; Horvath et al., 2016). These findings imply that accelerated immune-associated aging (Hannum-EEAA) can be accompanied by slower "intrinsic" aging (Horvath-IEAA), consistent with theorized tradeoffs between immune function and development (Urlacher et al., 2018). More work is needed to establish if early life infectious environments shape the trajectory of epigenetic aging, and if so, how.

3 | METHODS AND IMPLEMENTATION OF EPIGENETIC CLOCKS

3.1 | Design and sampling considerations

3.1.1 | Sampling, bias, and confounding

Epigenetic clocks are providing a set of highly versatile tools for studying tradeoffs in health, development, reproduction, and aging. However, like any method used to study human health and variation, the robustness and generalizability of findings using epigenetic clocks hinges on addressing individual research questions while minimizing biases and confounding introduced through study design (Michels, 2012). Here I outline basic protocols for sampling that aim to avoid bias and confounding in studies of epigenetic age.

Sampling bias occurs when samples collected do not represent a random selection from the population of interest (Kahn, Kahn, & Sempos, 1989). In cohort studies, for example, attrition of individuals with higher SES could bias the sample so that lower SES individuals are overrepresented. Because SES is in turn associated with nutrition, lifestyle, and psychosocial stress, all of which are known to alter epigenetic clocks, attrition of higher SES individuals will be reflected in changes to epigenetic age in the remaining sample. Resampling, following-up lost participants, or statistical approaches such as inverse probability weighting can minimize the impact of sampling biases.

Confounding—when an observed relationship is distorted by another, unaccounted for measure—is another challenge faced by studies of health and aging, including those using epigenetic clocks. Confounding can attenuate, amplify, falsely create, or obscure a relationship between the variables of interest and epigenetic age (Michels, 2012). For example, a study comparing the effect of physical activity on epigenetic age between urban and rural participants might be confounded by differences in diet, reproductive patterns, SES, or exposure to pollutants. Recognizing the sensitivity of epigenetic clocks to environmental factors and including those in surveys and statistical models can help minimize the issue of confounding in studies of epigenetic age.

Epigenetic variation, including epigenetic clocks, is partly explained by genetic variation. Although most epigenetic clocks using hundreds of CpG loci appear be fairly robust to such genetic variation, cross-cultural comparison studies should consider the potential for population stratification. Including genome-wide genetic data and testing for gene x environment interactions is one way to address this issue but can be costly. Other approaches for reducing the impact of genetic variation on epigenetic age is through restricting analysis to a fairly homogeneous sector of the population, or by using statistical approaches such as mixed model association methods to model population substructure (Yang, Zaitlen, Goddard, Visscher, & Price, 2014).

3.1.2 | Sample collection and storage

All commonly used epigenetic clocks described here involve the quantification of DNAm. While all cells in the body contain more or less identical genomic DNA, DNAm varies widely between tissue and cell type. For this reason, it is important to consider the tissue and cell types sampled for any epigenetic study, including epigenetic age. With the exception of Horvath's clock and McEwen's pediatric clock, most epigenetic clocks have been trained and validated using DNAm measured in blood. Even Horvath's clock, which was trained using 27 different tissues, shows some variation between tissues of the same individual. For example, obesity and Alzheimer's are most strongly linked with Horvath-AgeAccel in the liver and brain, respectively (Horvath et al., 2014; Levine, Lu, Bennett, & Horvath, 2015). In some cases, findings for one tissue type (eg, buccal cells) may not apply to another (eg, whole blood) (Levine et al., 2016; Slieker, Relton, Gaunt, Slagboom, & Heijmans, 2018). Unless researchers are interested in conducting their own intra-individual tissue comparisons, using the tissue on which the clock of interest has been trained or otherwise validated is the safest approach.

While most epigenetic clocks have been trained or tested using whole-blood, venipuncture can be technically demanding, often requiring a trained medical technician or phlebotomist. This can be a major limitation in many field settings, where additional challenges arise

from the transportation, shipping, and storage requirements of whole blood (McDade, 2014). Fortunately, dried blood spots (DBS) collected on filter paper or "Guthrie cards" provide an accurate and low-cost, field-friendly alternative when measuring DNAm and epigenetic clocks (Dugué et al., 2016; Ramagopalan & Rakyan, 2013). Samples are easily collected using a finger prick from small lancet and after air drying can be stored at room temperature for long periods of time. Samples stored at room temperature on Whatman FTA cards—which include a chemical treatment specifically developed for DNA and RNA analysis—yield stable and accurate measurements of DNAm for at least a decade (Joo et al., 2013; Walker et al., 2019). DBS therefore provide researchers with the opportunity to combine new waves of survey and data collection with banked samples from long-standing studies around the world (Ghantous, Hernandez-Vargas, & Herceg, 2018). Alternatively, researchers with ongoing studies can collect and store DBS with an eye toward including epigenetic clocks in future research.

