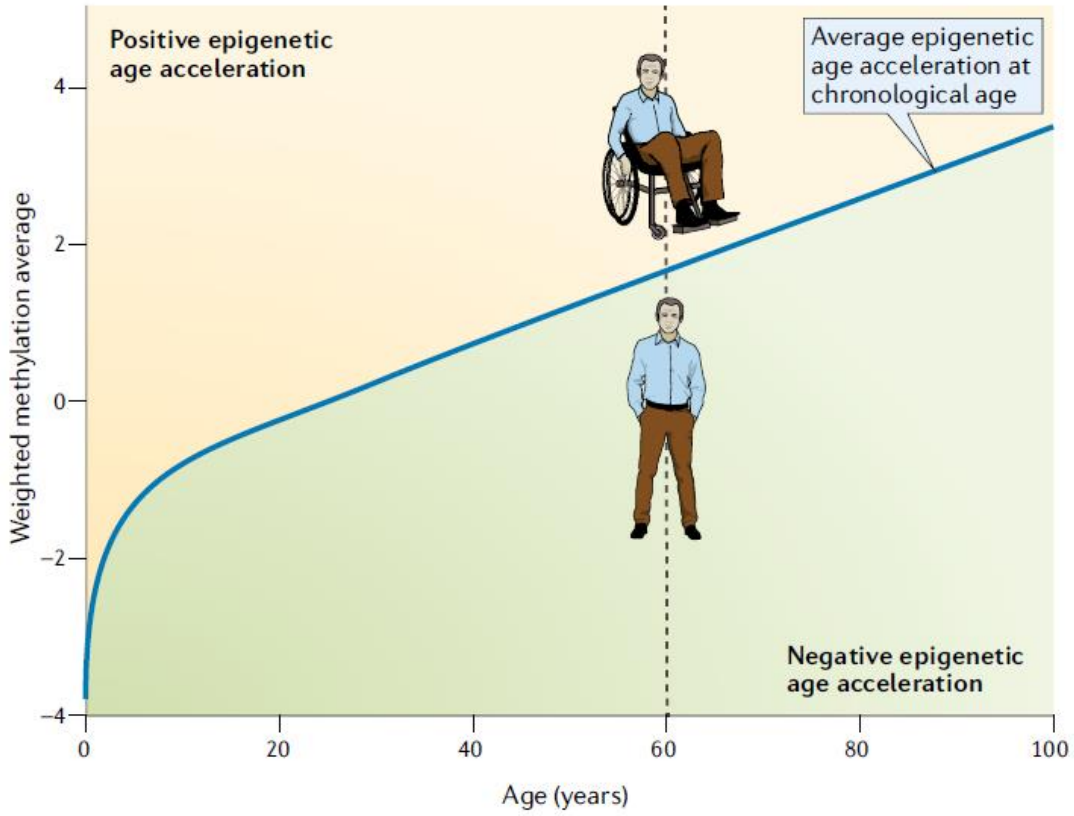
New perspective on aging: the epigenetic clock



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Abstract

Aging is a natural process that leads to morphological and functional changes of the cells. Research regarding aging is expanding rapidly due to the increasing number of individuals over 65 of age, resulting in a burden on health care costs and a dramatic increase in age-related diseases (ARDs) like Alzheimer's disease, cancer and other chronic diseases. In order to reduce the health costs and improve the quality of elderly by preventing ARDs, more reseach focuses on the epigenetic mechanism: DNA methylation (DNAm). DNAm involves the process of adding a methyl-group to a 5' cytosine at a CpG site and is associated with genomic stability and regulation of gene expression. Changes in methylation of the CpG sites are observed regarding to age. Several epigenetic 'clocks' have been composed allowing the prediction of chronological age of an individual based on the methylation status of a selection of age-related CpG sites. Factors like smoking, education, gender and disease have been found to influence these epigenetic clocks. Intriguingly, it is now possible to calculate all-cause mortality risk based on the difference between the predicted and chronological age and even time-to-death with an epigenetic clock. Furthermore, DNAm could be reversed using a recombinant growth hormone, opening up opportunities to slow down aging and extend lifespan. Future perspective of the applications of the epigenetic clocks might be regarding the fields of personalized medicine, forensics and generate possible to ways to slow down aging; improving health and elongate lifespan.

Table of contents

1. Introduction to aging

The amount of elderly is ever increasing. In the Netherlands in 2017, 2,4 million people which accounted for respectively 14% of the population was above 65 years old and in 2060 it is estimated that this number will increase to 5,2 million people (28% of the population) (CBS, 2017). When individuals reach the age of 65, it is estimated that they live for another 20 years (CBS, 2017). Importantly, life expectancy does not equal health expectancy (HALE) e.g. how long an individual will live in good health. The WHO estimated that in 2016, men were healthy till the age of 77.8 and women till 79.9 years when they had reached the age of 60 (WHO, 2018). This results in a discrepancy of 7.2 and 5.1 years respectively.

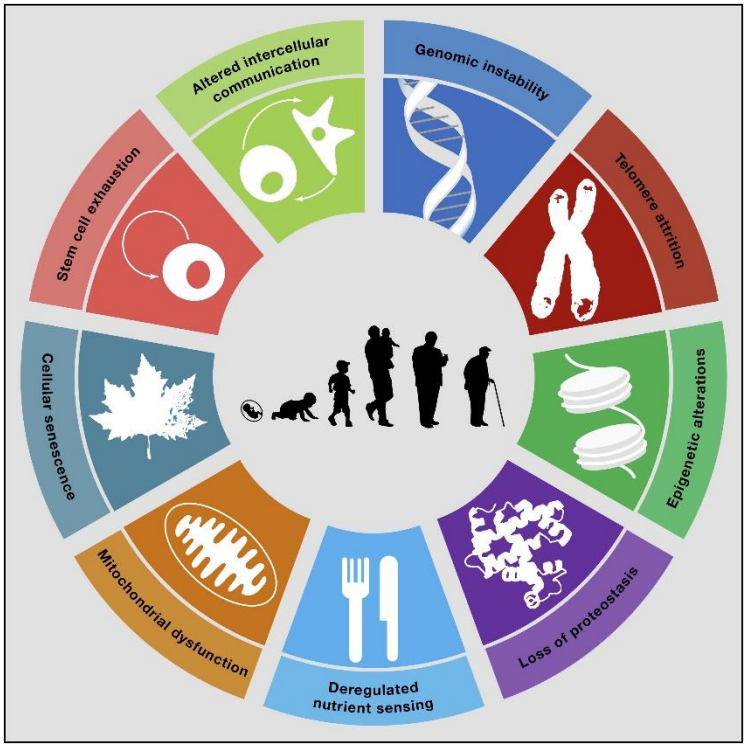


Figure 1. The nine hallmarks of aging. Adapted from López-Otín *et al.* (2013).

