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Back to the future: epigenetic clock plasticity towards healthy aging

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Highlights

- The epigenetic clock DNA methylation signature has outperformed other biomarkers in predicting age
- Age associated DNA methylation drift is highly conserved across mammalian species
- Epigenetic clock acceleration promotes lifestyle diseases and mortality risk
- Epigenetic clock acceleration is associated with mitochondrial DNA copynumber but not with telomere length
- Lifestyle interventions are developed to extend healthy lifespan by slowing down the epigenetic clock progression

Abstract

Aging is the most important risk factor for major human lifestyle diseases, including cancer, neurological and cardiometabolic disorders. Due to the complex interplay between genetics, lifestyle and environmental factors, some individuals seem to age faster than others, whereas centenarians seem to have a slower aging process. Therefore, a biochemical biomarker reflecting the relative biological age would be helpful to predict an individual's health status and aging disease risk. Although it is already known for years that cumulative epigenetic changes occur upon aging, DNA methylation patterns were only recently used to construct an epigenetic clock predictor for biological age, which is a measure of how well your body functions compared to your chronological age. Moreover, the epigenetic DNA methylation clock signature is increasingly applied as a biomarker to estimate aging disease susceptibility and mortality risk. Finally, the epigenetic clock signature could be used as a lifestyle management tool to monitor healthy aging, to evaluate preventive interventions against chronic aging disorders and to extend healthy lifespan. Dissecting the mechanism of the epigenetic aging clock will yield valuable insights into the aging process and how it can be manipulated to improve health span.

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1. Introduction

Aging can be seen as an inevitable inherent biological program by which the maximal lifespan of each species of the animal kingdom is time restricted and to a large extent predetermined. Aging is characterized by the progressive loss of function at the molecular, cellular, tissue and organ level, eventually leading to death. Why all living species age and senesce, and how to extend lifespan are questions that have intrigued a lot of people and scientists throughout history. The risk of many human diseases, including cancer, cardiovascular diseases, neurodegenerative diseases and diabetes increases with age. Knowledge in the underlying mechanisms which drive species specific aging may therefore also improve our understanding about multiple age-related diseases. Furthermore, ways might be found to interfere with the aging process to extend healthy lifespan.

Recently, nine cellular and molecular hallmarks of aging were described [1]. These hallmarks are: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. It is clear that these hallmarks are highly interconnected and that further research needs to clarify the causal relationships between the hallmarks and the relative contributions of each hallmark to aging .

While every living organism senesces and eventually dies, the rate of aging can be variable. Individuals with the same chronological age may have different risk profiles for age-related diseases. In other words, the chronological age of two individuals may be the same, however there could be significant differences in their biological age. Studies in monozygotic twins have shed light on the relative contribution of genetic and non-genetic (shared and unique environmental) factors in aging [2, 3]. The heritability of longevity lies around 25%, suggesting an important role for environmental and lifestyle factors [4, 5]. The variability in rate of aging is therefore a consequence of both genetic, lifestyle, environmental and interaction factors.

A predictor in rate of aging or biological age would be helpful in determining an individual's health status and aging disease risk. Such a biomarker will also be a valuable tool to test therapeutic interventions that increase healthy lifespan. A well-known and studied "biological clock" is the progressive loss of telomere lengths during aging [6, 7]. Telomeres are repeat sequences at the chromosome ends. Every cell division and DNA replication round, the telomere shortens which eventually leads to cell cycle arrest and replicative senescence. Telomere length in blood was found to be a marker for chronological age, and interestingly, was also associated with diseases, risk factors and mortality [6-8]. However, conflicting results have been reported [6, 9, 10].

In the last years, DNA methylation has been put forward as a more accurate predictor for chronological age [11, 12]. DNA methylation is an epigenetic regulatory mark which affects phenotype without a change in genotype. DNA methylation refers to the biochemical process of adding a methyl group to a cytosine nucleotide leading to the formation of a 5-methylcytosine (5mC). In humans and most other animals, methylation is almost exclusively found on cytosine nucleotides followed by a guanine nucleotide (the so called CpG-sites).

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CpG sites are unevenly distributed across the genome. Most of the CpG sites are clustering together in regions of around 1 kb with a higher density of CpG sites compared to the rest of the genome. These regions are called CpG islands (CGIs) [13]. About 50% of the CGIs are located at promoter sites of known annotated genes. Furthermore, about 70% of annotated promoters possess a CGI. Based on studies in the 70'-80', a dogma emerged which associates DNA methylation predominantly with transcriptional repression [14]. Although, later studies based on genome-wide data confirmed this negative association in promoter regions, a more complex picture is currently emerging in which DNA methylation also plays roles in gene activation, alternative splicing, nucleosome positioning, and the recruitment of transcription factors [15]. The function of DNA methylation is therefore dependent of the context and genomic location [16, 17]. CGIs are most often unmethylated, even when the regulated gene is not expressed. Regions just outside these CGIs, which are called CpG shores (< 2kb flanking CpG Islands) and shelves (<2kb flanking outwards from a CpG shore), are more variable in DNA methylation and were found to have a stronger impact on gene expression [18]. An important aspect of DNA methylation is that changes are cumulative with age in response to various environmental stimuli [19-21]. Furthermore, aberrant epigenetic changes (epimutations) are stochastic and associated with multiple diseases and disease risk factors upon reaching specific thresholds [22]. As such, an individual's DNA methylome is a perfect track record of past environmental exposures and read-out to monitor health status to estimate future disease risk or life expectancy.

In this review, we will first summarize dynamics of DNA methylation during aging. Next, we will discuss the possibility to use DNA methylation as an "epigenetic clock" readout to estimate chronological and biological age. In addition, we will give an overview of studies linking epigenetic age acceleration with mortality, diseases and lifestyle factors. We will conclude with possible hypotheses and future directions.

