“Biological” clocks: a peek into the future or a haphazard guess of mortality?

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One of the biggest challenges in aging science is that we don’t have any biomarkers that can reliably track the aging process. Such biomarkers – were they to exist – would have obvious uses in predicting lifespan, but perhaps more importantly, they would finally allow us to assess the efficacy of *interventions* intended to *slow* the aging process. When you take a drug to lower blood glucose, apoB, or blood pressure, you can titrate the dosage up or down as needed because you have a readout, a *biomarker*, to monitor the magnitude of the drug’s effect. But when it comes to the most interesting geroprotective molecules – rapamycin, metformin, or NR/NMN, to name a few (we use the term “geroprotective” to denote that such drugs target the basic hallmarks of aging rather than specific diseases) – we have no biomarker to tell us (1) if the drug is having any effect, and (2) if we are taking too much or not enough.

However, in the past decade, a technology has emerged that might offer a clue. Several consumer-facing “biological clocks” claim to give the user insight into their “biological age,” which may be different from their “chronological age.” If biological clocks accurately predict future lifespan and could demonstrate that an intervention postpones mortality, they might prove to be a useful tool in determining the efficacy of geroprotective therapies.

What do biological clocks measure?

These so-called biological clocks use a DNA sample from blood or saliva to analyze epigenetics, a term that encompasses a variety of factors that affect how DNA is expressed but are not encoded in the DNA sequence itself. One of the most popular types of biological clocks analyzes one such factor in particular – DNA methylation patterns – to estimate aging. DNA methylation occurs when a methyl group is added to cytosine (the “C” nucleotide in DNA, which is composed of “As,” “Cs,” “Gs,” and “Ts”). This is a normal, at times reversible, cellular process that affects gene expression either positively or negatively depending on the exact methylation site, and it plays a major role in cell differentiation and regulation.

Although methylation can occur at any cytosine nucleotide, in somatic cells over 98% of methylation occurs at a CpG site (a dinucleotide of cytosine followed by guanine – “G”). In humans, about 70% of gene promoters – regions of DNA that are involved in the initiation of gene expression – contain CpG “islands,” a location with higher-than-expected frequencies of CpG sites. Methylation that occurs at the site of a gene promoter either directly impedes gene transcription or attracts proteins that bind and prevent the attachment of transcription factors, thus reducing the expression of the associated genes.

How does methylation relate to age?

During each cell division, DNA is replicated, followed by duplication of the “methylome” – the whole pattern of methylation across the entire genome. Although this epigenetic inheritance process is generally stable, as the number of cell divisions increases (as occurs with increasing age), occasionally passive loss of methylation occurs such that the methylation pattern on the new DNA doesn’t exactly match the original. Termed “epigenetic drift,” this is a proposed mechanism for the accumulation of epigenetic changes observed with aging. Using a method to detect methylation sites called “whole genome bisulfite sequencing” (WGBS), researchers showed that DNA from a centenarian had significantly fewer methylated sites than that of a newborn. This change over a lifetime adds up to nearly 500,000 fewer methylated sites, approximately 3% of the total number of methylated sites in a newborn.

However, the *rate* of this accumulation of methylation changes does not appear to be constant throughout life. In addition to examining two extremes of the aging spectrum, above, the researchers also analyzed the entire methylome from a healthy 26-year-old as an intermediate. Given that 26 years is roughly one-quarter of a century, we would expect – if methylation changes were linear over time – that the difference in methylated sites between the newborn and the 26-year-old would be roughly one-quarter of the difference between the newborn and the centenarian, or approximately 125,000. Instead, the investigators found that the 26-year-old had only about 67,000 fewer methylated sites than the newborn, suggesting that the rate of

methylation changes may accelerate over the course of one's life. Additionally, in younger genomes, neighboring CpG sites are often highly correlated in their methylation status, but this too decreases with age, indicating both a change in the absolute number of DNA methylation sites and a pattern to how they change with time.