This discrepancy results in rising health care costs as aging is associated with certain age-related diseases (ARDs) including diabetes, cancer, cardiovascular diseases, neurological diseases like Alzheimer's disease, Parkinson's Disease and other chronic diseases (Bana and Cabreiro, 2019; Gadecka and Bielak-zmijewska, 2019). For instance, the annual medical costs for elderly was 17 billion in 2015 and is estimated to increase to 43 billion in 2040 in the Netherlands (RIVM, 2018).

In order to improve the quality of life by reducing ARDs and reduce health costs, healthy aging needs to be top priority of the government. Before ARDs can be reduced and health improved, first a thorough understanding of what aging actually is needs to be established (Gadecka and Bielak-zmijewska, 2019). To investigate aging, a clear definition has to be established. For instance, common age-related health problems like impaired vision and hearing, impaired wound healing, osteoporosis and increased vulnerability to infections are consequences of aging but do not define aging itself (Gadecka and Bielak-zmijewska, 2019).

To define aging, nine hallmarks of aging were described by López-Otín *et al.* (2013) including: genomic instability, telomere attrition, epigenetic alterations, loss of proteostatis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intracellular communication (See Figure 1).

The past few years, extensive research has been conducted regarding epigenetic alterations (Bocklandt *et al.*, 2011; Koch and Wagner, 2011; Hannum *et al.*, 2013; Horvath, 2013). Several studies reported that epigenetic alterations could be correlated with chronological age (Bocklandt *et al.*, 2011; Hannum *et al.*, 2013; Horvath, 2013). Based on these epigenetic alterations, a 'clock' could be established to predict the

chronological age of an individual (Hannum *et al.*, 2013; Horvath, 2013). Furthermore, this clock could also be used to calculate all-cause mortality risk (Zhang *et al.*, 2017; Levine *et al.*, 2018) and even time-to-death (Lu *et al.*, 2019) (See **Figure 2**). Research also shows that different factors like smoking, education, ethnicity and diseases affect the epigenetic clock (Gao *et al.*, 2016; Horvath *et al.*, 2016). In the future, epigenetic clocks could possibly be used to develop personalized medicine based on the individual clock. Furthermore, the epigenetic clock has shown potential for forensics to estimate the chronological age based on a biological sample on a crime scene. Lastly epigenetic clocks will help clarify the process of aging and generate possibly to ways to slow down aging; improving health and elongate lifespan.

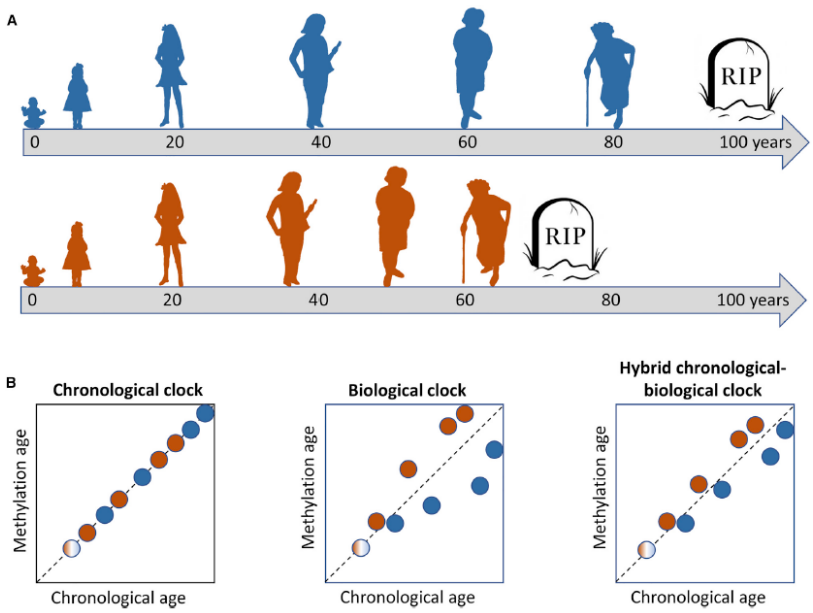


Figure 2. Visual representation of the chronological, biological and hybrid chronological-biological clock. Adjusted from: Field et al. (2016) A) Two timelines are visualized of two different individuals with the same chronological age at baseline result in different time-of-deaths. B) The chronological clock is the same for blue and orange. The biological clock however shows differences between orange and blue that eventually will result in the different time-of-death which is combined in the hybrid clock.

This essay will first discuss DNA methylation, the epigenetic alteration that is the basis for the epigenetic clocks and how age influences DNA methylation. Second, the important studies regarding the development of the epigenetic clocks and their applications will be described. Third, examines the future perspectives regarding epigenetic clocks and aging.

2. Aging, DNA methylation and the epigenetic clock

2.1 History of epigenetics

The field of epigenetics is a hot topic nowadays, but the term was coined over half a century ago. Conrad Waddington was the first to use the term epigenetics in 1939 in an attempt to link genotype with phenotype (Waddington, 1939). Importantly, the molecular structure of the DNA was not elucidated until at least 14 years later. Epigenetics for Waddington was involved in developmental processes and the differentiation of cells (Waddington, 1939; Villota-salazar *et al.*, 2016). Years later, David Nanney stated that there were two cell regulatory mechanisms, one was based on the DNA (genetic) and the other determined which information would be expressed in a particular cell (epigenetic), which is more in agreement with the current definition (Nanney, 1958). Both Waddington and Nanney tried to answer the question how genetics was involved in phenotypic development, but looked at it from different levels (Villota-salazar *et al.*, 2016; Nicoglou and Merlin, 2017). Waddington was more focused on Mendelian genetics while Nanney focused on molecular genetics which was possible because of the advances on molecular level (e.g. the elucidation of the DNA structure) (Nicoglou and Merlin, 2017). Although Nanney had more insight in the molecular level, he still thought that the epigenetic mechanisms took place in the cytoplasm (Nanney, 1958).

