



Review

Aging biomarkers and the brain

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ABSTRACT

Quantifying biological aging is critical for understanding why aging is the primary driver of morbidity and mortality and for assessing novel therapies to counter pathological aging. In the past decade, many biomarkers relevant to brain aging have been developed using various data types and modeling techniques. Aging involves numerous interconnected processes, and thus many complementary biomarkers are needed, each capturing a different slice of aging biology. Here we present a hierarchical framework highlighting how these biomarkers are related to each other and the underlying biological processes. We review those measures most studied in the context of brain aging: epigenetic clocks, proteomic clocks, and neuroimaging age predictors. Many studies have linked these biomarkers to cognition, mental health, brain structure, and pathology during aging. We also delve into the challenges and complexities in interpreting these biomarkers and suggest areas for further innovation. Ultimately, a robust mechanistic understanding of these biomarkers will be needed to effectively intervene in the aging process to prevent and treat age-related disease.

1. Introduction

Across diverse organ systems—from cardiovascular to neurological—the single biggest driver of pathology is aging. The likelihood of developing a disease like sporadic Alzheimer's Disease (AD) in early or midlife is infinitesimal. However, this risk grows exponentially with aging, with AD incidence doubling every five years after age 65. Decades of research in aging biology indicate the link between aging and disease is not merely correlative. Instead, the molecular and cellular changes that occur over an organism's lifespan are causal contributors to the etiology of the diverse conditions that occur as individuals grow older. The Geroscience paradigm postulates that our ability to develop interventions to slow, modify, or reverse these changes will drastically reduce disease burden across the board, including neurodegeneration, stroke, cancer, heart disease, diabetes, osteoporosis, and susceptibility to infectious diseases. But before we can successfully intervene, we need the ability to define and measure biological aging.

Traditionally, chronological age has been used as a proxy to estimate the degree of changes our bodies have incurred throughout our lives. However, chronological age is an imperfect measure of biological aging for two main reasons. First, individuals vary in their rates and manners of molecular, cellular, and phenotypic change over time. People have

diverse genetic susceptibilities and different environmental risk exposures throughout their lives that influence aging, such as stress, socio-economic factors, smoking, alcohol, and diet. Some individuals will develop age-related conditions before age 60, while others may celebrate their 100th birthday free from disease and disability. Second, chronological age is not modifiable. Studies in animal models have demonstrated that the rate of aging can be intentionally accelerated or slowed, which is a critical consideration when it comes to disease prevention and health promotion. By quantifying and monitoring biological processes that change with age, we can develop novel strategies to target them and extend healthy life expectancy.

Numerous groups in the aging field have spent the past few decades attempting to model age-related changes and produce valid and reliable estimates of “biological age.” These biomarkers will be critical for (1) informing our understanding of basic mechanisms of aging; (2) facilitating secondary and tertiary interventions by identifying at-risk groups; and (3) providing clinical endpoints within a short follow-up for assessing therapeutic and behavioral interventions aimed at slowing the aging process.

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2. Aging and the brain

A long life spent in good health offers the opportunity to further cultivate relationships with friends and family, use accumulated wisdom to care for grandchildren and lead communities, and otherwise engage in meaningful work, hobbies, and other activities. It is worth stating the obvious: that all this requires a working brain and mind, with all of the associated complex functions: consciousness, personality, emotions,

abstract thinking, planning, memory, knowledge, sociality, language, and sensorimotor abilities. Pathological aging may threaten these abilities by predisposing to neurodegenerative diseases and other disorders leading to cognitive impairment [1]. Late-life depression or psychosis can be manifestations of biological aging processes and contribute to cognitive deficits, though psychiatric disorders are less common in older adults than younger adults [2,3]. While some circumvent these complications as they grow old, a certain degree of cognitive decline remains