Since there are known sites and temporal patterns of methylation, a characterization of an individual's methylation pattern might serve as a surrogate for estimating age. Although the previously mentioned research analyzed the entire genome, DNA methylation "biological clocks" rely on assessing methylation status at a limited number of CpG sites. One of the original clocks (invented by Steve Horvath in 2013) analyzed the methylation of 353 CpG dinucleotides. Although there is a net loss of methylation sites that occurs with aging, some sites lose methylation (hypomethylation) while others gain methylation (hypermethylation). In the original clock, of the 353 CpG sites, 193 sites were positively correlated with age and were hypermethylated, although with no discernable pattern. The other 160 CpG sites were negatively correlated with age and were hypomethylated. (Interestingly, the hypomethylated sites tended to be located on "island shores" or distances within approximately two kilobases from CpG islands, reinforcing the concept that the pattern of methylation errors correlates with age.)

From a database of DNA methylation datasets, a subset was used to construct and train the "biological" age predictor based on the known chronological ages of healthy tissue. Unlike other biological clocks that only use one tissue type (usually white blood cells) to train the predictive model, this biological clock used training datasets from more than 50 tissue types in subjects of all ages. The remaining, non-overlapping datasets were used to test the predictive value of epigenetic age against chronological age. In many types of healthy tissue, there was a 95% correlation between biological age (what the epigenetic predictor says) and chronological age (what the calendar says), with an average error between predicted and chronological age of a little more than 4 years. However, the predictive value of this "biological clock" was poor in certain tissue types including breast, endometrium, skeletal, and cardiac tissues, which may indicate that other factors such as hormones, high tissue turnover, and the recruitment of satellite or stem cells may all be factors that influence a tissue's DNA methylation "age."

While the first round of DNA methylation clocks can relatively consistently predict chronological age (within a few years), most people already know their chronological age (obviously!). The more striking problem with this and more recently developed biological clocks is the lack of evidence to support the enormous leaps that people make in interpreting what it means to be "biologically" younger. The Horvath clock is based on healthy tissue samples that came from publicly available individual data sets *without* longitudinal data. These individuals very likely had a wide variety of mortality and long-term health outcomes, but in the absence of this information, there is no plausible way to differentiate the epigenetics of those who lived shorter from those who lived longer than average lives. One of the reasons people assume that a prediction of a younger age has some correlation with a longer life is that the opposite was true – accelerated epigenetic aging was observed and an older epigenetic age was predicted in cancerous tissue. But just because diseased tissue is epigenetically "older," that doesn't mean that an epigenetic age 10 or more years "younger" than your calendar age will equate to a longer lifespan.

Next-generation tests

The key test is whether a biological clock can predict rates of cellular aging, susceptibilities to age-related diseases, or mortality risk. Recall that the training datasets used to make the Horvath clock were all from healthy tissue without longitudinal data, so despite their success in correlating with chronological age, they were less reliable for these other, more relevant predictions for health.

The subsequently developed epigenetic clocks sought to improve the predictive model of epigenetic age by including more phenotypic health information in the training model. To make a model predictive of both lifespan and healthspan, a newer version of this test ("PhenoAge") examined 513 CpG sites (only 41 of which overlapped with the original 393 sites) from whole blood. In this newer test, the predictive models were trained on methylation data and data on "phenotypic age," a composite of chronological age along with nine serum blood biomarkers. In other words, the models were not designed to predict chronological age exclusively but instead also factor in variables related to *health* at a given chronological age. Predictably, age predictions from these models based on DNA methylation had a lower correlation with chronological age than older tests but had significant associations with *mortality* risk. For each one-year increase in predicted PhenoAge, there was an associated 4.5% relative increase in the risk of all-cause mortality (ACM).

PhenoAge, a calculator based on multiple large biobank samples, also estimates an epigenetic "acceleration" parameter based on differences in methylation patterns among individuals of the same chronological age. While the PhenoAge predicts a "phenotypic" age from both chronological age and other biomarkers, the PhenoAgeAccel parameter is calculated as the difference between an individual's predicted PhenoAge from a linear model comparing chronological age and PhenoAge. Essentially, the linear model is a description of how the population's chronological age aligns with the predicted "PhenoAge" and PhenoAgeAccel indicates whether an individual is aging slower (and is therefore "younger") or faster (and is therefore "older") than the average person of the same chronological age. To demonstrate how aging speed correlated with mortality, the developers of this biological clock used samples from the Women's Health Initiative from women ages 50-79. In these women, the PhenoAgeAccel parameter predicted a nearly 2.6-fold difference in risk of ACM between the top 5% of the fastest and the bottom 5% of slowest agers.