More insight in the epigenetic mechanisms came in the 70's when the structure of DNA was further elucidated and the importance regarding condensed DNA (euchromatin) which is linked with repression of genes opposed to open chromatin (heterochromatin) which is linked with activation of genes was, discovered. Nucleosomes, proteins with DNA wrapped around it, were discovered and it was found that modification of these proteins, histones, result in euchromatin or heterochromatin formation (Allfrey *et al.*, 1964). DNA methylation, cytosine residues to which a methyl-group was attached, appeared to be another epigenetic mark contributing to genome stability (Holliday and Pugh, 1975; Riggs, 1975). Much later, RNA-based mechanisms were identified as the third epigenetic mechanism regulating gene expression (Lee *et al.*, 1993). All of the epigenetic mechanisms have in common that they do not alter the DNA sequence (Villota-salazar *et al.*, 2016). Epigenetics nowadays is defined as the study of heritable changes in gene expression that do not involve changes to the underlying DNA sequence (See Figure 3) (Villota-salazar *et al.*, 2016; Nicoglou and Merlin, 2017). This essay will only discuss DNA methylation and its relation to aging.

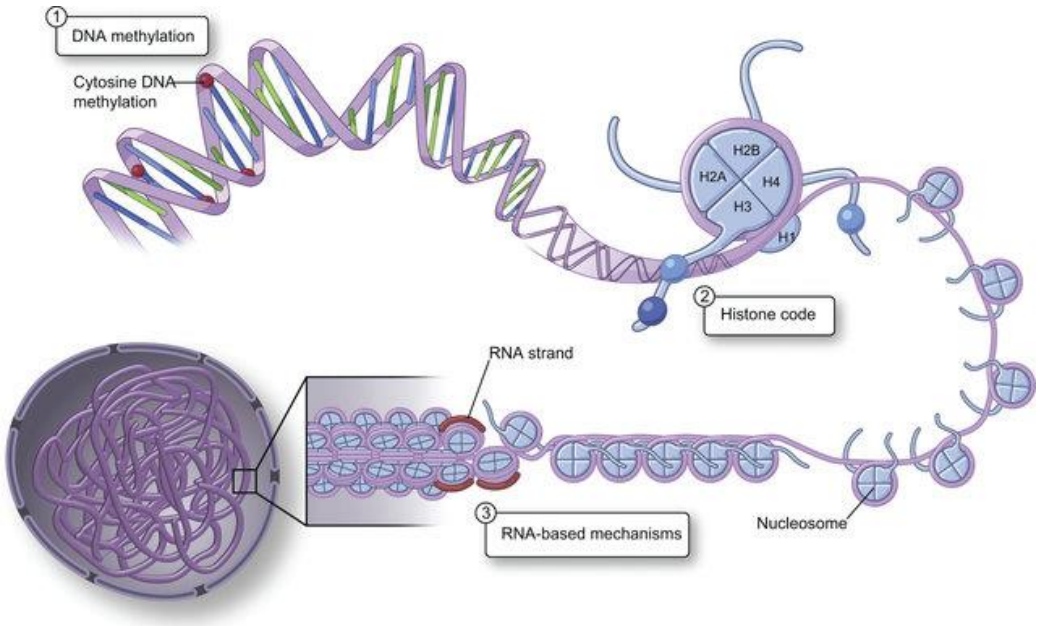


Figure 3. Schematic overview of epigenetic mechanisms. Adapted from: (Yan *et al.*, 2010)
DNA is wrapped around proteins called nucleosomes, that consists of 8 histones. The histone tails can be modified which results in a more condensed chromatin (euchromatin), linked to the repression of genes or more accessible chromatin (heterochromatin) linked to the activation of genes. DNA methylation takes place on the cytosine of the DNA and is associated with the regulation of gene expression. The third epigenetic mechanism is RNA-based mechanisms.

2.2 DNA methylation

DNA methylation (DNAm) is essential for the following, first DNAm ensures DNA stability by silencing transposable elements (transposons) that can cause instable DNA. Second, DNAm is also involved in X chromosome inactivation of females and regulating gene expression. DNAm regulates gene expression by methylating promoter regions, thereby repressing a gene. Third, gene repression is especially important in the germ cell line to activate and repress the appropriate gene sets from paternal and maternal origin (genomic imprint) (Benayoun *et al.*, 2015; Edwards *et al.*, 2017). DNAm gene repression is also important in tissues, whereby non-essential genes are methylated which results in tissue-specific gene expression (Benayoun *et al.*, 2015; Edwards *et al.*, 2017).

DNAm entails the process whereby a methyl-group is added to the fifth position of a cytosine (5mC) in a cytosine-phosphate-guanine (CpG) dinucleotide site The CpG sites can be located in CpG islands (CGIs). CGIs are regions of high CG density (over 200 basepairs) and are often found in promoters. CGIs are often not methylated as transcription factors (TFs) are then unable to bind and transcribe the gene (Bana and Cabreiro, 2019; Gadecka and Bielak-zmijewska, 2019) (See Figure 4). However, a change in CGI methylation in promoters does occur in pathologies like cancer and aging, silencing tumor supressor genes for example (Christensen *et al.*, 2009; Kim and Costello, 2017).

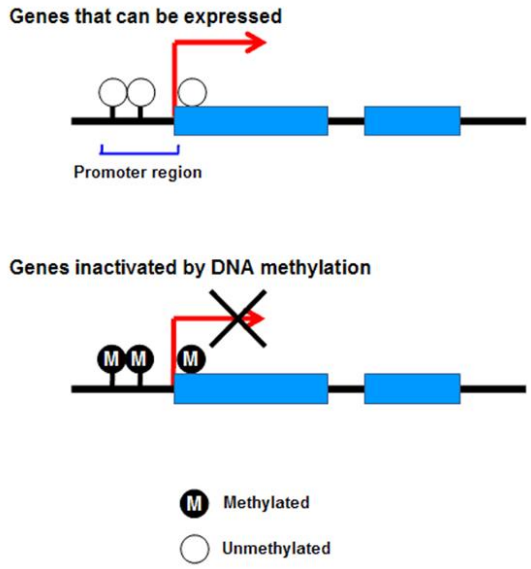
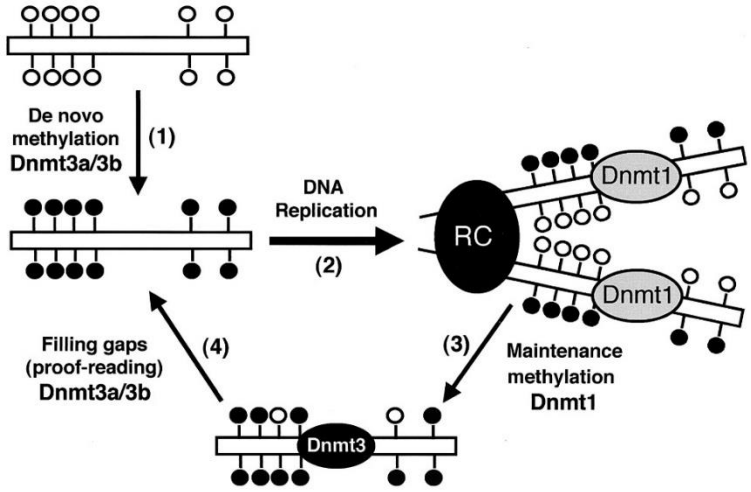


Figure 4. Repression of gene expression by DNA methylation.
Adapted from: Ushijima *et al.* (2003)
Cytosines, indicated with white or black coloured bulbs, are present in the promoter region. When the cytosines are not methylated (as shown above) genes can be expressed whereas cytosines that are methylated (as shown below) repress gene expression.