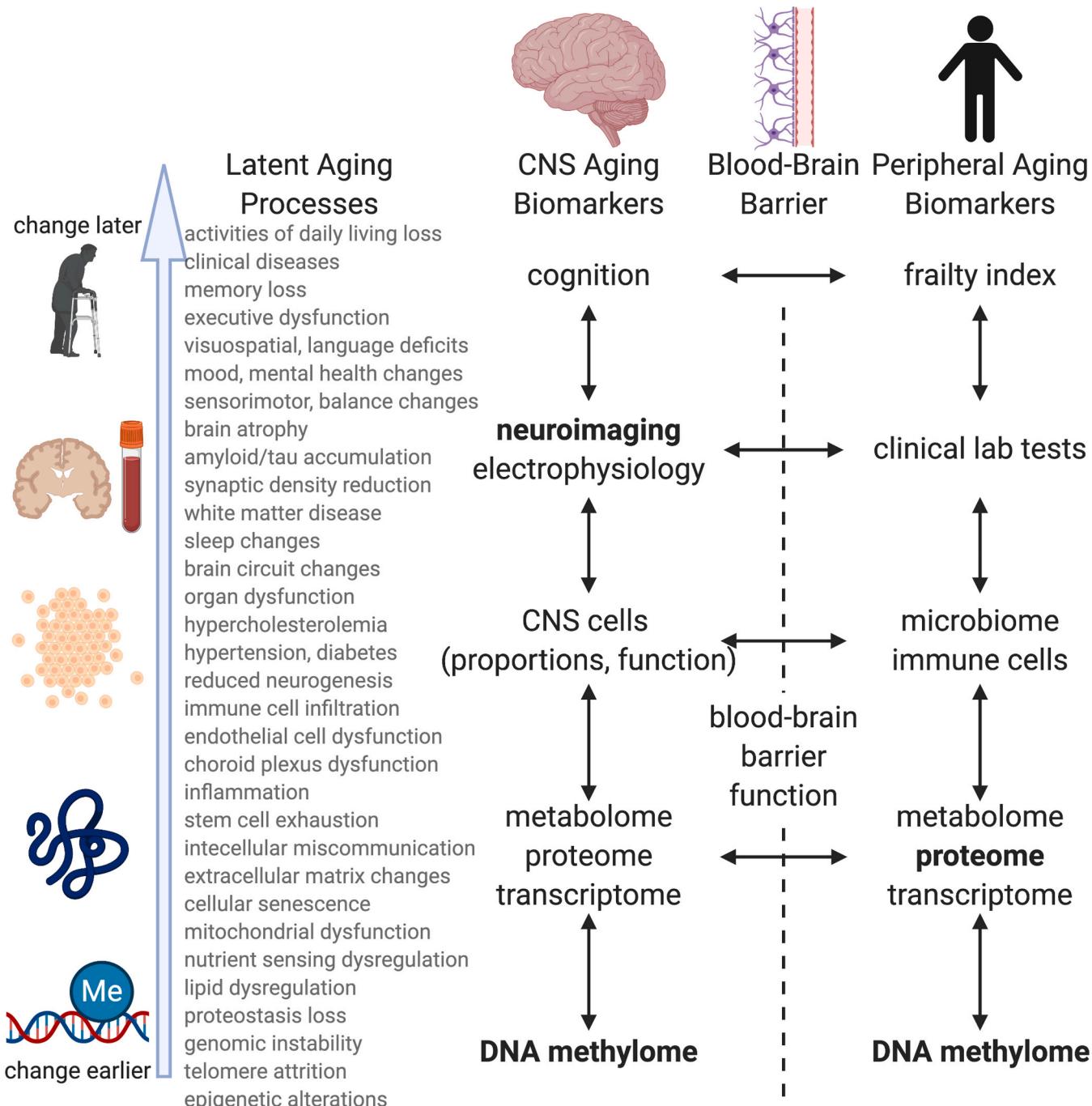


Fig. 1. A hierarchical framework for aging biomarkers. Aging involves a series of changes that propagate upwards across molecular, cellular, physiological, and functional levels. These latent aging processes can be detected using data on observable variables collected at each of these levels and modeled using aging biomarkers. Many types of variables are needed due to the vast and encompassing effects of aging. Brain aging can be captured by data from both the CNS and periphery due to interactions between organ systems, regulated by the blood-brain barrier. We list here the types of observable variables that have been used to create aging biomarkers that are relevant to the brain, but this list is non-exhaustive and will expand in coming years. The bolded variables are those that we discuss in detail, selected because they are the best-studied with respect to the brain. These biomarkers are highly interconnected and can capture overlapping sets of latent aging processes. Created with BioRender.com.

inevitable. Thus, identifying and targeting pathological aspects of aging to preserve brain function is of the utmost importance for biomedical research and society in general.

But where should we start in our quest to conceptualize and quantify biological age, and how do we understand aging's impact on an organ as complex as the brain? Aging has pervasive effects on the organism, changing numerous molecular, cellular, physiological, and functional characteristics. Because so many biological processes change with age, there is little agreement in the field about what types of variables to use for biomarkers. Thus, researchers have developed aging biomarkers from a wide variety of data types, such as DNA methylation, the microbiome, brain imaging, and even facial photos.

We have organized existing aging biomarkers in a framework to highlight how they are interconnected in the larger context of aging and brain function (Fig. 1). Age-related changes can be considered according to a hierarchy of biological organization that explains the temporal sequence of such changes [4]. Early in aging, even during development, changes are detectable at the lowest level of biological organization—the molecule. Yet, due to redundancy and homeostatic mechanisms, these changes are typically tolerated for an extended period. However, once molecular dysfunction reaches a given threshold, it can overwhelm the system and manifest as changes observed at the next highest level—cells and tissues. Again, these changes initially have a minimal overall impact, but over time they will emerge as dysregulation in the physiology of organ systems. Organismal function may not be appreciably affected up to this point. However, function may decline dramatically after physiological resilience factors are exhausted. Of course, higher-order changes can also affect the lower levels due to compensatory mechanisms or system feedback. Nevertheless, the overall pattern trends toward an upward propagation of aging.

At each of these levels, different biological processes change with age. We term these “latent aging processes,” detectable via their effects on a range of observable variables. We cannot directly observe the entirety of these age-related changes and instead can only approximate them. Yet, we can use these measured effects to create aging biomarkers (Section 3). At the molecular and cellular levels, latent aging processes include the famous “hallmarks of aging” or “pillars of aging” [5,6]. These are intertwined phenomena that include epigenetic alterations, genome instability, mitochondrial dysfunction, loss of proteostasis, stem cell exhaustion, cellular senescence, and inflammation. Not only do these processes change with age, but they can also exacerbate or ameliorate a wide range of age-related pathologies when manipulated. Thus, they play causal roles in aging and disease. These aging hallmarks have also been implicated in brain aging and neurodegenerative diseases [7]. Next, at the physiological level are aging processes that can be detected by clinical tests, from high blood pressure to the accumulation of amyloid and tau in the brain. Finally, at the functional level, aging affects cognition, mental health, and activities of daily living. Different sets of biomarkers capture aging processes at each of these different levels.

Ultimately, the level at which we choose to define biological aging will depend on the scientific question at hand. Based on this hierarchy, molecular and cellular changes could be considered more causal or mechanistic drivers of aging, with effects that lead to physiological and functional changes. Thus, if the goal is to understand the root causes or intervene in the aging process, molecular estimates of biological aging may be advantageous. However, when predicting the consequences of aging (disease, frailty), aging measures at the physiological or functional level may be more informative as they are directly proximal to the outcome of interest.

In addition to this vertical hierarchical structure, we must consider the horizontal relationships within levels, such as feedback between the brain and peripheral tissues. Directly studying the aging brain is often challenging, as access to human brain tissue is mostly restricted to post-mortem and rare neurosurgical samples. Not only does this preclude longitudinal studies and introduce artifacts, but it also results in biases

in the data related to mortality (e.g. dementia, suicide, substance use). Thus, we often must infer the state of the living brain by multiple indirect measures, including biomarkers from blood, CSF, or other accessible tissues. In particular, proteins, metabolites, and cells can enter and exit the CNS through highly regulated routes such as the blood-brain barrier. The CNS and periphery can also interact at the physiological and functional level; for example, peripheral pain can inhibit sleep and therefore cognition. Furthermore, because the brain controls behavior, it can indirectly impact aging in other systems by altering individuals' exposure to environmental and lifestyle factors.

The interconnectedness of aging across and within biological levels presents an opportunity and a problem for the biomarker field. There is no shortage of informative biomarkers that can be constructed to capture various aspects of aging, and these have taught us much about the brain. However, integrating insights from these biomarkers into a complete picture of aging will be challenging.

3. Principles of developing and interpreting aging biomarkers

Here we briefly review how aging biomarkers are developed and discuss the major implications for their interpretation. Ideally, an aging biomarker should reflect the fact that aging impacts numerous biological pathways simultaneously. Single measured variables cannot capture these systems-level phenomena and are subject to idiosyncratic variation unrelated to aging. Thus, composite measures that integrate many diverse variables are preferred. Aging biomarkers should also reflect the heterogeneity of aging, i.e. aging proceeds at different rates in different individuals and even within the same individual in different tissues and biological pathways. It is often useful (though not always necessary) to derive a “biological age” marker for determining the age best reflected by a given person's biological profile. For example, a person with a chronological age of 50 may have the biology (based on the parameters used) of the average 40-year-old in the population, and thus their biological age is said to be 40. Since aging involves many processes that may not change in concert with one another within an individual, it is often useful to calculate many different biological ages that each capture a unique aging signal.