2. DNA methylation dynamics during aging

Epigenetic drift

Although DNA methylation patterns are retained by maintenance DNA methyltransferases (DNMTs) every cell division, DNA methylation changes with age. For example, older individuals having more variable DNA methylation patterns compared to younger individuals [23]. The same was observed in mesenchymal stem cells and blood cells, which shows higher DNA methylation variability in older people [24]. However, methylation of some of the CpG sites were shown to converge during lifetime [24]. Similarly, in a small-scale longitudinal study of twins, intra-pair drift was seen in only a subset of the twins between birth and 18 months of age, while in other pairs no differences or even converging methylation patterns were observed [25]. In other monozygotic twin studies, discordance in methylation was found to be higher in older twins than in younger twins [23, 26]. This observation was observed in cross-sectional as well as longitudinal study designs [23, 26, 27].
Multiple studies have shown that aging is accompanied with a progressive loss of DNA methylation. However, in the first years of life this correlation has been less well studied. A longitudinal study from birth to three years of age showed a small but significant increase in global DNA methylation using an ELISA-based method [28]. Global methylation measured

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by pyrosequencing of LINE-1 and Alu repetitive elements in whole blood was found to be lower in 9 years old children in comparison to corresponding fetal cord blood [29]. However, the absolute difference was small (0.01 – 0.05% 5mC). Alu DNA methylation was also progressively decreasing in adults and elderly subjects [30-32]. In another study using monozygotic twins with ages ranging from 18 to 89, LINE-1 DNA methylation was slightly lower in old individuals [23]. In adults, global DNA methylation in leukocytes was decreased using an ELISA-based method [33]. Comparison of newborns and nonagenarians using a whole genome bisulfite sequencing and 450k Illumina methylation array approach, resulted in a similar loss of DNA methylation [34]. In contrast, using a LUMA approach, global DNA methylation changed over time, but didn't show a general decrease in global methylation, suggesting that outcomes could depend on the methodology [35]. Overall, different studies points to a progressive decrease in global DNA methylation. However, the effect size is rather small, especially compared to the large inter-individual variation. Furthermore, most of the studies focused on blood cells, and therefore this global hypomethylation may not be valid in other tissues.
The observation that the variation in DNA methylation increases apparently randomly with age indicates a stochastic process, which originally was called "epigenetic drift" [26, 36]. A possible explanation could be a decline in the maintenance of DNA methylation patterns over cell division [37]. Another reason is the multitude of environmental exposures which could trigger epigenetic changes. Especially prenatal and developmental stages are susceptible to DNA methylation changes by environmental stimuli. The developmental origin of health and disease paradigm (DOHaD), highlights the importance of both genetic, intrauterine environment and their interaction in establishing neonatal DNA methylomes which affect the risk of later pathophysiological processes associated with chronic, especially noncommunicable disease (NCD) [38-44]. These DNA methylation changes, by mitotic transmission, may persist in adulthood and increase the risk for age-associated diseases [45, 46]. During lifetime, twin studies showed that DNA methylation variation by age is mainly attributed by unique environmental factors [23, 47]. A recent study measured the contribution of genetic and environmental factors in the inter-individual variation of DNA methylation in a large sample of individuals from twin families [48]. They found a strong effect of environmental and stochastic influences on DNA methylation. Using interaction models, they demonstrated that environmental or stochastic influences, rather than genetic influences, were more important in explaining age-associated DNA methylation variation. In addition, the effect of stochastic and environmental influences increases with age, supporting the epigenetic drift model [48]. A similar conclusion could be drawn from a 10 year longitudinal study in elderly twins, where almost 90% of the longitudinal changes could be attributed to unique individual or stochastic environmental factors [49].

Overall, from these studies we can conclude that a complex interplay between genetic, environmental and stochastic influences results in an epigenetic drift during aging. In addition, the contribution of environmental and stochastic influences seem to increase with age.

Specific age-associated methylation changes

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With the rise of the Illumina methylation arrays (Infinium 27k, 450k and EPIC), an age-specific methylation pattern could be demonstrated, suggesting that epigenetic drift is not completely stochastic but seems to be restricted to specific sites in the genome. Age-associated hyper- and hypomethylated CpG sites could be consistently detected in different epigenome-wide association studies (EWAS), indicating that besides epigenetic drift due to environmental and stochastic factors, also more age-specific regulatory mechanisms could be involved. Hypermethylated CpG sites were mainly found in CpG islands [50-55], whereas hypomethylated sites were enriched in CpG poor regions just next to CpG islands (CpG shores/shelves) [50, 54, 55]. When looking to histone modifications and chromatin states, hypermethylated CpG sites were mostly located at promoters containing repressed (H3K27me3/H3K9me3) and bivalent (both H3K27me3 and H3K4me3) histone marks [24, 53]. In addition, hypermethylated CpG sites most often located in Polycomb group target genes [56]. In contrast, hypomethylated CpG sites were found to be enriched for the active histone mark H3K4me1 [24], which was also found to be associated with distal upstream regulatory regions (enhancers). In a study in human epidermis, methylation was found to erode with age, where strongly methylated sites tend to decrease methylation level,s whereas weakly methylated sites increase in methylation levels with age, suggesting that DNA methylation progresses towards intermediate (50%) methylation level with a reduced dynamic range [57].

Interestingly, methylation of most of the age-associated differentially methylated positions (DMPs) didn't correlate with transcriptional expression of the DMP-associated genes and did not induce an expression change [55, 58]. Furthermore, these genes were mainly lowly expressed [51, 53, 58], which is in agreement with the enrichment of repressive histone marks. In another study focusing on promoter regions, a negative correlation was found with gene expression [59]. Different studies showed an enrichment of these genes in developmental processes, morphology, transcriptional regulation and DNA binding [50-52, 55]. Especially genes associated with hypermethylated CpG sites were found to be enriched in biological pathways such as organismal growth & development, cellular growth and proliferation and development [55].