DNA methylation occurs by DNA methyltransferases (DNMTs) that transfer the methylgroup from S-adenosylmethionine (SAM) to the cytosine. DNMT1 methylates new synthesized strands and is therefore involved in mainting global DNA methylation, whereby DNMT3A and DNMT3B are involved in *de novo* methylation as shown in Figure 5 (Chen *et al.*, 2003; Anderson et al., 2012).

Figure 5. Maintenance and *de novo* methylation during DNA replication.
Adapted from: Chen *et al.* (2003).
DNA methyltransferases (DNMTs) 3A and 3B are involved in *de novo* methylation and proof-reading of newly methylated DNA strands, whereas maintenance methylation after DNA replication is carried out by DNMT1. Dark coloured balls represent methylated cytosines and white balls unmethylated cytosines.



DNA demethylation takes place through passive and active mechanisms. Passive DNA demethylation occurs when the DNA is replicated but no maintenance methylation takes place. Active DNA demethylation occurs through Ten-eleven translocation (TET) enzymes, which remove or modifies the methyl-group from 5mC as shown in Figure 6 (Bana and Cabreiro, 2019; Gadecka and Bielak-zmijewska, 2019).

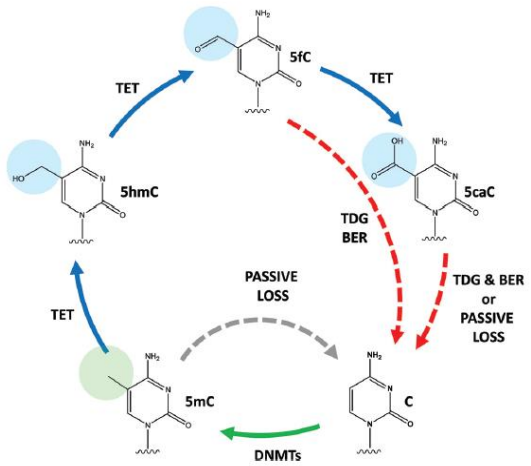


Figure 6. Cycle of DNA methylation and demethylation in mammals. Adapted from: Ravichandran et al. (2017). DNA methyltransferases (DNMTs) add a methyl-group to cytosine. Active demethylation is carried out by ten-eleven translocation (TET) enzymes which remove the methyl-group from the cytosine in several steps. Base excision repair (BER)/ Thymine DNA glycosylase (TDG) can also be involved in the demethylation process. Passive demethylation takes place in the absence of maintenance methylation DNMTs during DNA replication.

2.2.1 Factors that influence DNAm

Besides DNMTs and TET enzymes, diet plays an essential role in DNAm. Folate is converted to dihyrdofolate (DHF) to tetrahydrofolate (THF) by vitamin B_6 and vitamin B_2. THF is converted to methionine by vitamin B_{12}.. Methionine is then converted to SAM; the universal methyldonor (Bana and Cabreiro, 2019; Gadecka and Bielak-zmijewska, 2019) (See Figure 7). In Figure 7, in yellow indicated are the micronutrients that are needed from the diet to ensure a functioning one-carbon metabolism (Anderson *et al.*, 2012). Studies have shown that the availability of the micronutrients can alter DNA methylation (Anderson *et al.*, 2012; Mahmoud and Ali, 2019). Besides diet, studies have shown that smoking, stress and exercise influence DNAm also (Ambatipudi *et al.*, 2016; Bakusic *et al.*, 2017; Mcgee and Hargreaves, 2019).

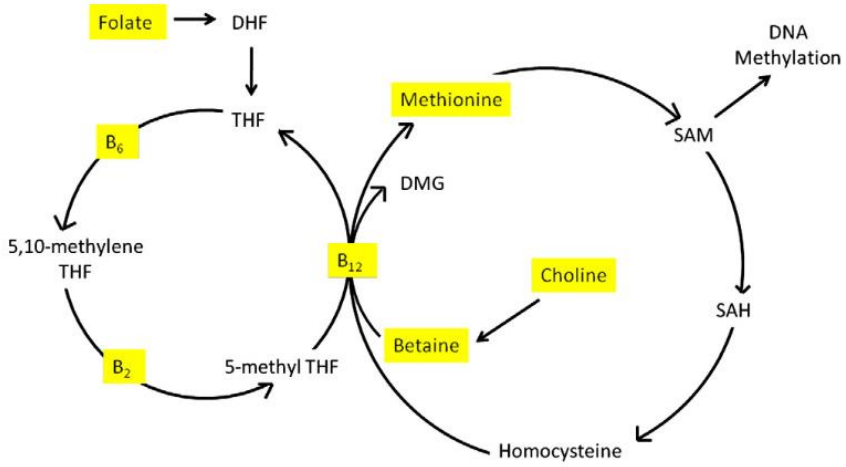


Figure 7. Micronutrients involved in one-carbon metabolism. (Adapted from: Anderson et al. (2012))
The (dietary) micronutrients involved in one-carbon metabolism are highlighted in yellow.
THF: tetrahydrofolate, DMG: dimethyl glycine, SAH: S-adenosylhomocysteine, SAH: S-adenosylmethionine

2.2.2 DNA methylation & analysis

DNAm can be examined with various methods, many of them make use of a bisulfite (BS) conversion step. (Kim and Costello, 2017). Sodium bisulfite converts the unmethylated cytosine to uracil, which becomes a thymidine upon PCR amplification and sequencing. The cytosine of 5mC does not convert to an uracil and can therefore be distinguised from unmethylated cytosine (See Figure 8) (Frommer *et al.*, 1992). Nowadays high-throughput and whole genome BS sequencing are applied. The Infinium methylation 450k microarray is a cost-effective, high-throughput method for detecting DNAm and is suitable for human samples which is now replaced by the MethylationEPIC (EPIC) BeadChip which covers over 850,000 CpG sites (Pidsley *et al.*, 2016). The advances in technique facilitated the opportunity to examine individual CpGs. In turn, the focus of studies shifted to specific DNAm instead of global methylation. Because of the amount of data, bioinformatics play a bigger role nowadays, for example in extracting CpGs that correlate significantly with aging (Kim and Costello, 2017). This essay will, however, not discuss these bioinformatic analyses.