Biomarker construction requires a set of observable biological variables (Fig. 2), ranging from clinical laboratory tests (multi-system; often on the order of 10^1 to 10^2 variables) to individual genes or DNA methylation sites (usually on the order of 10^5 to 10^7 variables). Some subset of these variables contains information about latent aging processes (Fig. 1), but *a priori* we often do not know which ones. After selecting which types of measures to consider in the biological aging score, we apply machine learning (ML) or other computational methods to arrive at a final equation for biological age. This can be carried out in one of two ways; either via a supervised (or prediction) approach, in which an outcome is selected with the goal of predicting it, or via an unsupervised method, in which patterns are quantified without tethering them to a specific outcome variable.

For aging biomarkers, the supervised approach is utilized more often. For this, we begin with a training dataset with many samples. For each sample, we have measured all of our observable biological variables as well as some outcome variable—typically either chronological age or an age-related phenotype like mortality risk (time-to-death). Various computational algorithms can then create composite predictors of the outcome. These algorithms must overcome two significant problems. The first issue is multicollinearity, where many variables are correlated with each other, possibly due to effects from a latent aging process. The second is overfitting, which is a major risk when there are many more variables than samples. Overfitting can result in an overly complicated model that primarily explains idiosyncrasies in the training data and does not generalize to other data sets. Many popular ML techniques such as elastic net regression will only include a sparse subset of variables in the final prediction model, selected after extensive cross-validation, to ameliorate these issues. Latent aging processes related to

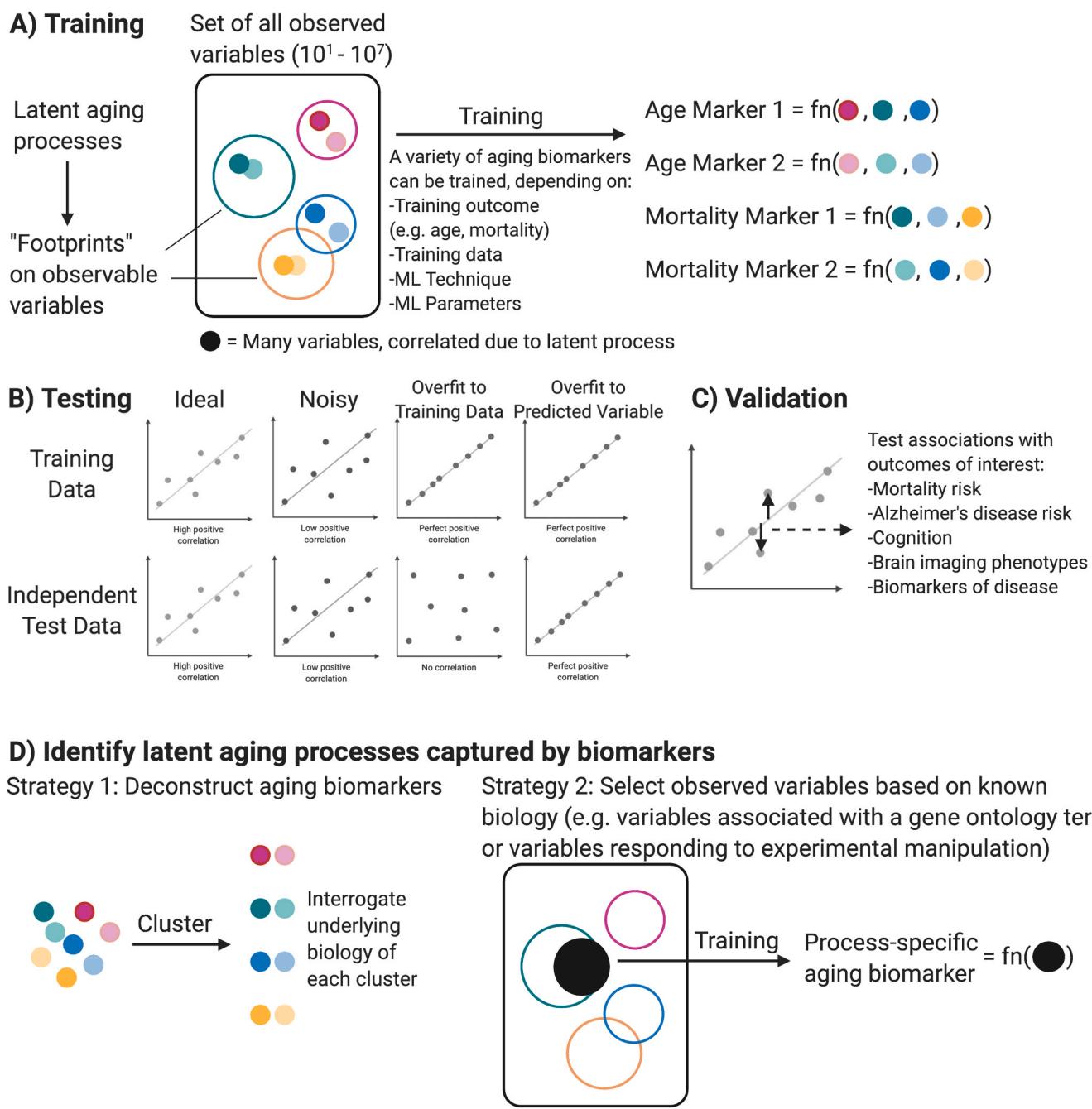


Fig. 2. Framework for developing and interpreting aging biomarkers. See Section 3 text for details. Many different aging biomarkers can be trained that may incorporate completely different sets of variables in their models, but actually capture overlapping aging signals. Testing and validation are critical to ensure the models generalize to other datasets and capture meaningful information. Studying the underlying latent aging processes may lead to a mechanistic understanding of these biomarkers. ML = Machine learning; fn = function. Created with BioRender.com.

the outcome may be captured by these final variables, though not necessarily. Models incorporating different variables in their final equations might be associated with the same latent aging processes due to redundancy. Because one is using a measurable outcome to train the biological age, selecting an informative outcome is critical as this will influence the validity of the final aging measure.