An interesting observation is that this age-specific methylation pattern is rather tissue-independent and a large fraction of age-associated DMPs are overlapping in multiple different tissues and cell types [51, 53, 60, 61], including stem cells [24]. Besides, tissue-specific age-associated DNA methylation changes have also been reported [24, 54, 60]. Tissue-specific CpG sites were mostly hypomethylated with age, not located in CGI and enriched for expressed genes, while the opposite was seen for common CpG sites across tissues [60]. The observation that age-associated hypermethylated DMPs were mainly located in lowly expressed genes and hypomethylated DMPs in highly expressed genes was further confirmed by a study of Yuan et al [62]. It seems that tissue-specific sites were located in genes involved in cell type specific functions and pathways. For example, genes which showed significant correlations with age-associated methylation and gene expression in blood were enriched in more specific functions involved in immunological processes and cytoskeletal remodeling [55]. In skeletal muscle, unique negative age-associated CpGs showed a strong enrichment in muscle contraction [60]. Similarly, although age-associated CpG sites in CD8+ T-cells seem not to affect gene expression of nearby genes, CpG sites that did induce gene expression changes were located in genes enriched for T-cell mediated immune responses [63]. Although a large overlap was found in age-associated CpGs between T cells and monocytes, the ones that showed correlation with gene expression were cell-type specific [64]. In addition, the

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genes harboring these methylation-expression correlated sites were enriched in antigen processing and presentation .

In conclusion, age-associated methylation changes can be divided in a part which occurs randomly in the genome, called epigenetic drift, whereas another part consists of age-specific methylation changes which occur at specific genomic locations and are often shared across different tissue and cell types.

3. Epigenetic clocks

In the last few years, multiple efforts have been made to use DNA methylation signatures as a predictor for chronological age. Using the methylation status of multiple age-associated CpG sites, different epigenetic clock applications have been developed. Below, we will describe the main epigenetic clock models developed.

Hannum's epigenetic clock

The epigenetic clock constructed by Hannum and coworkers was built using 450k Illumina methylation profiles of two cohorts (N=482 and 174) from whole blood samples [12]. Using Elastic net regression models, methylation of 71 CpG sites were used to predict chronological age in a highly accurate manner. They found a correlation between chronological age and DNA methylation age of 96% with an error of 3.9 years (Root-mean-square-deviation, RMSE), and 91% and an error of 4.9 years in the validation set. Furthermore, with minor adjustments, the predictive power of the clock was also valuable in other tissues instead of whole blood. However, because this clock was only trained using whole blood, the predictor may be affected by the underlying cell blood count. Interestingly, the clock was also found to be biologically meaningful. For example, the aging rate (ratio predicted methylation age to the chronological age) was higher in men compared to women [12]. Tumors had a higher methylation age compared to matched normal tissue [12].

Horvath's epigenetic clock

Hannum's epigenetic clock already demonstrated that DNA methylation could be used to predict age in multiple tissues [12]. Using a much larger collection of 27k and 450k Illumina DNA methylation datasets, containing 7,844 non-cancer samples from 51 different tissue and cell types, Steve Horvath was able to design a multi-tissue age predictor [11, 65]. The predictor is based on 353 age-associated CpG-probes, of which 193 CpGs are positively correlated with age and 160 CpGs negatively. The correlation between chronological age and epigenetic age measured by the Horvath epigenetic clock in the test dataset was remarkably high, with a correlation of 0.96 when taking all the cell types and tissues in consideration. Furthermore, the median absolute difference (MAD) between the DNA methylation age and chronological age was low: 3.6 years. In other words, in 50% of the individuals the absolute difference between DNA methylation and chronological age is less than 3.6 years.
Although a small error was found when taking into consideration all tissue types, some individual tissues such as breast tissue, uterine endometrium, dermal fibroblasts, skeletal muscle tissue and heart tissue showed bigger error rates. In a follow-up study, epigenetic age

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was found to be higher in breast tissue compared to matched blood samples from the same donor [66]. Further research is needed to evaluate whether hormonal effects may be a possible explanation. Studies examining age-related changes in skeletal muscle tissue demonstrate a small overlap with other age-associated CpG sites in other tissues [60, 67]. Furthermore, skeletal muscle age-associated CpG sites were more associated with tissue-specific gene expression compared to other tissues [60].

In general, the 353 clock CpG sites have the same characteristics as the age-associated specific CpG sites described by Hannum *et al.* [12]: positive correlated CpG sites were enriched in Polycomb-group target genes and poised promoters (however no enrichment in CGI was found), while negative correlated CpGs are enriched in CpG shores, weak promoters and strong enhancers. Surprisingly, clock CpG sites did not overlap with genes of which its expression level is correlated with age. However, whether methylation of these clock CpG sites correlates with age associated gene expression or is able to induce age related expression changes was not yet examined. In addition, genes harboring age-associated CpG sites were enriched for cell death/survival, cellular growth/proliferation, organismal/tissue development, and cancer biofunctions. The age-related methylation changes in these clock CpG sites are remarkably small, with an average difference in beta-value of 0.032. Six CpG sites were in common between the Horvath and Hannum epigenetic clock.

Unlike the clock of Hannum, this clock is less likely to be influenced by the heterogeneity of different cell types because of the use of different tissues to construct this epigenetic clock.

Weidner's epigenetic clock

Weidner and colleagues developed a predictor for chronological age using only three CpG sites [68]. Based on 575 blood 27k Illumina DNA methylation profiles, 102 age-associated CpGs were selected, and three of them were used to create an age predictor with a MAD of 4.5 years using bisulfite pyrosequencing. The CpG sites are located in the genes ITGA2B, ASPA and PDE4C.

The advantages of this epigenetic clock model are the low assay cost, no need for bioinformatics, and the independence of specific microarray platforms. Therefore, it is a possible way to predict age for forensic investigations, as recently shown in saliva samples [69-82].

Similarly, as seen with the Horvath clock, embryonic stem cells (ESCs) and iPS cells had an epigenetic age of zero. DNA methylation age tends to be higher in men and obese people (however not significant due to sample size limitations), with alcohol consumption and number of children [68].

A disadvantage of the Weidner's epigenetic clock is that is much less accurate when applied to Illumina 450k methylation arrays, probably because it is trained using pyrosequencing data [83]. Furthermore, the association with mortality seen for the Horvath and Hannum epigenetic clock (discussed below) was not found using the Weidner predictor, even when the predictor was adjusted upon using microarray data [83, 84]. This suggests that Weidner's clock is probably less useful for predicting mortality and disease risk notwithstanding that a few or even single CpG markers may be enough to estimate biological age [84].