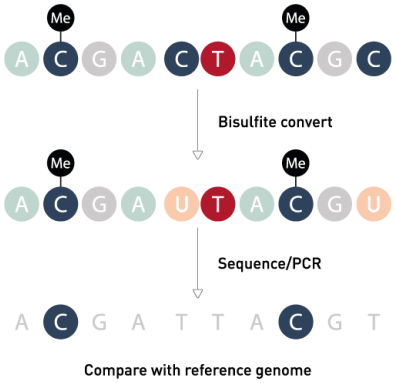


Figure 8. Schematic overview of bisulfite sequencing. (Diagenode, 2020)

Sodium bisulfite converts the unmethylated cytosines to uracil, but not the methylated cytosines. After sequencing, the methylated cytosines can then be distinguished from the unmethylated cytosines.

2.2.3 DNA methylation & aging

The link between aging and DNAm was established almost 50 years ago. It was hypothesized that organisms that lived longer should have a more stable DNAm pattern then shorter-lived organisms, since DNAm ensures DNA stability (Richardson, 2003). Several studies showed that DNAm decreased with age in different tissues in salmon, rats and mice, but in the liver of the rat, an increase in methylation was observed (Berdyshev *et al.*, 1967; Vanyushin *et al.*, 1973; Wilson *et al.*, 1987). These studies measured the total DNAm, but it was not clear what the effect was on gene expression.

To invesitage the effect of DNAm on gene expression, methylation sensitive endonucleases, which recognized CG sites but would only cleave the site if the cytosine was unmethylated, were used. This allowed the comparison of the methylation of restriction sites between young and old individuals. Tissue-specific methylation of certain restriction sites were observed (Richardson, 2003). For example, the β-actin gene was found to be demethylated with age in the spleen of rats but not brain or liver. Besides changes in DNAm in coding sequences of the DNA, there were also changes found in non-coding DNAm. Demethylation of repetitive DNA sequences were found in liver, thymus and heart, which could result in chromosal translocations with aging (Romanov and Vanyushin, 1981; Mays-hoopes *et al.*, 1986; Rath and Kanungo, 1989; Lengauer, Kinzler and Vogelstein, 1997; Richardson, 2003).

With the development of BS sequencing and especially high throughput BS sequencing (Frommer *et al.*, 1992) DNAm could be more accurately studied. Instead of the global methylation, the focus shifted to specific DNAm of CGI in promoters from certain genes and especially in humans. A study with monozygotic (MZ) twins showed that DNAm was altered in humans by age. Whereby, young MZ had similar DNAm and the DNAm differed more with age in older twins (Fraga *et al.*, 2005). Therefore, underlining the environmental influences on DNAm. Several studies showed that certain genes were hypermethylated, for example, tumor suppressor genes in elderly compared to young indiviuals and a global loss of methylation was also observed (Lopatina *et al.*, 2002; Christensen *et al.*, 2009; Ashapkin, Kutueva and Vanyushin, 2015).

2.3 Epigenetic clocks

Despite the new advances in techniques and analyzing methods, it was still not clear which genes were involved in the aging process and what role DNAm plays in aging. In 2011, the link between DNAm and aging was confirmed when Bocklandt and collegues showed that DNAm could be correlated with chronological age (e.g. an individual's actual age) (Bocklandt *et al.*, 2011). This accellerated the understanding of the role of DNAm in aging and was used to generate epigenetic clocks (e.g. a 'clock' that can predict the chronological age and later all-cause mortality risk and time-to-death based on biomarkers) (Bocklandt *et al.*, 2011; Hannum *et al.*, 2013; Horvath, 2013; Zhang *et al.*, 2017; Levine *et al.*, 2018; Fahy *et al.*, 2019; Lu *et al.*, 2019).

In order to understand how and why epigenetic clocks are important regarding the research of aging, several epigenetic clocks that had a significant influence in the research regarding epigenetic clocks and their results are highlighted.

2.3.1. Epigenetic predictors of age: correlating DNA methylation with chronological age

Bocklandt *et al.* (2011) were the first group of researchers who correlated DNAm with chronological age and their results can be acknowledged as a predecessor of the epigenetic clock. The methylation status of over 27,000 CpG loci in saliva of 34 pairs of biological twins between the age of 21-55 years were quantified. A total of 88 novel loci that correlated with age were identified, whereby 19 of the CpGs were negatively correlated, and thus hypomethylated and 69 were positively correlated and therefore hypermethylated with age. Further analysis indicated that, the gene-loci correlated with age were mainly involved in cardiovascular disease, neurological disease and genetic disease. After validation of the model, the error between the predicted age and chronological age concerned 5.3 years for males, 6.2 for females and 5.2 years combined. The error was even reduced to 3.5 years after adding another toppredictor of age.

2.3.2. The epigenetic clocks of Hannum and Horvath

To create a model that can be used to predict age based on DNAm, Hannum *et al.* (2013) used methylome-wide profiles of whole blood of 426 Caucasian and 230 Hispanic individuals aged between 19 and 101 years. Out of the 485,577 CpG markers, 71 methylation markers were found that were highly predictive of age. The correlation between chronological age and predicated age was 96% with an error of 3.9 years. Almost all the markers of the model were within or in close proximity of genes that are known for functions in age-related conditions, including Alzheimer's disease, tissue degradation, DNA damage, oxidative stress and cancer. A second cohort was included to validate the model, the correlation between chronological age and predicted age was still high: 91% but the error increased to 4.9 years. Besides the age-related markers, the study also found that gender significantly contributed to the aging rate. The methylome of men appeared to age 4% faster than that of women.

To test whether the model was also suitable for other tissues Hannum *et al.* (2013) tested breast, kidney, lung and skin samples with the same model. Although the correlation between chronological age and predicted age was still high (R=0.72), a clear linear offset from the expectation was seen in every tissue. The effect that men age quicker than women was, however, found again in these tissues. Lastly, Hannum *et al.* (2013) created *de novo* models for breast, kidney and lung tissues. Most of the markers in the model differed, although some were the same.

DNAm is shown to differ between tissues, however studies had found that there were also age-dependent DNAm changes independent of tissue (Teschendorff *et al.*, 2010; Koch and Wagner, 2011). In an attempt to create a multi-tissue age predictor, Horvath (2013) assessed the DNAm in more than 7000 healthy samples corresponding to 51 tissues and cell-types.