We then apply the model to independent datasets to test whether it generalizes beyond the training data (Fig. 2B). For example, if we were training a chronological age predictor, we would use the resulting equation and then plot known chronological age against the model's predicted age in both the training and test dataset. Ideally, the correlation within the test sample should not be substantially different from

that of the training sample. If the model is poorly fit, it may contain a lot of noise and cannot meaningfully capture information relevant to the predicted trait. If there is a good fit in the training data set but a poor fit in the test data set, the model was likely overfit to the training data (akin to type I errors). On the other hand, very tightly fit models in both training and test (e.g. $R^2 = 1$) are also not helpful, as they are effectively overfit to the predicted variable. This is especially true when using chronological age as an outcome variable. Even though chronological age is used during biomarker construction, our actual goal is to estimate biological age, so we would want to capture variability in biological aging among individuals of the same chronological age. However, DNA methylation- and neuroimaging-based age predictors with near-perfect

predictions of age often show greatly attenuated prediction of mortality or other aging outcomes [8,9]. Thus, models should not be solely judged by their accuracy (e.g. mean absolute error or age correlation) but instead by their associations with relevant aging phenotypes (Fig. 2C).

Because an age prediction model represents the normative or average aging trajectory, we can calculate deviations from this trajectory for every sample. The simplest method is subtracting chronological age from biological age, but this can generate biased estimates if the association between chronological age and biological age is not perfectly linear with slope = 1. Thus, a better method is by estimating the residual of biological age regressed upon chronological age. Positive and negative residuals (or deviations) are taken to represent age acceleration or deceleration, respectively. Indeed, age acceleration—using various popular age predictors—is linked to risk factors for pathological aging (e.g. smoking, obesity) and future risk of age-related mortality and morbidity (including cognitive aging or neurodegenerative disease) [10].

Notably, this biomarker construction process does not reveal the biomarkers' underlying mechanisms or imply any causal roles in aging. In the sections ahead, we will review the major aging biomarkers and discuss their relevance to brain aging and disease, as well as efforts to explore the latent aging processes for these biomarkers (Fig. 2D).

4. Epigenetic clocks

Epigenetic clocks are among the best-studied aging biomarkers. They are thought to capture molecular aging that precedes physiological and phenotypic changes. Epigenetic clocks estimate a sample's biological age based on DNA methylation (DNAm) at specific sites across the genome. In mammals, DNAm typically refers to the addition of a methyl group (CH_3) to a CpG dinucleotide (5'-C-phosphate-G-3'). Often, increases in DNAm within a region, particularly at gene promoters, are associated with transcriptional repression via its effect on chromatin organization. DNAm, along with other epigenetic modifications, controls various critical cellular processes, including differentiation, replication, X-chromosome inactivation, stress response, and genomic imprinting. More than thirty years ago, researchers reported that the levels of DNAm changed dramatically with age. This included a gain of DNAm at gene promoters and loss of global DNAm, representing trends towards hypomethylation in intergenic regions associated with dispersed retrotransposons, heterochromatic DNA repeats, and endogenous retroviral elements [11]. The underlying mechanisms of these age-related methylation changes remain unclear and are likely heterogeneous [12]. Nevertheless, given the predictability of these age-related changes, coupled with the influx of publicly available, large-scale DNAm data, DNAm represented an ideal resource for generating molecular age predictors.

In 2011 a scientist at UCLA published the first DNAm biological age measure (or epigenetic clock) using data from the saliva of twins [13]. Through supervised machine learning, the authors generated an age predictor that accounted for over 80% of the variance in chronological age and produced age estimates with an average absolute difference from the observed age of just over 5 years. Over the years, dozens more epigenetic clocks have been developed. These include the “Hannum clock” (for the first author, Gregory Hannum) [14], which was trained using blood DNAm and predicts age based on 71 CpGs, as well as the famous “Horvath Clock,” for which Steve Horvath incorporated data from 51 different tissues and cell types to produce one of the most accurate human age predictors based on 353 CpGs [15].

These first-generation clocks were trained to predict chronological age, but chronological time is an imperfect proxy for the heterogeneity of biological aging. Thus, researchers more recently switched to developing clocks to predict aging correlates. The first such clock, commonly referred to as either DNAmPhenoAge or the Levine clock, was published in 2018 by Levine et al. It was trained to predict a variable called “phenotypic age,” which was derived from clinical chemistry measures

(e.g. glucose, albumin, CRP) [16]. Based on 513 CpGs, the Levine clock strongly correlated with chronological age but was also optimized to capture physiological differences among persons of the same age. Using a similar line of thinking, Lu et al. developed the GrimAge clock in 2019 [17]. To do so, the team generated DNAm predictors of individual plasma proteins and smoking pack-years, and then combined these estimates with age and sex in a model aimed at predicting mortality risk. As such, GrimAge remains one of the best mortality predictors to date. However, it is also distinct from other clocks in that (1) its derivation requires chronological age and sex, and (2) it was only developed for blood samples. Finally, mitotic clocks have been designed to estimate the number of stem cell divisions in a tissue and can be used to predict cancer risk [18,19]. Although these various clocks share relatively few CpGs, they still capture overlapping aging signals for metabolism, immunity, autophagy, cellular senescence, and mitochondria [20]. These observations are consistent with the framework presented in Fig. 2 and Section 3, where many different observable variables may relate to the same latent aging processes.

4.1. Epigenetic clocks in blood

Due to the accessibility of blood DNA methylation data in large longitudinal cohorts, blood epigenetic age is one of the best-studied aging biomarkers, even in the context of brain aging. However, it is also the most indirect indicator of brain health that we will discuss, and therefore its interpretation is the most complicated. Blood epigenetic age can reflect DNAm changes within individual leukocyte cell types or changes in leukocyte population dynamics. It has been associated with a wide range of aging outcomes, including all-cause mortality, cardiovascular disease, cancer, diabetes, physical functioning, and cognitive decline [21]. It also appears to be modulated by a wide range of behavioral factors and environmental exposures [21,22]. Blood DNA methylation can even be used to directly predict smoking, alcohol, BMI, and even educational attainment [23]. Because the brain controls behavior, this adds complexity to the various studies that relate blood epigenetic age to neuropsychiatric traits, dementia, cognition, and brain structure.

Psychiatric disorders are illustrative of this complexity. Blood epigenetic age acceleration has been associated with stress, depression, schizophrenia, and alcohol use disorder [24–27], but this literature is complex and often seemingly conflicting. For example, epigenetics is hypothesized to be one mechanism through which stress drives aging through methylation of glucocorticoid or inflammatory pathway genes [24]. However, while some studies have reported associations between epigenetic age acceleration and specific stressors or PTSD, others have failed to replicate these associations [25,28–32]. This may point to heterogeneity in the stress response while also reflecting complex interactions between stress, behavior, psychosocial, and cultural factors. For example, different study populations may show differential associations between stress and smoking or alcohol, leading to inconsistent results between studies concerning epigenetic age acceleration.