Methylation is affected by more than just age

Of course, biological clocks based on epigenetics would be useless if chronological age and genetics were the only variables that affected DNA methylation. The entire concept behind these clocks is that other, *modifiable* factors may hasten or slow the process of epigenetic drift in a manner corresponding to changes in mortality risk. For instance, cigarette smoking alters the DNA methylome and induces hypomethylation at several known sites. This epigenetic change is even posited as a causative factor in smoking-related diseases, especially for the disease risks that remain elevated even after smoking cessation, since some epigenetic alterations can persist for decades despite the attenuation of other smoking-related changes in that time.

Problematically, in PhenoAge's initial analysis, there was no association between pack-years (the number of packs smoked per day multiplied by the number of years spent smoking) and DNA methylation age, and it wasn't until further stratification by smoking status (i.e. non-smoker, former smoker, current smoker) and adjustments by number of pack-years were added into the model that an increase in DNA methylation age was associated with a small increase in lung cancer.

The need for this adjustment to the model raises more questions about what *other* known behaviors are, like smoking, known to cause changes to DNA methylation patterns *and* elevate risks of all-cause mortality. For example, sleep deprivation is known to elevate the risk of ACM and results in changes to several methylation sites. Should models be accounting for sleep or shift work, too? While it might seem tedious to adjust for all of the negative health behaviors that affect DNA methylation, it's important to take these factors into account to achieve accurate predictions of mortality risk. Without specific adjustments for behaviors and variables that we *know* shorten lifespan, such variables tend to get lost in the process of data averaging, resulting in a risk prediction that fails to account for their clear impacts on longevity.

Discerning signal from *biological* noise

In order to use biological clocks as a tool for testing geroprotective molecules, it's also important to understand how day-to-day behaviors may cause *acute* fluctuations in predicted DNA methylation age on a relatively short timescale, as this fluctuation creates noise that may mask any potential positive or negative changes associated with various interventions. For example, if your predicted age changes by several years every time you get a bad night's sleep, eat a piece of cake before bed, gain a few pounds, or have a stressful month at work, it will be much harder to discern the effects of a geroprotective intervention. Thus, more longitudinal studies investigating changes in epigenetic age with changes in behavior (e.g., smoking cessation, weight loss/gain, increased exercise, improved sleep) are needed to rule out potential confounding factors of daily living on both long and short timescales.

Existing literature hints at the scope of the noise problem attributable to fluctuations in human behavior. For instance, a pilot study testing whether lifestyle interventions could reverse "biological" age as measured by epigenetic clocks required subjects to avoid consuming alcohol, nicotine, and cannabinoids in the week before scheduled study visits. This requirement suggests that acute factors such as alcohol consumption may outweigh epigenetic changes of lifestyle interventions (e.g., diet, exercise, sleep, relaxation interventions) or geroprotective therapies. A different study of two, healthy, Japanese men in their 30s analyzed epigenetic age 24 times over three months, with each blood draw taken after an overnight fast and no consumption of alcohol in the prior 24 hours. Without any intentional lifestyle alterations, the predicted PhenoAge fluctuated by 8-12 years, with *daily changes between 5-6 years*. This is a profound amount of biologic noise!

The Japanese study also illustrates another source of biological noise in epigenetic clocks: variations across different types of cells and tissues. Data from that study showed that monocytes (a type of white blood cell) were predicted to be nearly *20 years younger* than the PhenoAge estimated from whole blood, which includes other types of white blood cells, along

with red blood cells. With such an enormous discrepancy between whole blood and one of the components of whole blood, how much more of a discrepancy might be possible when comparing two entirely different tissues?

Indeed, most current epigenetic clocks are trained on only one type of tissue (usually a subset of white blood cells) but are then applied to test the "biological age" of countless other cells and tissues in the body. This practice relies on the assumption that different cell types and tissues experience the same methylation changes and age at the same rate, yet as we've seen from the Japanese study as well as other published data, *this assumption is incorrect*. Tissue-specific disease (e.g., breast cancer), for instance, changes DNA methylation and locally "ages" tissue, and even in healthy tissue, cross-tissue correlation of predicted age has been reported, at best, as only 0.46 to 0.68. Thus, reducing biological noise and increasing the accuracy of epigenetic clocks will ultimately require the development of clocks that are specific to particular cell or tissue types.