The amount of DNA needed for epigenetic clocks depends partly on the method of measurement (array-based vs pyrosequencing), as well as the efficiency of DNA extraction, purification, and bisulfite conversion. The bisulfite conversion step in particular can degrade genomic DNA, making methods for efficiently extracting and purifying DNA from DBS essential (Ghantous et al., 2018). For array-based methods, 250 ng of bisulfite converted starting DNA is required (www.illumina.com/documentation). Sufficient quantities of bisulfite converted DNA (300-800 ng) can typically be obtained from 1 to 2 6 mm punches (600-800 ng prior to bisulfite conversion). Pyrosequencing-based methods for locus-specific clocks may require a slightly lower amount of starting DNA to accurately measure DNAm (Busato, Dejeux, Gut, & Tost, 2018).

3.2 | Technical considerations

3.2.1 | Choosing a clock and measuring DNAm

As discussed, epigenetic clocks can be characterized based on the method used to generate them, which also largely corresponds to the number of sites required for their calculation. The most widely used and well-validated epigenetic clocks also use the greater number of CpG sites, and were derived through the application of supervised machine learning to high-dimensional array-based datasets. These datasets come from a family of microarrays designed by Illumina (Illumina, Inc, San Diego, California). Illumina arrays are able to provide a reproducible, low bias, high density, single base resolution DNAm for hundreds of thousands of sites across the genome (Bibikova et al., 2011). Most early clocks were trained on legacy Infinium HumanMethylation27 (~27 k CpG sites) and Infinium HumanMethylation450 (~485 k sites) BeadChip arrays, but appear to be nearly as accurate on the newer Infinium methylationEPIC Beadchip array (~850 k CpG sites) (Logue et al., 2017; Solomon et al., 2018). Other methods, such as whole-genome bisulfite sequencing, may provide alternatives for measuring clocks with many CpG sites, but are typically more expensive and often technically more challenging to work with than array-based methods. More affordable alternatives, such as microdroplet PCR (Komori et al., 2018) may eventually prove useful in the study of epigenetic age, but have yet to be validated for use with epigenetic clocks. Any variation or biases tied to the Illumina BeadChip technology that are not present when using these other methods could affect the accuracy of the clocks derived from them, making such alternatives less desirable than currently available array-based methods (Box 1).

Array-based methods do have some drawbacks. Although less expensive and technically challenging to work with than next-generation sequencing, processing Illumina arrays still requires costly equipment (IScan System, Illumina, Inc), trained technicians, and strict measures of quality control. For most human biologists and anthropological geneticists, leveraging collaborations or capitalizing on university genomics core facilities is likely the most economical and efficient way to successfully meet these demands. While the quality and cost of using core facilities can vary, most universities offer a discounted rate for larger orders or researchers affiliated with the university. At the time of writing, costs ranged from between $200-350 USD per sample, depending on the scale and researcher affiliation. Although not inexpensive, the added advantage of using array-based clocks is a wealth of additional information about the methylome. Covering over 850 000 CpG sites, the Infinium methylationEPIC Beadchip array provides researchers with many opportunities to study the relationship between DNAm and health, development, and the environment beyond those offered by epigenetic clocks.

The second major method for deriving epigenetic clocks comes from epigenome-wide association studies. These clocks concentrate on predictable changes in a small number of CpG sites (ie, <11), making them amenable to bisulfite pyrosequencing or other targeted approaches to measuring DNAm. While bisulfite pyrosequencing is an affordable, highly accurate method for measuring DNAm, it is not applicable to array-based

clocks due to the large number of CpG sites that must be measured simultaneously—beyond a certain number of loci, the cost of labor, reagents, and primers exceeds those of array-based alternatives. These targeted clocks show great promise as measures of chronological age (Garagnani et al., 2012; Slieker et al., 2018), frailty, and mortality risk (Y. Zhang et al., 2018, 2017), but have not been as well-validated as array-based clocks. As such, these clocks may provide opportunities for researchers to run pilot studies or student projects that can later be scaled up to genome-wide, array-based methods.

When measuring DNAm using either array-based or targeted approaches, researchers should be cautious to avoid confounding the phenotype of interest with batch effects. While methods like surrogate variable analysis (Teschendorff, Zhuang, & Widschwendter, 2011) can successfully account for batch effects by themselves, analyzing samples from distinct groups, timepoints, or geographical locations together can confound technical batches with sampling procedures or the phenotypes of interest. Furthermore, batch correction during preprocessing is not recommended for some epigenetic clocks (see Box 2). To minimize technical confounding, samples that can be clearly grouped by time, exposure, geographical location or phenotype of interest should be processed and assayed in a random fashion (ie, day, technician, chip, batch). In some cases, studies that originally utilized whole-blood for estimating epigenetic clocks may later have easier access to DNA from DBS. At other times, researchers conducting longitudinal studies may find that methods have changed since their original sample; follow-up research for samples originally run on the discontinued 450 k array may need to be run on the current EPIC array, for example. Changes in either blood collection method or array platform may confound with batch effects and should be avoided if possible. When such changes are unavoidable, researchers can run a small subset of replicate samples using both methods to demonstrate the repeatability and lack of bias within samples. Although this can be costly for array-based methods, it is important for establishing validity and continuity in a study over time.