Comparison with other molecular age predictors

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From the epigenetic clocks described above, it is clear that DNA methylation is a highly accurate way to estimate chronological age. Compared to other well-known age predictors including mRNA, T-cell specific DNA rearrangements and telomere length, DNA methylation markers achieved the highest correlation with chronological age and strongest predictive power in blood samples [85]. The epigenetic clock constructed by Weidner and coworkers based on only three CpG markers was much more accurate compared to telomere length prediction with a MAD of 18.2 years and RMSE of 23.1 years [68]. Similarly, DNA methylation age predictors of Hannum and Horvath did not correlate with telomere length, but represent independent predictors for chronological age [86, 87]. Recently, also an age predictor based on gene expression in blood was built, but also here, the prediction was much less accurate compared to Horvath's epigenetic clock [88]. Interestingly, the age-associated genes didn't contain more age-associated CpG sites than other genes, however, they were enriched for CpG sites (mainly located at enhancers) which were correlated with gene expression [88, 89].

Although no large-scale comparison of different molecular age predictors is yet performed, from these studies it is clear that DNA methylation is a remarkable accurate way of estimating chronological age.

4. The biological relevance of epigenetic clocks

Measures of age acceleration

Although age-associated clock CpG sites are located at specific genomic locations, the biological role of these CpG sites is not yet unveiled. For example, methylation at these sites doesn't induce gene expression changes, which raises questions about the functionality. However, the age-specific methylation pattern shows some remarkable similarities with the methylation profiles seen in tumor cells. For example, hypermethylated age-associated CpG sites are mainly located at bivalent promoter sites and Polycomb group protein target genes. The same pattern is also seen in multiple cancers, suggesting a link between aging and cancer through DNA methylation [90]. Indeed, several studies showed a large overlap between the hypermethylated sites related to age and cancer associated hypermethylation [53, 56, 91-95]. Furthermore, similar as in cancer, large hypomethylation blocks were identified during aging in whole blood [62]. Furthermore, there was a significant overlap between these blocks and hypomethylation in cancer. However, in contrast to cancer, only a small subset of these blocks harbor CGI hypermethylation [62]. Although less well investigated, also methylation differences in obesity samples were found to be overlapping with age-associated DMPs [96]. These studies indicate that age-associated methylation patterns are biologically relevant and might be useful biomarkers to evaluate health and disease risk.

By measuring the differences between the DNA methylation age and the chronological age (Δage), one can estimate age acceleration. While chronological age advances linearly, biological DNA methylation age may vary in time. A positive age acceleration means that one is biologically older compared to their chronological age, while a negative age acceleration means that one is biologically younger. A recent study developed different age acceleration measures which occur independently of chronological age [97]. A universal age acceleration measure was defined by using the residual result from a linear regression model between the Horvath age predictor and chronological age. For blood samples, two additional

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measurements were defined. The intrinsic epigenetic age acceleration (IEAA) uses the Horvath epigenetic clock adjusted for cell blood counts. In this way, a predictor was obtained which is independent of blood cell counts and therefore measures only the real epigenetic effects in blood cells. In contrast, the extrinsic epigenetic age acceleration (EEAA) is based on the Hannum epigenetic clock whereby the contribution of the blood cell counts was increased. Because the Hannum clock was only trained using whole blood datasets, the predictor will be more affected by blood cell counts compared to the Horvath clock, which was trained using multiple tissues. EEAA therefore measures both the effect of the change in blood cell counts and intrinsic DNA methylation during aging [97]. It is known for example that the number of naïve CD8+ T-cells decreases with age, while senescent CD8+ T cells increases. In this way EEAA also measures the contribution of immune-senescence.

Using twin cohorts, DNA methylation age acceleration was found to be highly heritable, with decreased heritability later in life, suggesting that the importance of non-genetic factors increases with age [11]. Heritability for both the Horvath and Hannum age acceleration was estimated to be around 40% in adults [83]. However, part of this 40% is, beside genetic factors, probably also attributed to shared non-genetic factors [98]. Using longitudinal cohorts, it was demonstrated that DNA methylation age acceleration is remarkable stable throughout adulthood suggesting that the differences in age acceleration are primary set before adulthood [99]. In another cohort study in children, an increase in variation of age acceleration from birth to 7 and 17 years of age was seen, but the within-subject correlation of epigenetic age increases with age further confirming that DNA methylation age is already set early in development [100]. These results are also in agreement with the observation that methylation changes more rapidly in children compared to adults [101], and that rate of methylation changes in the clock CpG sites is faster from newborns until adulthood, indicating that the ticking rate of the epigenetic clock is the highest during organismal growth [11]. Interestingly, associations with age acceleration and prenatal and early life factors, including sex, birth weight, birth by caesarean section, and maternal smoking, weight, BMI, selenium and cholesterol levels were found suggesting that early life factors may impact the DNA methylation age, however, causality is not yet proven [100].

Age acceleration in health and diseases

In the last few years, couple of studies proved the usefulness of DNA methylation age acceleration as a measure for biological age (Figure 1). For example, multiple studies confirmed that men have significant increased age acceleration compared to women, which is in agreement with the average longer lifespan of women [12, 102]. In addition, long-lived individuals are younger than would be expected from their chronological age [103, 104]. Furthermore, the offspring of semi-supercentenarians (105-109 years of age) have a lower epigenetic age than age-matched controls [104]. These results again confirmed the biological relevance of DNA methylation as an age acceleration measure.