Discrepancies may also reflect differences between epigenetic clocks, as many studies only use one clock and often not the same one. In PTSD, divergent results were found for different clocks [28,33]. In schizophrenia, we recently performed a comprehensive analysis of 14 epigenetic clocks in 3 independent cohorts [26]. We found schizophrenia was consistently associated with a large acceleration in clocks designed to predict mortality (e.g. GrimAge and PhenoAge) related to smoking and age-associated serum protein levels. Meanwhile, mitotic clocks that correlate with the number of cell divisions were decelerated in schizophrenia. These observations matched epidemiological studies showing greatly increased mortality albeit paradoxically reduced cancer risk in schizophrenia. This study and others [34] also report clocks trained to predict chronological age can be modulated by psychiatric medications used for schizophrenia and bipolar disorder. Because different covariates may influence different clocks, interrogating many age

biomarkers simultaneously can provide a framework for understanding why specific biomarkers are associated with psychiatric disease or psychosocial stress, and may help reconcile different studies.

It is likely these complexities also apply to blood DNAm's relationship with age-related brain disorders and phenotypes. Blood epigenetic age is increased in Alzheimer's disease, stroke, Parkinson's disease, frontotemporal dementia, amyotrophic lateral sclerosis, Huntington's disease, and multiple sclerosis [16,35–39]. Increased blood epigenetic age is also related to decreased brain volume (both white and gray matter), reduced white matter integrity, increased white matter hyperintensities, increased vascular lesions, and cortical thinning [40–42]. It is inversely associated with general cognitive ability, various measures of fluid intelligence, crystallized intelligence, and reaction time [41, 43–45].

However, interpreting these associations can be complicated, as the mechanistic relationships between blood epigenetic age and brain phenotypes are often unclear. On the one hand, covariates like smoking or cardiometabolic factors that affect blood DNAm are etiologically informative as risk factors for future age-related brain disease. Indeed, epigenetic age is associated with AD risk factors [46]. Some studies have found blood epigenetic age predicts future cognitive decline, dementia, or stroke [47–49], though others have not [43,50]. However, reverse causation is also possible. Age-related diseases could cause behavioral or other environmental changes that lead to observed cross-sectional associations with epigenetic age. For example, dementia is often accompanied by various neuropsychiatric symptoms, including eating abnormalities, sleep disturbance, irritability, anxiety, and hallucinations, and these symptoms are often treated with medications [51]. Physical activity, socialization, self-care, and engagement with community and home activities also decline [52]. Any of these factors could significantly alter blood epigenetic age, even within a short time frame.

Thus, the associations between blood DNAm and neuropsychiatric traits are complex and indirect, and caution is warranted when interpreting them. Studies with more detailed behavioral and phenotypic characterization are needed, ideally with designs that allow for longitudinal analysis and causal inference.

4.2. Epigenetic clocks in the brain

Applying epigenetic clocks to brain tissue can provide a direct molecular measure of brain aging and may be useful for dissecting the mechanisms of brain aging and age-related disease. However, as discussed in Section 2, generating and evaluating biomarkers in brain tissue is hampered by tissue accessibility—postmortem and neurosurgical sampling can introduce artifacts, mortality bias, and limitations like lack of longitudinal data or smaller sample size. Despite these obstacles and the much smaller number of studies examining epigenetic age in the brain, there is still much to learn from brain epigenetic clocks.

The majority of epigenetic aging clocks were developed solely in blood, with some still predicting age in a wide variety of tissues quite well [20]. However, the salient exception is brain tissue, where clocks often underpredict age both in terms of absolute age and the rate of increase in biological age [20,53]. This underprediction is likely multifactorial. Given that epigenetic aging includes mitotic signals [18, 19], it is plausible that this is partly related to the fact that neurons are post-mitotic. Neurons and glia appear to have markedly divergent epigenetic signals with respect to aging and Alzheimer's disease [54]. Consistent with this, the epigenetic clock measures the cerebellum as particularly young [55,56], and the cerebellum contains a far larger proportion of neurons compared to other brain regions [57]. As this underprediction could lead to misleading associations between epigenetic age and other factors, a recent clock was developed using only cortical tissue across a wide range of ages that improves the accuracy of brain age prediction in the human cortex [53]. This clock's age residual is highly negatively correlated with neuronal proportions, capturing neuron attrition with age. Also, mouse hippocampus and cortex-specific

clocks have been developed that are accelerated in transgenic AD mouse models [58]. Moving forward, it will be important to determine to what extent differences between blood- and brain-trained clocks are attributable to quantitative calibration to a specific tissue, or if they reflect different epigenetic aging processes.

With those caveats in mind, studies have reported associations between brain epigenetic age and disease. The majority utilized Horvath's "pan-tissue" clock, which included some brain samples among its various training tissues [15]. Pre-mortem HIV-associated neurocognitive disorders demonstrate accelerated epigenetic age over HIV-positive neuropsychiatric controls [59]. When applied to brain tissue, epigenetic age acceleration is also associated with bipolar disorder [60] and Alzheimer's disease [16,61]. Epigenetic age acceleration has also been reported in Huntington's disease (HD) in patients with mild to moderate cognitive impacts [62]. However, the same study found epigenetic age *deceleration* in patients with severe HD. Deceleration has also been reported in postmortem brain tissue of heroin users [63]. Rather than truly reflecting slower aging, these effects may instead stem from the impact of heterogeneous cell proportions (particularly of neurons) on the epigenetic age measures.

Specific CpGs within the epigenetic clocks also provide important insights into brain aging. For instance, **ELOVL2** is included in multiple clocks [20], and ELOVL2 hypermethylation is itself a strong predictor of age across tissues and cell types, including both neurons and glia [54, 64–66]. ELOVL2 is an elongase critical for the synthesis of long-chain polyunsaturated fatty acids, especially docosahexaenoic acid (DHA), the primary omega-3 fatty acid in the brain. DHA plays essential roles in neuronal membranes, signal transduction, neuroplasticity, neuronal survival, and protection from neuroinflammation, and reductions in ELOVL2 expression contribute to CNS aging phenotypes in mice [67–69]. Some evidence suggests **DHA** is reduced in dementia and that supplementation is beneficial, but more studies are needed [67]. Somatostatin (**SST**) is another significant protein in nervous system aging. During brain aging, the SST promoter becomes hypermethylated while gene expression decreases [70,71]. In the Hannum clock, 2 CpGs are found within the SST gene body [14]. SST serves as a neuropeptide expressed in a class of GABAergic inhibitory neurons distributed throughout the neocortex and hippocampus which play essential roles in fine-tuning firing patterns in cortical networks [72]. Decreased **SST** expression or decreased **SST+** neuron populations contribute to cognitive dysfunction in aging, dementia, and neuropsychiatric disorders [73]. SST promotes the degradation of A β -42 [74], and SST expression is reduced in individuals with AD [73,75] or the APOE ϵ 4 allele [76].