Using a mathematic model, 353 CpGs were selected that combined could best predict age, also called clock CpGs. A weighted average of the clock CpGs was calculated, put in an self-learning algorithm and then calibrated by using training data. Three prediction measures were used: first the correlation between the clock CpGs (DNAm age) and the chronological age, second the median difference between the predicted age and the chronological age. Lastly, average age accelleration defined by the difference between the predicted and chronological age to determine whether the age of a tissue was higher or lower than expected. The epigenetic model of Horvath (2013) had an error rate of approximately 3.6 years and proved highly accurate in heterogenous tissue, even in tissues/fluid that have a high turnover rate. The DNAm model was however less accurate in breast tissue, uterine endometrium, dermal fibroblasts, skeletal muscle tissue and heart tissue.

193 clock genes were hypermethylated with age and 160 hypomethylated. The hypomethylated CpGs varied more than the hypermethylated CpGs across tissues which was also found in Weidner *et al.* (2014). Further analysis indicated that hypermethylated CpGs were more likely to be in poised promoters, meaning that the promoters can be repressed as well as activated, whereby the hypomethylated CpGs are more likely to be in either weak promoters or strong enhancers.

The DNAm model of Horvath (2013) showed some more insight in aging. The 353 clock CpGs vary greatly across ages, it was then proposed that DNAm is logarithmic until adulthood and after adulthood it slows to a linear dependence (See Figure 9). Horvath (2013) proposed a theory for the switch from logarithmic to linear dependence. The switch is based on the epigenetic maintenance system (EMS) which is crucial regarding the growth of an individual, which Horvath (2013) called the ticking rate. The ticking rate corresponds with the 'work'of DNMTs, especially DNMT1. In early life during development, DNMTs' activity is required to maintain epigenetic stability, since there is high cell turnover which results in a high ticking rate and a logaritmic dependence. However, once development is completed, the 'workload' and therefore the ticking rate, can decrease to a linear dependence to maintain epigenetic stability.

Following the EMS theory, Horvath (2013) proposed that DNAm age should be accelerated by many perturbations that affect epigenetic stability, for example cancer. Each tissue affected by cancer showed evidence of significant age acceleration with an average age of 36.2 years. Furthermore, the number of mutations per cancer sample tends to be inversely correlated with age acceleration, which would fit the hypothesis that DNAm age acceleration results from processes that promote genome stabiltiy.

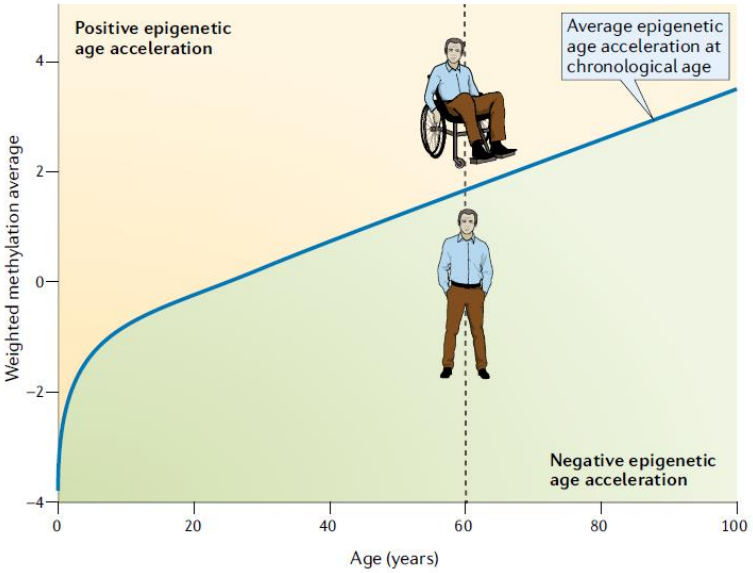


Figure 9. Deviations of epigenetic age regarding to chronological age. Adapted from: Horvath and Raj (2018)
When the epigenetic age is higher than the chronological age, it is associated with comorbidities and worse health than when the epigenetic age is lower than the chronological age.

2.3.3. Insights of centenarians

With regards to longevity, centenarians are an interesting subject to study because assing the differences between centenarians and elderly could give more insight in which genes and methylation patterns are involved in longevity. Studies including centenarians are scarce, because there are not many centenarians. The largest study conducted, was by Horvath *et al.* (2015b). 75 participants between the age of 99-113 years, 63 offspring of the centenarians between the age of 50-89 years and 46 participants between the age of 52-85 years old (control) participated. Previous research showed that DNA methylation is for 40% heritable (Fraga *et al.*, 2005), in this study the offspring of the centenarians had a predicted age 5.1 years younger than age matched controls. Analysis showed that the centenarians were 8.6 years on average younger than expected based on their chronological age. Moreover, the DNAm age of centenarians differed significantly from the DNAm age of the controls, but not compared with their offspring. It has to be noted that confounders can play a role since there are no suitable controls for participants over 105+ years and it has to be repeated with particpants with a different genetic background, lifestyle and cultural habits.

Other studies with centenarians found enriched DNAm regions in pathways associated with ARD, like type-2 diabetes, cardivascular and Alzheimer's disease, which when suppressed could lead to longevity (Xiao *et al.*, 2016). Moreover, a slower cell growing/metabolism, better control in signal transmission and a better preservation of DNAm status is proposed to contribute to human longevity. Preservation of DNAm status in centanarians was found as the DNA global hypomethylation associated with age, was delayed in centenarians (Gentilini *et al.*, 2013). Furthermore, it was found that age-related DNA hypermethylation occurs predominantly in genes involved in the development of anatomical structures, organs and multicellular organisms and genes involved in nucleotide biosynthesis, metabolism and control of signal transmission were differently methylated between centenarians' offspring and offspring of non-long-lived parents (Gentilini *et al.*, 2013).

2.3.4. Factors that influence the epigenetic clocks

Several studies investigated factors that influence the epigenetic clock as shown in Table 1. The influence of ethnicity and education was also assessed but with a different method. Horvath *et al.* (2016) used whole blood, whereby they calculated the intrinsic epigenetic age accelleration (IEAA) which captures the DNAm and extrinsic epigenetic age accelleration (EEAA) which also captures the changes of the composition in blood. The study found that Hispanics and Tsimane (an indigenous population of the lowlands of Bolivia) based on DNAm aged slower than Caucasians however if the changes in blood where taken into account Caucasians aged slower than Hispanics and Tsimane. African Americans had the lowest EEAA. Furthermore, higher education was associated with decreased EEAA in every ethnic group.