Recent studies showed that age acceleration can also be used to predict disease risk and mortality (Table 1 and Figure 1). All-cause mortality was associated with an increased epigenetic age in blood based on four independent longitudinal cohorts both measured with the Hannum and Horvath epigenetic clock [83, 87]. In a follow-up study using 13 cohorts, both IEAA and EEAA was associated with all-cause mortality, with EEAA showing the most significant association [97]. Similar results were found in a Danish longitudinal twin study

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[105]. In addition, the twin partner with the highest DNA methylation age died first in 69% of the cases. Furthermore, the larger the differences between the twin pairs, the higher the probability of mortality in the biological oldest twin [105]. Also in a German case cohort, all-cause mortality was positively associated with age acceleration (Δage) [106]. Furthermore, also cancer and cardiovascular disease (CVD) mortality was significant associated with an increased age acceleration [106].

The main risk factor for cancer is aging. Cancer incidence and mortality was found to be associated with an increased age acceleration (IEAA) in blood [107]. Furthermore, the association was independent of telomere length [107]. In a study examining the association with multiple DNA methylation age predictors and breast- and colorectal cancer incidence, only ELOVL2 single CpG marker in blood was associated with breast cancer incidence and not the other markers [108]. For colorectal cancer, the age acceleration based on Horvath and FHL2 single marker was increased only in females [108]. A weak but significant association was found with IEAA in blood and the development of postmenopausal, but not premenopausal breast cancer in a case-control study [109]. IEAA measured in blood was associated with lung cancer incidence [110]. However, IEAA was not associated with smoking, but the association with lung cancer was the strongest in current smokers, while the association was not significant in never-smokers [110]. Another study confirmed the non-significant effect of smoking on age acceleration [111].

An increased age acceleration (residuals) in the liver tissue of obese subjects was found, but not in blood, adipose and muscle tissue [112]. Bariatric surgery and exercise intervention did not alter DNA methylation age in the short term [112]. In another study an association with obesity was found in blood cells (Δage), but only in middle-aged (40-49 years) and not in young (15-24 years) and elderly (90 years) people [113]. In addition, a more recent study confirms that BMI was associated with an increased age acceleration (both IEAA and EEAA), however smaller effects were found as compared with liver [114]. Also in buccal cells increased age acceleration was associated with BMI [115].

Also, links between DNA methylation age acceleration and neurological diseases were recently demonstrated. Interestingly, epigenetic accelaration was found to correlate with mitochondrial DNA copy number rather than telomere length in bipolar disorder, suggesting mitochondrial control of the epigenetic clock [116]. In individuals with Alzheimer's disease, epigenetic age acceleration (residual) measured in dorsolateral prefrontal cortex was positively associated with multiple neuropathological measurements including diffuse plaques, neuritic plaques, and amyloid load, and a decline in global cognitive functioning, episodic memory and working memory [117]. The association of increased epigenetic age acceleration in Alzheimer's disease patients was recently confirmed [118]. In another study, however, general cognitive function was not found to be associated with age acceleration (Δage and residual) in blood samples from monozygotic twins [119]. Also in Parkinson's and Huntington's disease, IEAA and EEAA associations were reported with methylation age in blood samples and/or different brain tissues [120, 121]. DNA methylation age acceleration was not found to be associated with schizophrenia in blood and brain tissues [122-124]. Increased epigenetic age acceleration (IEAA) in blood samples was also found to correlate with reduced white matter integrity in the brain indicating that blood can be used as a surrogate. White matter hyperintensities on brain magnetic resonance imaging are a marker for cerebral small vessel disease and are associated with an increased risk of stroke, cognitive and functional impairment, dementia, and death. Raina and colleagues found an association with epigenetic age measured in blood and white matter hyperintensities burden independent

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of chronological age [126]. In another study, ischemic stroke outcome was better predicted using blood epigenetic age than chronological age [127]. These studies indicate that DNA methylation of surrogate blood samples can be used to estimate biological age of the brain. Interestingly, two recent GWAS studies discovered genomic loci associated with age acceleration in brain tissues. In cerebellum, two loci containing the genes DHX57 and MLST8 were associated with epigenetic age acceleration and transcriptional expression of the genes. Interestingly, they found a significant overlap with GWAS of Alzheimer's disease, Parkinson disease and age-related macular degeneration [128]. In another study, associations were found in prefrontal cortex and across five brain regions [118]. Again, GWAS results demonstrated a significant overlap with those of cognitive decline, dementia, Alzheimer's disease and onset of Huntington's disease.

Chronic HIV-infected patients have higher risks for multiple age-related disorders, including neurodegeneration and cancer and therefore seem to age faster. HIV-associated neurocognitive disorders have been associated with an increased age acceleration (residual) compared to HIV+ neurocognitively normal people in occipital cortex samples [129]. HIV positive individuals have higher DNA methylation age compared to HIV-negative individuals measured in blood, using a consensus method of Hannum and Horvath [130]. Similar results were found both in brain and blood samples of HIV+positive individuals [131]. Beside HIV infections, also cytomegalovirus infections have been associated with epigenetic age [132]. Recently, *Helicobacter pylori* infections, which is involved in gastric cancer was also found to accelerate epigenetic age both using the Horvath as the Hannum predictor .

Interestingly, also lifestyle factors may impact DNA methylation age. A recent study investigated the effect of different lifestyle factors to epigenetic aging [114]. Fish intake, moderate alcohol consumption, education and fruit/vegetables intake was associated with a decreased EEAA and poultry intake with a decreased IEAA, although the observed effects were weak [114]. Another study failed to demonstrate a significant association of epigenetic age or age acceleration, with different biological and lifestyle factors, including smoking, education, childhood IQ, social class, APOE, cardiovascular disease, blood pressure and diabetes [83]. Recent studies highlight the importance of stress-related factors in influencing DNA methylation age. In 96 male soldiers, trauma was associated with an increased Horvath age acceleration in blood samples. Surprisingly, post-traumatic stress disorder (PTSD) symptoms were inversely correlated with epigenetic age [134]. In contrast, in two papers of Wolf *et al.* PTSD severity and symptoms in military veterans were found to be positively associated with DNA methylation age acceleration [135]. These associations were only found significant for the Hannum clock and not for the Horvath age predictor however. Also in children stress was found to have an impact on the epigenetic clock. Experienced violence, but not witnessed violence, was associated an increased age acceleration measured in saliva samples. The results remained significant after correction for epithelial buccal cell proportion. Cumulative lifetime stress seems to have the largest effect on epigenetic age, while childhood maltreatment and trauma or current stress alone were not found to be associated with an accelerated epigenetic age [136]. It was suggested that aberrant glucocorticoid signaling due to cumulative stress could be a mediator of these effects. Many of the epigenetic clock CpG sites were found to be located within glucocorticoid response elements, and were changed in methylation after treatment with the glucocorticoid receptor agonist dexamethasone [136]. A recent study further supports this hypothesis. In adolescent girls, Jovanovic and colleagues an association between diurnal cortisol production and accelerated epigenetic age. Furthermore, accelerated epigenetic age was found to be a mediator between diurnal cortisol levels and