Many CpG sites that become hypermethylated with age, especially in mitotic clocks, are located in or near binding sites for Polycomb repressor complex-2 (PRC2) [18,19,77]. PRC2 generally modulates chromatin by methylating H3K27 and regulates cell identity, growth, and tissue homeostasis. In the brain, PRC2 is involved in neuronal identity maintenance, neural stem cell differentiation, and microglial activity [78,79]. Loss of brain PRC2 can cause de-repression of genes in neurons and glia and contribute to neurodegenerative processes in mice [78,80]. In humans, genomic regions differentially methylated in Alzheimer's disease are enriched for PRC2 binding sites [81]. Overall, the epigenetic clocks capture at least some biological processes that are functionally relevant to brain aging.

We note current age predictors might omit significant epigenetic aging processes in the brain due to the distinct methylation landscape compared to other tissues. CpG hydroxymethylation, which has unique functions compared to CpG methylation, is particularly enriched in the brain, may show unique aging patterns in the brain, and is altered in AD [82–85]. CpH (non-CpG) methylation also has distinct functions, is enriched in neurons, decreases during aging and AD, and can be used to predict age in neurons [86–88]. These epigenetic marks are not captured by existing human methylation data primarily obtained through Illumina array-based technology. It will be interesting to determine if aging biomarkers based on CpG hydroxymethylation or CpH methylation will

be informative for unique aspects of brain aging.

5. Proteomic clocks

Recently, human proteomic clocks analogous to epigenetic clocks have been developed [89–93]. Many proteins change with age in various tissues, summarized by a systematic review identifying 1128 age-related proteins [93]. Proteomics technologies, such as aptamer-based SOMAscan or mass spectrometry methods, have matured sufficiently to enable the accurate and reliable measurement of thousands of diverse proteins across a large dynamic range, with reduced interference between proteins [91,94]. While CSF or CNS proteins could be used as brain aging biomarkers [95,96], these are limited by tissue accessibility. Thus, the majority of aging biomarker research using proteins has focused on plasma proteomics.

Like epigenetic clocks, proteomic clocks are built by applying machine learning techniques to predict age or aging phenotypes using a composite score of many proteins. A study of 240 healthy adults (22–93 years old), without any diseases or functional impairments, found chronological age could be accurately predicted from 76 plasma proteins measured by SOMAscan [90]. Another large study of 4263 adults (18–95 years old) found that nearly half of the approximately 3000 measured plasma proteins changed with age [91] and that a robust age predictor could be estimated based on 373 proteins. Furthermore, accelerated proteomic aging (higher predicted age compared to chronological age) was associated with reduced executive function, processing speed, visuospatial abilities, and physical function. In both of these studies, the clocks could be restricted to as few as 9 proteins without substantial loss of predictive power for age (Spearman's correlation remained above 0.9). However, different models showed varying associations with clinical and functional parameters. It is unlikely any single linear model is ideal for capturing biological age for all purposes. Accordingly, a variety of clocks can be built from subsets of proteins annotated as reflecting distinct pathways [92], using the strategy in Fig. 2D. In the future, it will be important to determine if these clocks are useful for different purposes.

Though currently more expensive and less studied than epigenetic clocks, proteomic clocks have several potential advantages. Plasma proteins have many different tissues of origin and could be used to monitor aging in various tissues simultaneously and determine how aging in one organ affects another [97]. Protein activity can be more directly modified by pharmacologically targeting or supplementing specific proteins, which is important for assessing causation. Any given age-related change may serve pathological, incidental, or compensatory roles in the aging process, and it is possible for proteins that increase with age to protect against pathology and for proteins that decrease with age to exacerbate aging. Many human protein-age associations are also conserved in mice [91], and mechanistic studies reveal many proteins that correlate with age in humans can extend lifespan or attenuate cognitive decline when manipulated in various model organisms [92]. Proteomics is also more interpretable; specific proteins can be linked to biological processes more easily than specific CpGs, considering the myriad and often indirect ways that methylation influences gene expression. Age-associated proteins are enriched in functions related to the immune system, coagulation, intercellular signaling, extracellular matrix, angiogenesis, stress responses, cellular senescence, and axon guidance [90,91,93].

Changes in the plasma proteome with age due to altered peripheral tissue activity contribute to brain aging. This has been demonstrated by various studies exposing young animals to aged blood factors and vice versa via heterochronic parabiosis (aged and young animals surgically connected to share a circulatory system) or plasma transfusion (without cells). Young blood factors can restore cognitive function, synaptic plasticity, and neurogenesis to aged animals, while aged blood has progeroid effects on young animals [98–100]. Transfusion or blockade of individual proteins can also partly recapitulate the effects of

heterochronic parabiosis [101,102], such as beta-2-microglobulin [103], VCAM-1 [104], or CCL11 [98]. Of note, many of these proteins influencing neurogenesis and cognition are immune system proteins. Immune system proteins are greatly enriched among plasma proteins that increase with age, are associated with mortality and frailty, and are especially strong at predicting age compared to other subsets of proteins [90,92,105,106]. This fits with findings suggesting that reshaping of the peripheral immune system with aging may modulate brain aging, Alzheimer's progression, and cognitive decline [107].

Interestingly, the plasma proteome shows significant non-linear changes with age, with "aging waves" centered around ages 34, 60, and 78 [91]. Cognition and Alzheimer's disease are associated with proteins that change in the latter two waves. For example, a cluster of 60 proteins enriched for ephrin signaling and axon guidance functions show no change until about age 60, followed by exponential growth. Ephrins and ephrin receptors are expressed throughout the body and engage in bidirectional signaling via cell-cell contact to regulate growth and cell migration. In the brain, the attractive and repulsive effects of these proteins play critical roles at synapses to regulate axon guidance, synaptic plasticity, neurogenesis, and memory formation [108]. Ephrin receptors are reduced in Alzheimer's disease, disrupting these processes, and may also play a role in neurotoxicity induced by amyloid-beta oligomers [109]. Also, pleiotrophin, one of the proteins most associated with age, increases in serum primarily after age 50 [90,91], though it decreases with age in CSF [95]. Pleiotrophin is named after its diverse pleiotropic roles in functions such as cell growth, migration, and survival in brain, bone, the immune system, and other tissues [89,110,111]. Thus, it likely plays complex, tissue-specific roles in aging, and it could exacerbate pathology or serve as a compensatory response to aging. Pleiotrophin is made by pericytes at the blood-brain barrier and provides critical neurotrophic support to neurons. Loss of pleiotrophin is necessary for neurodegeneration following pericyte death, which occurs in various neurological disorders such as Alzheimer's [112]. The significance of plasma pleiotrophin and ephrin signaling proteins in late-life brain aging should be explored further.