However, as many cell types (such as neurons) cannot logistically be sampled from living humans, developing such tissue-specific clocks for commercial viability is not a trivial task. Ideally, we would need methylation clocks that can use samples from accessible tissues (e.g., blood) to provide insights on *other* tissues, and at minimum, this would require analysis of a greater number of methylation sites and a far deeper understanding of methylome differences and changes across tissues than we currently possess. (The obvious way to do this is to borrow a chapter from the liquid biopsy world and sample plasma for cell-free DNA, which of course requires much more technical horsepower, as discussed below.)

Discerning signal from *technical* noise

Beyond the fluctuations introduced by biological noise, there is also a degree of *technical* noise inherent in measuring methylation levels. Commercially available biological clocks use DNA methylation microarrays, which are an attractive approach for the high-throughput, targeted analysis used by their algorithms for predicting "biological" age. (To understand why these methods are suboptimal, you need to understand at a basic level how they work. Apologies in advance.)

Microarrays measure DNA methylation through a few steps: 1) DNA is extracted and purified from the tissue sample; 2) DNA strands are separated and fragmented into segments of a few hundred base pairs; and 3) the fragments are run through a microarray which uses fluorescence to indicate the level of pattern matching between the unknown DNA sample and known DNA methylation patterns of probes on the microarray. This technique provides an *average* methylation level at a given area but does *not* have the resolution to be CpG site-specific. Newer generation microarrays can evaluate tens or even hundreds of thousands of CpG sites by using smaller, 50-base pair DNA fragments, but even these smaller sections still may contain anywhere from 4-10 CpG sites per fragment.

In contrast, whole genome bisulfite sequencing (WGBS) evaluates the entire methylome (analogous to whole genome sequencing), base pair by base pair, which is estimated to contain about 28 million CpG sites across the genome. WGBS has single base pair resolution,

as well as the ability to detect both methylated and unmethylated sites, making it a far more comprehensive analysis. In this regard, sequencing is like having a detailed road map showing how and where roads are laid out in relation to each other and to other landmarks or destinations, whereas microarrays are like knowing how many stoplights exist. Although initially prohibitively costly, declining costs in the past few years have made WGBS more feasible. One of the disadvantages of this methodology is that the treatment with sodium bisulfite can cause DNA degradation, with a bias towards unmethylated regions of DNA. When the DNA is subsequently amplified, this preferential degradation leads to an overrepresentation of highly methylated regions and an underrepresentation of unmethylated CpG sites. It will be important to minimize these types of biases if site-specific DNA methylation loss is going to be used in predicting mortality from epigenetic aging.

Newer methods use enzymatic treatments to detect methylation without amplification bias in small samples of DNA (e.g., cell-free DNA). These methods have demonstrated the ability to better preserve DNA than bisulfite treatments but are in early development stages and are still being improved to accurately detect all methylation sites. As it stands, the continuing development and higher costs of whole epigenome analysis may be prohibitive for a commercially-available “biological” clock but finer and more comprehensive resolution of DNA methylation is one of the best roads forward for increasing the utility and meaning of an epigenetic age.

Missing links for lifespan prediction

Other missing pieces of information will also need to be elucidated before we should take any of these biological clocks too seriously. One of the considerable unknowns, and I would argue the most important, is the ability of an aging clock to predict *remaining* years of life *better* than chronological age can. Testing this at least in animal models would be fairly straightforward, since, unlike humans, genetically homogeneous mice have lifespans on a scale that permits studies on longevity. And yet, to date, even in mice we lack data demonstrating that a predicted “biological age” correlates with mortality (i.e., the mouse with the “youngest” biological age at mid to late life also lives the longest, and perhaps even lives to a predictable number of weeks knowing the maximal lifespan of the species).

Laboratory experiments are conducted in the most ideal setting possible, in which researchers control variables (e.g., diet, sleep cycles, and environmental exposures) that they could never control in human studies, so the current absence of proof-of-concept data even in the highly controlled setting of animal experiments make it clear that, as of yet, these clocks are still far from primetime-ready. However, by *starting* from a tightly controlled setting, we might learn something that will help to translate this methodology into a meaningful predictive tool in people, with the many uncontrolled variables of free living.