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reduced left hippocampal volume [137]. Interestingly, meditation could provide a beneficial effect on epigenetic age. While there was no difference in IEAA between long-term meditators and meditation-naïve controls, years of meditation was negatively associated with IEAA .Other studies report significant associations with menopause , physical development , low income [141], self-control and socio-economic status [142], physical and cognitive fitness [115, 143], frailty [86], insomnia symptoms , Werner syndrome [145] and Down syndrome [146].

Overall, we can conclude that age acceleration could be a useful marker for estimating disease risk. However, also negative results were obtained, for example no relation with coronary heart disease incidence [102], schizophrenia [122-124] and some cancers risks were found [11], indicating that DNA methylation age is not a universal health-disease marker. One explanation could be tissue-specificity. For example, BMI was only associated with an increased age acceleration in liver tissue. In another study, the cerebellum ages slower than other tissues, suggesting that age acceleration could be different in different tissues [147]. One study also concluded that a frailty index based on 34 health items was better in predicting mortality compared with DNA methylation age measured in blood which was not found to be correlated with mortality [148, 149]. This lack of association is probably due to a lower statistical power compared to the more large-scale studies finding a clear association with all-cause mortality [83, 87, 97, 105, 106].
Although lifestyle factors, like stress and diet, might have an impact on the DNA methylation age, prolonged longitudinal studies in big cohorts of different ethnicities maybe required to identify significant effects. Moreover, future studies should investigate possible causal links between lifestyle factors, epigenetic age, and health and disease conditions.

5. Possible underlying mechanisms of epigenetic aging

So far, many questions remain unanswered i.e. "What is the cause of these age-associated DNA methylation pattern? Does DNA methylation causes/accelerates the aging process or is it just a consequence of aging?" More research is needed to further unravel the molecular mechanisms behind the age-related DNA methylation changes. For now, we can only speculate about possible underlying mechanisms of age-associated DNA methylation pattern. Moreover, the chicken-egg discussion has not yet been resolved: does DNA methylation drives the aging process or is DNA methylation a consequence of aging? It has been proposed that epigenetic drift is the result of the accumulation of DNA methylation errors during cell division and DNA replication [37]. DNA methylation patterns are maintained after each cell cycle by DNMTs. Although this system is relatively reliable, mistakes may occur and can lead to incomplete transmission of the DNA methylation pattern. However, this explanation does not account for the clock CpGs which are tissue-independent. For example, also in postmitotic tissues, like brain, chronological age was accurately predicted [11]. Furthermore, also different blood cells have similar epigenetic age despite a huge variation in turnover rate between different blood cells [11]. Remarkably, the rate of methylation changes in the clock CpG sites is faster in newborns than in adulthood, indicating that the ticking rate of the epigenetic clock is the fastest during organismal growth [11, 101]. Recently, a study constructed a mitotic clock based on DNA methylation to estimate the stem cell division rate,

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an important cancer risk factor [151]. Unlike the epigenetic clock, the authors showed that this clock was universally accelerated in cancer, pre-cancerous lesions, and normal buccal cells from smokers [151]. One can argue that most reproducible age associated methylation changes must have an origin in stem cells, and that the methylation drift seen is a result of stem cell division, as alterations in DNA methylation have been reported to contribute to age-associated hematopoietic stem cell decline [37, 152].

DNA methylation age was also found to occur independently of cellular senescence. Induction of senescence by DNA damage didn't induce an increase in epigenetic age [153]. In addition, cells immortalized by telomerase showed an increase in epigenetic age after long-term passaging, but didn't show any sign of cellular senescence [153]. These results indicate that cellular aging is a process distinct from cellular senescence. Furthermore, telomere length didn't correlate with DNA methylation age, but were found to be independent predictors of chronological age and associated with mortality, indicating that telomere length and the epigenetic clock are measuring different aging processes [87]. Similarly, in another age acceleration model, no association was found with telomere length [86].

Horvath proposed a more molecular epigenetic maintenance system, which helps to maintain epigenetic stability, of which the activity can be tracked through age-associated DNA methylation changes [11]. In this way, an accelerated age reflects an increased activity of the epigenetic maintenance system. However, for now, it is unclear which components are involved in this system or how such a system should be regulated.

It will be interesting to study why some CpGs are more variable and others relatively stable during lifespan. There exists some evidence that both genetic and environmental exposures can limit epigenetic drift at specific CpG sites [154]. In another study, many CpG sites associated with common genetic variants were stable between different tissues indicating that genetic variants may constrain inter-tissue epigenetic drift [155]. Whether the same is true for aging epigenetic drift should be further investigated. As described before, hypermethylated age-associated CpG sites are enriched in Polycomb group protein target genes. One hypothesis could be that PRC1 and PRC2 protects unmethylated DNA from DNA methylation and that during aging these complexes become degraded leading to *de novo* DNA methylation by DNTM3A and -3B .