The plasma proteome can also reflect brain outputs during aging or neurodegeneration, such as changes in neuroendocrine hormones that occur with hypothalamic aging [113]. Peripheral biomarkers of CNS pathology include neurofilament released in response to neuronal damage, or peripheral amyloid-beta and tau isoforms as specific biomarkers of Alzheimer's [114–117]. In mice, the astrocytic marker GFAP increases with age in plasma in a trajectory that is well-correlated with brain Gfap mRNA expression [97].

One complicating factor for plasma proteomics and brain aging is that protein trafficking between blood and brain changes with age. Brain function appears to be directly influenced by some plasma proteins penetrating the brain parenchyma by crossing the blood-brain barrier (BBB) [118]. Labeled proteins injected into mice at physiological plasma concentrations can subsequently be detected widely throughout the brain, including in neurons, glia, and endothelium, though the thorough characterization of which specific proteins cross the BBB has not yet been reported. During aging, receptor-mediated transcytosis declines in endothelial cells while non-specific transport increases [118], and pericytes that maintain the BBB are lost [112]. The choroid plexus and the glymphatic system also change with age [119,120], altering protein trafficking through these pathways. If the loss or gain of specific plasma proteins entering and exiting the brain contributes to brain aging and disease independent of plasma levels, biomarkers of this process may complement plasma proteomics. For example, BBB integrity can be measured using neuroimaging using contrast dye that indicates BBB permeability or by CSF markers of pericyte injury [121–123]. Of note, both imaging and molecular data implicate APOE in BBB integrity, and the Alzheimer's risk allele APOE4 contributes to cognitive aging by contributing to BBB breakdown independently of amyloid and tau accumulation [118,122].

It would be useful to develop plasma proteomic biomarkers that

reflect various aspects of brain aging or neurodegenerative risk, rather than predicting chronological age. Proteomics has already been used to predict smoking, alcohol consumption, and metabolic health risks [124]. Proteins that change with age in mid-to-late life are associated with executive function and Alzheimer's disease [91]. Some age-related proteins are also associated with dementia status, future cognitive decline in cognitively normal individuals, ApoE genotype, amyloid burden, brain volume, and brain atrophy rate [91,125,126]. In machine learning models, serum proteins can modestly enhance the prediction of some Alzheimer's-related traits when added to clinical covariates [127, 128]. It will be interesting to train different proteomic clocks to directly predict brain aging phenotypes such as atrophy, amyloid accumulation, microglial activation, cerebrovascular disease, or cognition.

6. Neuroimaging age predictors

Brain imaging offers one of the most direct observations of the brain that can be performed longitudinally in living subjects. An extensive literature concerns imaging biomarkers of age-related brain disorders such as Alzheimer's [129], but we will focus on brain age metrics developed using the rich set of neuroimaging features [130]. A variety of imaging modalities can be used for age prediction, such as structural or functional magnetic resonance imaging (MRI) [131,132]. Like methylation and proteomic biomarkers, brain imaging age is associated with mortality risk, frailty, disease, and cognitive decline [133–135]. Brain imaging age predicts conversion from mild cognitive impairment to Alzheimer's disease better than neuropsychological testing, and individuals that progress to Alzheimer's show greater increases in brain imaging age in longitudinal follow-up [136].

It is important to note that neuroimaging age prediction is technically sophisticated, requiring several preprocessing steps that demand significant expertise, effort, and processing power [130]. Scanners and image acquisition protocols can introduce bias that is non-linear and difficult to correct. Spatial registration to a standard brain template is performed to make variables comparable between subjects. The features used to predict age may be defined in various ways, such as using individual voxels or segmenting the brain using different predefined brain parcellations or networks [137,138]. All of these factors introduce significant complexity and can limit the generalizability and utility of imaging-based biomarkers.

These issues can be partially overcome by using a large training set of images from diverse cohorts and scanners, then re-processing this data in a harmonized pipeline [9,134]. However, there is also great interest in deep learning models that incorporate non-linear relationships and can define features in a data-driven approach without significant pre-processing [9,139]. Bashyam and colleagues recently developed a tool called DeepBrainNet trained using 11,729 MRI scans (age range 3–95) from 16 cohorts acquired with different scanners and protocols [9]. This large and diverse dataset enables deep learning to predict age using minimally preprocessed imaging data. Notably, by incorporating labeled data from multiple brain diseases such as Alzheimer's, depression, and schizophrenia, DeepBrainNet can also serve as a classifier for these diseases at the same time as predicting age. The authors found that tightly-fitted imaging predictors of chronological age showed worse prediction of these other traits, presumably because the models were trained to ignore relevant variation between individuals of the same age, mirroring findings with epigenetic clocks [8].

Beyond technical details, imaging age predictors also differ in the aspects of brain aging that they capture. For example, it is possible to create region-specific measures of brain age. An extensive structural MRI study of 45,615 individuals from many different cohorts developed age prediction models using only occipital, frontal, temporal, parietal, cingulate, insula, or cerebellar-subcortical features [134]. Regional age acceleration was associated with different diseases and cognitive measures, as well as different genetic variants in GWAS analyses. Beyond regional differences, various features of the brain can be detected by

neuroimaging, such as brain atrophy, geometry of structures, white matter integrity, hemosiderin deposition, vascular disease, lesions, brain activation patterns, functional connectivity, or amyloid accumulation. Smith and colleagues aimed to explicitly define distinct aging processes in the brain using a rich set of 3913 features derived from six brain imaging modalities obtained for each of 21,407 participants [135]. They used independent component analysis to identify 62 modes of variation within this data set across all modalities simultaneously, as well as 6 clusters of these modes. Most modes were associated with age. Interestingly, each mode was primarily driven by single imaging modalities, though clusters were more mixed. Separate GWAS analyses showed that 57 of the 62 modes and all 6 clusters were heritable with a number of associated SNPs. In contrast, GWAS analyses for all-in-one brain age models incorporating either all features or all modes identified no genetic associations. The modes also varied widely in their phenotypic associations. For example, the cluster that showed the most substantial aging effect was defined by lateral ventricle volume and various features of the choroid plexus, fornix, corpus callosum, and thalamic nuclei, all notably drained by the same veins. This cluster was associated with smoking, alcohol, diabetes, and non-fat body size, as well as genes involved in angiogenesis and blood vessel stability. Intriguingly, it was also associated with processing speed, raising the possibility that this cluster is a neurological substrate linking vascular aging to processing speed. Thus, it appears more informative to calculate many different brain age values rather than a single number, especially to interrogate the underlying biological mechanisms and link these to other aging biomarkers.