Factors that influence the epigenetic clock	Studies
Gender	(Hannum *et al.*, 2013)
Cancer	(Hannum *et al.*, 2013; Levine *et al.*, 2015a)
Genetics	(Horvath *et al.*, 2015a)
Obesity	(Horvath *et al.*, 2014)
Down's syndrome	(Horvath *et al.*, 2015b)
HIV infection	(Horvath and Levine, 2015)
Parkinson's disease	(Horvath and Ritz, 2015)
Alzheimer's disease	(Levine *et al.*, 2015b)
Life time stress	(Zannas *et al.*, 2015)
Smoking	(Gao *et al.*, 2016)
Coronary heart disease (CHD)	(Horvath *et al.*, 2016)

Table 1. Factors that influence the epigenetic clock.

2.3.5. All-cause mortality risk prediction based on the epigenetic clocks

After validation of the epigenetic clocks and assessing which factors influence the epigenetic clocks, Marioni *et al.* (2015) tested if all-cause mortality risk could be calculated by using the epigenetic clocks of Hannum *et al.* (2013 and Horvath (2013). Marioni *et al.* (2015) tested if Δage, the difference between predicted age and chronological age, or other DNA methylation biomarkers could be significant predictors of all-cause mortality using four cohort studies.

A 5-year higher Hannum Δage, which indicated that the predicted age was 5 years higher than the chronological age, was associated with a 21% greater mortality risk after adjusting for chronological age and sex, whereas a 5-year higher Horvath's Δage was associated with 11%. After creating a fully adjusted model which also controlled for smoking, education, childhood IQ, APOE, cardiovascular disease, high blood pressure and diabetes, the hazard ratio (HR) per 5-year difference was 1.16 for Hannum and 1.09 for Horvath. The study did not find a difference in the Δage and survival by sex. However they did find that women had a significantly lower Δage than men.

The study of Zhang *et al.* (2017) looked at mortality related DNAm signatures from blood samples and generated a new predictor to predict all-cause mortality risk based on DNAm. 58 CpGs were found in the baseline blood samples associated with mortality. Of the risk factors, 23 and 25 of the CpGs were associated with age and sex, 48 associated with smoking and 5 with alcohol consumption.

10 CpGs were eventually put in a model to calculate the mortality risk. Participants were then screened if they had aberrant methylation at the 10 CpGs and allocated a mortality score of 1-10. Whereas participants without aberrant methylation at any of the 10 CpGs had a score of 0. Participants that had a score of 1, 2-5 and 5 had a 2-, 3- and 7-fold risk of dying compared to participants with a score of 0. In the

fully adjusted model, the HR score for participants with a score of 5+ was 7.41. The all-cause mortality associations indicated stronger to be among women than among men.

Following these studies, other studies also investigated all-cause mortality risk based on DNAm. However, studies had indicated that biomarkers could be a better indicator for all-cause mortality than chronological age (Ferrucci *et al.*, 2010; Levine, 2013; Belsky *et al.*, 2015; Li *et al.*, 2015; Sebastiani *et al.*, 2017). Therefore, the following two studies that are highlighted here, will use biomarkers instead of chronological age.

2.3.6. Next generation epigenetic clocks based on biomarkers instead of chronological age

Levine *et al.* (2018) used the whole blood method based on IEAA and EEAA markers. Out of 42 biomarkers, 10 variables were selected for the phenotypic age predictor, one of the biomarkers was chronological age. They found that 1 year increase in phenotypic age was associated with a 9% increase in the risk of all-cause mortality from ARD, a 10% increase in the risk of CVD mortality, a 7% increase in the risk of cancer mortality, a 20% increase in the risk of diabetes mortality and a 9% increase in the risk of chronic lower respiratory disease mortality. The top 5%, the fast agers, had a mortality hazard of death 1.62 times that of the average person, opposed to 2.58 of the slowest agers. When compared to the epigenetic clocks of Hannum *et al.* (2013) and Horvath (2013), PhenoAge predicted 10- and 20-year mortality risk significantly better. 41 of the 513 CpGs were shared with the Horvath clock (Horvath, 2013), 5 CpGs were shared between all three (Hannum *et al.*, 2013; Horvath, 2013; Levine *et al.*, 2018). Besides ethnicity, education, the influence of exercise and dietary habits on PhenoAge were assessed. Whereby increased exercise and fruit and vegetable consumption were associated with a lower PhenoAge.

To identify the years left, based on all-cause mortality, DNAm GrimAge was created by Lu *et al.* (2019). To create the DNAm GrimAge, DNAm biomarkers of physiological risk factors and stress factors were determined. 12 out of the 88 corresponding plasma protein variables, generated by blood samples, were correlated with chronological age, sex and CpG levels from the training data. The 12 plasma protein variables were then put together with the DNAm estimator of smoking pack-years, chronological age and sex.

Before it was tested if the DNAm GrimAge was a better predictor of lifespan than chronological age, DNAm GrimAge was regressed on chronological age to define epigenetic age acceleration (AgeAccelGrim) and used in association tests with ARD, since age is a confounding factor. Using the blood samples of Caucasians, African Americans and Hispanics, it was found that AgeAccelGrim predicted lifespan better than chronological age. AgeAccelGrim was also accurate in predicting the lifespan of never-smokers and the surrogate marker was a better predictor of lifespan than self-reported smoke pack-years. Furthermore, AgeAccelGrim was also highly predictive of CHD incidence, which is also associated with hypertension, Type 2 Diabetes and physical functioning. Higher values of AgeAccelGrim were associated with lower physical functioning levels and menopause at an early age as well. In regards to dietary habits, AgeAccelGrim showed a strong relationship with mean carotenoid levels whereas a higher intake resulted in a lower AgeAccelGrim and a higher carbohydrate intake was associated with lower AgeAccelGrim this was the opposite for fat.

DNAm GrimAge was compared to the epigenetic clocks of Horvath (Horvath, 2013), Hannum (Hannum *et al.*, 2013) and PhenoAge (Levine *et al.*, 2018) and DNAm GrimAge outperformed the others with respect to predict time-to-death.

As showed above, epigenetic clocks can predict the chronological age using a biological sample and even calculate the all-cause mortality risk and time-to-death based on DNAm. Which leaves the next question, is it possible to reverse the epigenetic clock?