Since most of the age-associated CpGs and clock CpGs are not linked with a significant change in gene expression, a stochastic epigenetic drift model is favored for the clock CpGs. In addition, methylation changes are relatively small. However, no study yet examined whether there are *trans* effects or other biological consequences not directly reflected in the gene expression profiles (chromosome stability, alternative splicing). Furthermore, similarities with age-related DNA methylation profiles and methylation profiles of age-associated diseases could also provide us hints about the functionality of clock CpG sites. It was hypothesized that in general age-associated DNA methylation changes don't affect gene function, but that, through epigenetic drift, occasionally a key transcription factor could be targeted, leading to increased disease risk [36]. An interesting study from Zannas and colleagues showed a link between epigenetic age acceleration and glucocorticoid stress signaling [136]. Epigenetic age in blood was found to be accelerated with cumulative life stress. More interestingly, many of the epigenetic clock CpG sites were located within glucocorticoid response elements, and changed in methylation after dexamethasone treatment. Furthermore, also gene expression was affected [136]. The latter results suggest a TF-specific regulatory role of the epigenetic clock CpGs.

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How environmental factors may affect DNA methylation is far from completely understood. It is well known that inflammatory responses play crucial roles in age-related conditions and diseases, and are influenced by environmental factors, including diet, pollution and stress [157, 158]. It is believed that chronic low-grade inflammation and immunosenescence contribute to aging in a process called “inflammaging” [159, 160]. Of particular interest, links between inflammation, epigenetics and DNA methylation have been described [161, 162]. Methylation at multiple CpG sites was found to be associated with serum C-reactive protein (CRP), a marker of low-grade inflammation, indicating that DNA methylation plays a role in chronic inflammation [163]. The interrelationship with cellular metabolism is a possible way how inflammation may affect epigenetic patterns [164, 165]. Because epigenetic modifiers use key metabolites as substrates, a change in metabolite concentrations may affect their activity and result in altered histone and DNA modifications [164, 166, 167]. Another hallmark of aging is mitochondrial dysfunction which, in the traditional view, leads to an increased ROS production inducing global cellular damage [168]. Although recent studies led to a re-evaluation of the mitochondrial free radical theory of aging, the observation of increased ROS with age remains. Furthermore, oxidative stress leads to chronic inflammation [169]. There is a direct link between oxidative stress and the DNA demethylation process through the action of the TET enzymes. These enzymes catalyze an active demethylation via the oxidation of 5-methylcytosine to 5-hydroxmethylcytosine [170]. α-ketoglutarate is one of the substrates needed for this reaction, and is also involved in the Krebs cycle which is an important determinant of the cell redox state [171]. Global hydroxymethylation was found to decrease by oxidative stress, whereas paradoxically, an increase in hydroxymethylation levels could also be observed at specific loci [172]. Other mechanisms how ROS may affect DNA methylation are via changes in DNMT activity, DNMT expression, or DNMT recruitment on DNA [173, 174].

6. Conclusion

DNA methylation has outperformed other molecular biomarkers in predicting age. Although more research is needed, recent studies indicate that DNA methylation age may be better in estimating biological age rather than chronological age, and may therefore be a promising marker for health and disease status. Recently, also methylation-based predictors for gestational age in cord blood and placenta [177] were developed. Furthermore, gestational age acceleration was found to be associated with diverse maternal and offspring characteristics known to reflect prenatal environmental adversity , again demonstrating the power of DNA methylation as a biomarker of biological age. Until now, it is unclear whether DNA methylation drives aging and actively contributes to the aging process or whether it is rather a more passive and stochastic mechanism as a consequence of aging. Increasing our knowledge about possible underlying mechanisms and causal links between environment (lifestyle), DNA methylation, and diseases, may result in further improving the prediction of biological age. A recent study showed that age DNA methylation drift is highly conserved across three mammalian species (mouse, monkey and human). Age-associated DMPs displayed a same genomic distribution pattern, were located in genes involved in similar signaling pathways, and the same promoters were hypermethylated with age across the three species [59]. More interesting, a longer lifespan was found to be correlated with a lower rate of drift and caloric restriction, which is known to prolong lifespan, delayed age epigenetic drift in mouse and monkeys [59]. The conservation of age associated DNA methylation drift

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across species indicates the relevance of the use of model organisms to study epigenetic age. Indeed, accurate epigenetic clocks are now also being established for mouse, dogs and wolves or other vertebrates [116, 178-181]. Interestingly, initial experiments suggest that these clocks are also biological meaningful, since factors known to influence lifespan, including ovariectomy, caloric restriction, growth hormone receptor knock-out, maternal and offspring diet, dwarfism, rapamycin treatment also seemed to affect epigenetic age [178-182]. Therefore, these clocks can become useful tools to assess the effect of different interventions on aging and lead to increased mechanistical insight in the aging process [183]. The observation that mitochondrial functions are more associated with the epigenetic clock than telomeric length opens new therapeutic perspectives for epigenetic control of healthy aging through targeting of mitochondrial metabolism [171, 184-186].

In conclusion, although age-associated DMPs display a similar genomic distribution pattern, are located in genes involved in similar signaling pathways, and similar promoters are hypermethylated with age across the three species, epigenetic clock specific CpG sites in human are less accurate in predicting age across different species [59, 178-181]. This could be due to species-specific changes in metabolic rates, different drift rates and technical differences in clock design. Finally, by means of whole-genome bisulfite and novel single molecule realtime sequencing (Pacbio) approaches, it will be possible to build more sophisticated epigenetic age predictors in different species, integrating different nuclear and mitochondrial DNA modifications (5mC, 5hmC, 5fC, 5caC, 6mA, 8-oxoG, 8-oxoA).

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Figures

Figure 1: The epigenetic clock as a biological age predictor. Disease risk and lifestyle factors are shown known to be associated with either epigenetic age acceleration or deceleration. Check table 1 for a complete list of the health and disease factors associated with epigenetic age acceleration or deceleration.