7. Other biomarkers

There are numerous other biomarkers of aging and age-related phenotypes relevant to the brain, which we mention briefly here (Fig. 1). Telomere length was once a popular aging biomarker, but it has not been particularly predictive for mortality or other outcomes, and many confounders are challenging to account for [140]. Transcriptomics can be used to predict age, and transcriptomic brain age is increased in mental illness [141], but gene expression data tends to be noisy [142]. Serum or urine metabolomics can predict age, mortality, cardiovascular disease, metabolic syndrome, or cognitive decline risk [143–147]. Fatty acid metabolism is among the most dysregulated pathways for aging, mortality, and Alzheimer's disease [143,144,147]. The human gut microbiome can be used to construct an aging clock [148] and influences the CNS via signals through the enteric nervous system, blood metabolites, and immune system [149]. CNS and immune cell phenotypes such as cell type proportions change with age [70,150,151] and will likely be important for connecting molecular biomarkers to phenotypic and functional biomarkers. Electroencephalogram (EEG) signals can predict age, and a sleep EEG-based age measure predicts mortality [152]. Common clinical assessments of cognition (e.g. delayed recall, serial 7s) can be integrated into composite cognitive age measures that are accelerated by the ApoE4 allele and predict conversion to dementia [153]. Frailty indices count the number of health deficits (e.g. specific diseases, symptoms, physical exam findings, activities of daily living) and have been associated with various markers of Alzheimer's disease [154].

8. The future of aging biomarkers and the brain

Aging is not a single process and should not be treated as such; there is no single "best" aging biomarker. Thus, a plethora of diverse aging biomarkers has been produced in recent years due to the power of machine learning approaches applied to large aging cohorts. This naturally leads to the question: which biomarkers should be used, and when? Unfortunately, the answer is still unclear. While many of these biomarkers effectively prognosticate age-related morbidity and mortality, other basic questions remain about their nature and utility.

The complex hierarchy of aging biomarkers relevant to the brain (Fig. 1) makes it challenging to obtain a cohesive view of brain aging. These biomarkers likely capture overlapping latent aging processes, and there should be many regulatory and functional interactions between them. However, the majority of existing studies only examine one type of biomarker at a time. Thus, it is unclear if these measures are complementary or redundant. Even when multiple biomarker types are compared, the results are hard to interpret. For example, there is little correlation between metabolomic age and epigenetic age [145]. However, this does not rule out relationships between subsets of metabolites and DNA sites. Mapping the complex and dynamic connections of a multifactorial aging process will require enormous amounts of data, coupled with new and innovative computational techniques. This integration will be essential for the development of a rational framework to select variables from the wide variety of data types.

There is still much work to do to identify the underlying latent aging processes associated with each biomarker (Fig. 2D). This will likely be important for elucidating the mechanisms connecting aging to disease. For example, in Alzheimer's disease (AD), amyloid deposition followed by tau-mediated neuronal dysfunction is believed to begin decades prior to noticeable disturbances in memory or clinical function [129]. By selecting variables related to proteostasis, metabolism, or inflammation, or variables temporally associated with changes in amyloid or tau, we may be able to construct aging biomarkers for these stages of AD. Dissecting the molecular and cellular links between these biomarkers and AD pathology may yield novel insights into AD progression.

Understanding latent aging processes will also be critical in efforts to use aging biomarkers to monitor interventions that target pathological aging. For this application, “reducing biological age” is a tempting goal. However, not all age-related changes are maladaptive or causal. When faced with molecular and cellular changes that drive aging (e.g. the hallmarks of aging), an organism may employ compensatory mechanisms to maintain homeostasis or delay the onset of disease. Furthermore, aging may lead to observable changes that do not themselves lead to further alterations, acting as passengers, for example changes in hair color. Thus, aging biomarkers likely capture a mix of causative, compensatory, and incidental changes that represent “older” biological age, and reducing biological age could therefore have beneficial, harmful, or neutral effects on aging outcomes. Thus, future studies should focus on identifying the underlying mechanisms of aging biomarkers so we can anticipate the consequences of manipulating them.

We may also need to modify our assumptions about aging for future biomarker development. Most aging biomarkers are designed to capture a normative pattern of aging that applies throughout the entire population. However, individuals may age in qualitatively different manners involving different sets of variables. A striking example of this is seen in yeast, where individual cells are observed aging in one of two patterns: nucleolar aging or mitochondrial aging [155]. Inhibition between these processes results in two distinct phenotypes with very little overlap. Humans may also have such “ageotypes.” A multi-omics analysis of 106 individuals identified 4 general aging domains that change at repeated longitudinal follow-up (metabolic, liver, kidney, and immunity), and found some individuals only age in one or two of these pathways [156]. However, follow-up time was limited to 4 years or less, so it is possible those individuals would experience aging in all domains at some point in their lives, and it is unclear how distinct these aging trajectories might be. Nevertheless, it will be interesting to determine if certain ageotypes predispose to specific diseases like Alzheimer's, and if so, develop ageotype-specific biomarkers.

Finally, the brain is a highly dynamic, plastic organ, with many features that change with time as part of normal function, such as memories, skills, and adaptations to new psychosocial roles across the lifespan (e.g. parenthood). It is important to distinguish these from age-related changes that lead to disease and exercise caution about interventions potentially interfering with these functions. This raises a broader issue: the brain functions we aim to preserve are themselves

complex and our understanding of them is changing rapidly. Our very definitions and diagnostic schema of brain diseases are in flux, as exemplified by the Research Domain Criteria (RDoC) from the National Institute of Mental Health [157,158]. This evolving conceptualization will likely change how we connect aging biomarkers to the brain in the years to come.

Conflicts of Interest Statement

MEL is a Scientific Advisor for, and receives consulting fees from, Elysium Health. MEL also holds licenses for epigenetic clocks that she has developed. All other authors report no biomedical financial interests or potential conflicts of interest.

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