Many people today tout their biological age as being far lower than their chronological age as a sign of good health. Maybe this is the case, but let’s say a 60-year-old, who, from an actuarial perspective, could expect to live another 25 years based on chronological age, buys an aging

clock that tells him his biological age is 35. Does it really mean he can expect to live another 50 years? This seems almost impossible to imagine. And yet, this is the most important test we might have for such clocks.

Observationally, supercentenarians (105+ years) have been shown to have an average biological age of more than 8 years younger than their calendar age as measured from peripheral blood mononuclear cells, and their children have a lower "biological age" than their age-matched controls by about 5 years. Initially, it may seem that this would indicate that if your DNA methylation age is younger than your chronological age, you are likely to live longer. This may be true, but how much is "longer"? Centenarians tend to have a longer lifespan because they have delayed the onset of age-related diseases. If their lower "biological" age and slower rate of epigenetic aging are indicative of the deferral of chronic disease, it would not be surprising that their offspring might show some of the same trends. After all, family history is an important predictor of healthspan and lifespan. The DNA methylation clocks as they currently exist may be explanatory of a long lifespan on a population level, but if you look more closely at the data, even a few of the supercentenarians (<7%) have "biological ages" several years greater than their chronological age. What does an epigenetic age of 112 mean to a person who is 105 years old? They should already be dead? Conversely, what does an epigenetic age of 70 mean for a 105-year-old?

Looking forward

To this point, no one has tested whether chronological or epigenetic age is a better predictor of total lifespan, and in all likelihood, it will require more nuanced technology. A small set of methylation sites from a single tissue type just doesn't offer enough information to make any reliable predictions about the big picture of whole-body health, aging, and mortality risk, just as taking a handful of tiles from a massive mosaic tells us nothing about the overall image. Developing tissue-specific models and utilizing sequencing approaches over microarrays are promising avenues toward improving the reliability and utility of future iterations of epigenetic clocks, but current models just aren't there yet.

Future biological clocks must demonstrate that meaningful interventions known to alter risks of disease and all-cause mortality result in reliable changes in predicted age, while simultaneously demonstrating that predicted age is robust to *transient* changes that do *not* correspond to alterations in lifespan and disease. For example, if a person quits smoking, starts a regular exercise regimen, or loses weight by reducing fat mass (especially visceral fat), there would be an expected decrease in biological age. By the same token, the results need to be immune to the person who tries to metaphorically "cram for the test" by doing these types of behaviors only on the day before the blood draw. Making the distinction between transient and meaningful epigenetic changes will require uncovering which epigenetic markers are *causally* related to biological changes. Without this, it's hard to imagine such clocks having much value.

An important step toward demonstrating this robustness will be better source data. As in many of the studies we've cited above, most research assessing the performance of epigenetic clocks has relied on analysis of existing public datasets, but these datasets were built from studies that were not designed for the purpose of developing a biomarker of longevity. Building

longitudinal datasets specifically powered and controlled for use in developing epigenetic clocks is a more time- and cost-intensive approach, but it is critical for advancing our understanding of the relationship between DNA methylation, cellular aging, and mortality risk, which in turn is necessary for the creation of reliable epigenetic clocks that offer meaningful insights on the efficacy of geroprotective interventions and on expected lifespan and healthspan.

The bottom line

We all want to know the answer to the unknowable question, “How much time do I have left in my life?” As tempting as it might be to rely on an epigenetic “clock” for this answer, we simply don’t have nearly enough data yet for a “biological age” to have any meaning in terms of our overall lifespan and healthspan. In response to a predicted DNA methylation age four years greater than his chronological age, Steve Horvath himself said, “I will not jump to the conclusion that I will live four years less. Because humans are so complicated, and things can change.”

If an advanced “biological age” correctly predicts early mortality, we still need novel anti-aging interventions that would actively change or stall tissue aging and thus the DNA methylation prediction of age and mortality. Until that happens, there are no actionable takeaways outside conventional health advice: get sufficient sleep, don’t smoke, exercise, and eat a healthy, energy-balanced diet. While epigenetic clocks might someday be a vital tool for the field of aging science, as of now, they offer zero insight into either future lifespan or as a biomarker for geroprotective interventions. Instead, they are better suited as a research tool for continuing to develop better iterations of themselves and for investigating novel anti-aging therapies. But even in this capacity, we must exercise caution and remain aware of the significant noise that may mask the true signal from these tests.

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