2.3.7. Reversing the epigenetic clock

In response to this question Fahy *et al.* (2019) tried to reverse the epigenetic clock by using recombinant human growth hormone (rhGH) in 51-61 year old healthy men. At baseline the DNAm ages of the participants were lower than their chronological ages however after treatment the DNAm age decreased even further based on the Hannum, Horvath, PhenoAge and GrimAge clock (Hannum *et al.*, 2013; Horvath, 2013; Levine *et al.*, 2018; Lu *et al.*, 2019). After 12 months the mean Δage was approximately 2.5 years and even after 6 months discontinuation of the treatment, the Δage remained 1.5 years. Moreover, the GrimAge clock that predicts life expectancy, stayed even after 6 months on 2.1 years gain (Lu *et al.*, 2019). Interestingly, the rate of aging regression appeared to accelerate with increasing treatment time, respectively -1.56 +/- 0.46 years/year in the first 9 months to -6.48 +/- 0.34 years/year in the last 3 months of treatment.

3. Conclusions and future perspectives

To conclude, DNAm plays an important role in the aging process (Christensen *et al.*, 2009). Epigenetic clocks, based on DNAm, showed that DNAm could be used to estimate chronological age of an individual (Hannum *et al.*, 2013; Horvath, 2013), calculate all-cause mortality risk (Zhang *et al.*, 2017; Levine *et al.*, 2018) and time-to-death (Lu *et al.*, 2019). Lastly, studies also found that several factors influence the epigenetic clock (Hannum *et al.*, 2013; Gao *et al.*, 2016; Horvath *et al.*, 2016).

Although epigenetic clocks are very promising to study aging but there are still some limitations. First, epigenetic clocks need to be used accordingly, since the clocks are trained on different databases and tissues resulting in different results. For instance, Figure 10 shows the differences between the Hannum, Horvath and Levine's (PhenoAge) clock; because the clocks used different datasets and tissues this results in different outcomes with the same dataset.
Second, Caucasians are overrepresented in the used datasets which could result in a bias. Third, the epigenetic clock is not suited for single-cell or individual use yet. To make the epigenetic clocks clinically relevant, individual tissue-, disease- and mechanism specific clocks should be created. The strenghts and weaknesses should be indicated per clock, then improved in order to create the tissue-, disease- and mechanism specific clocks as needed (Bell *et al.*, 2019).

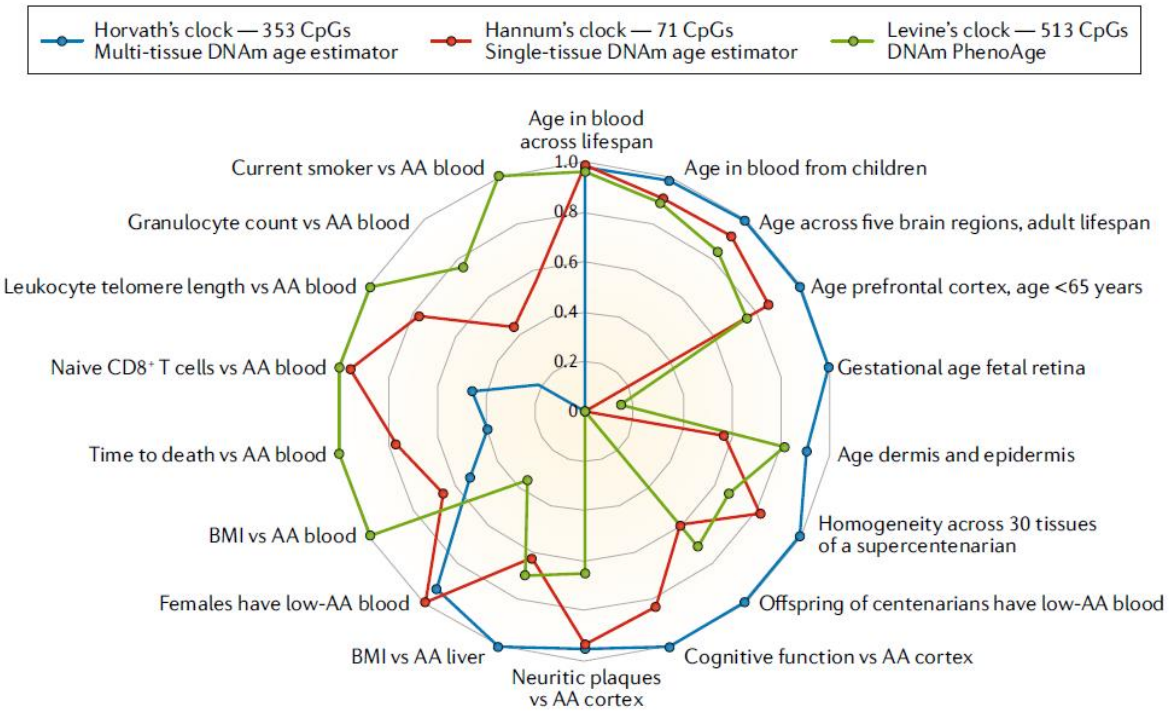


Figure 10. Comparison between Horvath's, Hannum's and Levine's clock.
Adapted from Horvath and Raj (2018)
This figure shows the differences in the Horvath's (Horvath, 2013), Hannum's (Hannum *et al.*, 2013) and Levine's clock (Levine *et al.*, 2018).

Besides technical improvements, the future of the epigenetic clocks lies in slowing down aging by rejuvinating the predicted age. Fay *et al.* (2019) showed that it is possible to slow down and even reverse the epigenetic clock with the use of a growth hormone. However, it is not likely that a growth hormone will be administerd to individuals. Therefore, research should focus on finding other compounds/things that could be used to rejuvinate the predicted age. One method that have been proven effective to extend lifespan in animals is caloric restriction (CR) (Hahn *et al.*, 2017). The effect of CR on lifespan is studied in yeast, worms, flies, mice and monkeys, in which animals that followed the CR diet had an increased lifespan (Fontana *et al.*, 2010). During World War 1 and 2 a drop in death rates was observed by the Danish and Norwegians that were forced to restrict their intake due to foodshortage (Hindhede, 1920; Strom and Jensen, 1951). In humans, the effect of a CR diet is only studied by coincidence since it is hard to find participants to study CR effects longterm, since CR is very invasive as the diet contains usually 10-40% less calories than the recommendations (Cava and Fontana, 2013; Most *et al.*, 2017). A drug that mimicks the CR effects would be optimal since it would not be as invasive. Metaformin is used to treat diabetes, but research showed that it could also be used to improve age-related symptoms, however no research has been conducted in combination with epigenetic clocks (Barzilai *et al.*, 2016).

To conclude, DNAm and especially epigenetic clocks are promising targets to study aging and can hopefully be used to improve the quality of life in elderly and to extend lifespan. However, to gain a broader understanding of aging, future research should also assess the influence of the epigenetic clock on the 9 hallmarks of aging (López-Otín *et al.*, 2013) as DNAm can not fully account for the aging process. So that in the future, the HALE can be equal to life expectancy.

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