3.2.2 | Processing DNAm and calculating array-based clocks

Compared to DNAm derived from targeted methods such as bisulfite pyrosequencing, DNAm from array-based methods requires rigorous quality control, background correction and signal normalization (Morris & Beck, 2015; Wang, Wu, & Wang, 2018). There are numerous programming tools and pipelines for carrying these procedures out, but quality control and normalization

BOX 2 Processing steps for Illumina BeadChip array data destined for array-based clocks

Quality control

- Do not remove SNP-associated probes (Pidsley et al., 2016; Price et al., 2013)
- Do not remove sex chromosome (XY) associated probes.
- Do not remove poorly performing probes (<3 beads per signal or with nonsignificant detection *P*-values) from the dataset entirely.
  - One option is to create a matrix with poorly performing participant/probe cells as "NA." Immediately prior to preparing beta-values for the calculator, use this matrix to only mask those cells for which it applies with "NA." This maximizes the retention of probes that perform well for most participants and allows for Horvath's calculator to impute from the missing values from the rest.

Normalization

- Noob or quantile normalization are the recommended preprocessing methods (Fortin, Triche, & Hansen, 2017). Horvath's calculator conducts its own normalization, based on a modified version of Teschendorff's BMIQ or beta-mixture quantile normalization (Teschendorff et al., 2013).
- Although samples should be processed to minimize confounding, batch effects should not be corrected for. Because it was trained on so many different samples from different sources, Horvath's clock is quite robust to the batch effects and correcting for them is thought to affect the normalization algorithms and epigenetic age estimates performed by the calculator. Still, researchers should avoid analyzing samples grouped by sex, age, exposure, or other potentially confounding variable.

Cell type correction

- When "Advanced Analysis" is selected, Horvath's calculator estimates white blood cell counts (CD4T, CD8T, Granulocytes, Monocytes, Natural Killer cells, etc.) and these are used for some measures of age acceleration (ie, Horvath-IEAA and Hannum-EEAA).

should be modified when output will be used for epigenetic clocks. It is currently possible to calculate epigenetic age for all array-based clocks (and several candidate gene clocks) using either openly available R scripts or a single online calculator developed and maintained by Steve Horvath at UCLA (http://dnamage.genetics.ucla.edu/). More detailed instructions for the preparation and processing of raw array-based data for epigenetic clocks can be found in Box 2.

For array-based clocks, Horvath's website provides a detailed tutorial on additional formatting for the calculator and interpretation of the results. Briefly, there is a "basic analysis" in which users simply provide a .csv file containing beta-values for roughly 30 k preselected CpG loci for each participant (columns). This will provide an estimate of BA using Horvath's clock and a standard quality statistic for each participant. There is also an "advanced analysis for blood," which requires an additional .csv file containing columns containing participant id, age, and sex. The additional file quality control checks for consistency between predicted and reported tissue type and sex (based on X chromosome DNAm). More importantly, the advanced analysis option automatically calculates clocks that include chronological age in their estimates (Horvath-IEAA, Hannum-EEAA, PhenoAgeAccel, GrimAgeAccel, etc.) as well as surrogate clocks for a number of blood biomarkers (eg, Telomere length, Leptin, PAI1, Cystatin), and estimates of white blood cell counts (eg, CD4T, CD8T, B cells, Natural Killer cells, Monocytes, etc.). The range of outputs lend Horvath's calculator great flexibility for a variety of research questions relevant to human biologists.

As discussed, most epigenetic clocks are trained directly on chronological age or measures closely tied to age-related decline. However, most researchers are interested in acceleration of BA compared to others of the same age: does this person look biological "older" or "younger" than we expect, and why? In other words, the raw output of an epigenetic clock is sometimes not the variable of interest. Estimates for age acceleration can be derived from the difference between epigenetic age and chronological age (epigenetic age—chronological age) or the residuals of a regression of epigenetic age on chronological age (epigenetic age $\sim a + b *$ chronological age, where a is the intercept and b is the slope). Alternatively, researchers can include chronological age with other variables in the model and epigenetic age as the outcome. As previously discussed, systematic differences between predicted and chronological age may arise from DNA degradation during storage or processing, making it important to randomize across chips and to maintain as close a sampling protocol as possible if comparing different populations.

For candidate gene clocks, genomic loci, coefficients, as well as primer sequences are available in the results or supplementary sections of the relevant sources (Weidner et al., 2014; Y. Zhang et al., 2017). Weidner's age estimate can also be predicted using a free online calculator (http://www.molcell.rwth-aachen.de/epigenetic-aging-signature/). Additional candidate gene clocks for chronological age, mortality, or other outcomes that are of interest to human biologists will no doubt continue to be developed.

4 | CONCLUSIONS

Epigenetic clocks are groundbreaking tools that are changing how researchers study human development, aging, and health. They are providing insights into fundamental molecular processes underpinning health and aging and are emerging as important biomarkers of those processes. But the pace of "aging" and how that plays out for any individual person is a complex interaction between evolutionary, social, and cultural forces. As a discipline, human biology strives to weave together these aspects of the human condition, and can both benefit from and contribute to our understanding of the epigenetics of aging and epigenetic clocks.

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AUTHOR CONTRIBUTIONS

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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