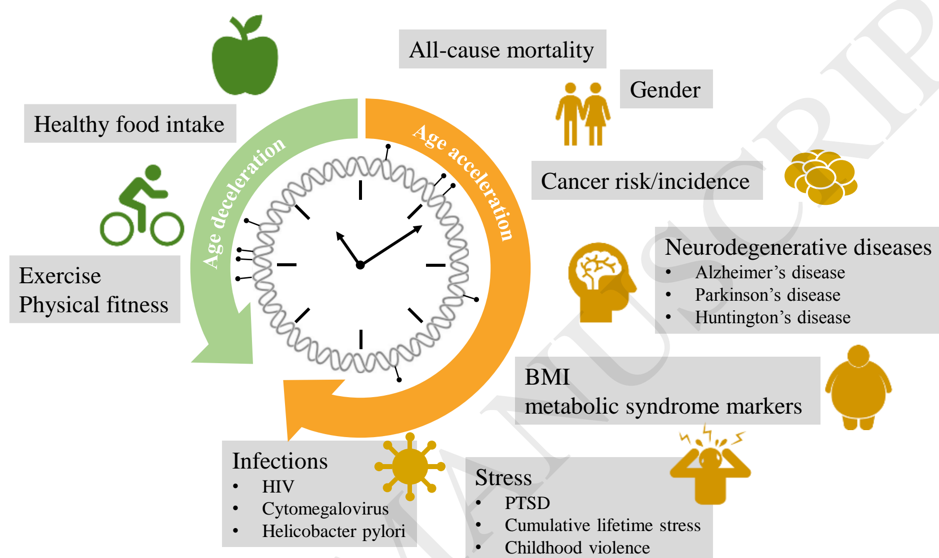

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Table

Table 1: Health and disease phenotypes associated with DNA methylation age.

Phenotype	Predictor	Tissue	Finding	Reference
All-cause mortality risk CVD mortality risk Cancer mortality risk	Hannum/Horvath	Blood	Associated with higher epigenetic age	[83, 87, 97, 105, 106] [106] [106]
Cancer mortality risk	Hannum	Blood	Associated with increased age acceleration	[107]
Cancer incidence risk	Hannum	Blood	Associated with increased age acceleration	[107]
Breast cancer incidence	Hannum/Horvath/Weidner/ELOV2/FHL2	Blood	Only ELOV2 marker associated	[108]
Colorectal cancer incidence	Hannum/Horvath/Weidner/ELOV2/FHL2	Blood	Only Horvath and FHL2 marker associated, only in females	[108]
Breast cancer risk	Horvath	Blood	Only in postmenopausal with IEAA	[109]
Lung cancer incidence	Horvath	Blood	IEAA was associated	[110]
BMI	Horvath	Blood, liver, adipose, muscle	Only age acceleration associated in liver	[112]
BMI	Horvath	Blood Blood, Buccal	Only association in middle-aged individuals. Associated in both tissues.	[113] [115]
Ischemic stroke outcome	Hannum	Blood	Epigenetic age is better predictor for stroke outcome compared to chronological age	[127]
Cerebral white matter hyperintensities	Hannum/Horvath	Blood	Age acceleration associated with white matter hyperintensities	[126]
Alzheimer's disease	Horvath	Dorsolateral prefrontal cortex	Associations with diffuse plaques, neuritic plaques, and amyloid load, and a	[117]

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		Prefrontal cortex	decline in global cognitive functioning, episodic memory and working memory Associated with increased epigenetic AA after adjusting for the proportion of neurons	
Parkinson's disease	Horvath	Blood	Increased IEAA and EEAA in PD patients	
Huntington's disease	Horvath	Brain tissue	Increased age acceleration	
Schizophrenia	Horvath	Superior temporal gyrus Frontal cortex Blood, dorsolateral prefrontal cortex	No difference in DNAm age between controls	[124] [123] [122]
White matter integrity	Horvath	Blood	Increased age acceleration with reduced white matter integrity	
HIV-associated neurocognitive disorders	Horvath	Occipital cortex	Increased age acceleration compared to control HIV+ subjects	
HIV	Hannum/Horvath Horvath	Blood Blood/brain	Increased AA in HIV+ compared to HIV-	[131]
Cytomegalovirus infection	Horvath	Blood	Increased age acceleration	
Helicobacter pylori infection and chronic atrophic gastritis	Horvath/Hannum	Blood	Increased age acceleration	[133]
Diet/lifestyle	Hannum/Horvath	Blood	EAA associated with fish intake, moderate alcohol consumption, eduction and carotenoid levels. IEAA associated with poultry intake	[114]
Menopause	Horvath	Blood, buccal,	Blood: increased AA associated with	

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		saliva	earlier menopause, bilateral oophorectomy, and a longer time since menopause Buccal: lower epigenetic age in women who underwent menopausal hormone therapy Saliva: higher epigenetic age in women who underwent bilateral oophorectomy	
Physical development during childhood and adolescence	Horvath	Blood	Positive AA at birth associated with higher average fat mass changes in weight and BMI from birth to adolescence. AA at age 7 associated with higher average height	
Low income	Hannum	Blood	Accelerated aging	
Self-control	Hannum/Horvath	Blood (PBMC)	In low-SES children self-control associated with accelerated age	
Werner syndrome	Hannum/Horvath	Blood	Increased IEAA and EEAA	
Down syndrome	Horvath	Blood, brain, buccal	Increased AA in blood and brain, but not buccal	
Posttraumatic stress disorder	Hannum/Horvath Hannum Horvath	Blood	Increased Hannum AA with PTSD severity. Increased Hannum AA with PTSD symptoms, but not severity or trauma. Increased Horvath DNAm age with trauma, but decreased DNAm age with PTSD symptoms.	 [135] [134]
Diurnal cortisol levels	Horvath	Saliva	Increased AA. AA as mediator between cortisol levels and	[137]

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			reduced left hippocampal volume	
Meditation	Horvath	Blood	No differences in IEAA between long-term meditators and controls. Increased IEAA in older controls compared to younger (not seen in meditators). Years of meditation negatively associated with IEAA	
Lifetime stress	Horvath	Blood	Cumulative lifetime stress associated with AA, no correlation with childhood maltreatment or current stress	
Childhood violence	Horvath	Saliva	Increased AA in children	
Physical and cognitive fitness	Horvath	Blood Blood, buccal	Increased AA with lower lung function, cognition and grip strength measures. Increased AA with decreased grip strength, only in blood. No associations with standing balance time and chair rise speed.	[115]
Frailty	Horvath	Blood	Increased AA with frailty measure	[86]
Insomnia symptoms	Hannum/Horvath	Blood	Increased EEAA	

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